



Contribution of neurotensin and its high affinity receptor to the response of chemotherapy in ovarian cancer

Jin Liu

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UNIVERSITE PIERRE ET MARIE CURIE

ED 394 PHYSIOLOGIE, PHYSIOPATHOLOGIE ET THERAPEUTIQUE

THESE

**CONTRIBUTION DU COUPLE NEUROTENSINE ET SON
RECEPTEUR DE HAUTE AFFINITE DANS LA REPONSE A LA
CHIMIOOTHERAPIE DANS LE CANCER DE L'OVAIRE**

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ABBREVIATIONS

AA	Amino acid
ACE	Angiotensin-converting enzyme
ADPR	ADP ribose moieties
AIF	Apoptosis-inducing factor
AP-1	Activator protein-1
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-Related
AUC	Area under the curve
BAD	BCL-2-associated death promoter
BAK	BCL-2 homologous antagonist killer
BAX	BCL2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma-extra large
BID	BH3 interacting-domain death agonist
Bim	BCL-2-like protein 11
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domain
CCC	Clear cell carcinomas
CDDP	Cisplatin
CDKN1A	Cyclin-dependent kinase inhibitor 1
cDNA	Complementary deoxyribonucleic acid
CHEK1	Checkpoint kinase 1
CHEK2	Checkpoint kinase 2
CI	Confidence interval
CNS	Central nervous system
CRE	cAMP response element
CREB	cAMP response element-binding
CTR1	Copper transporter 1
cyt-c	Cytochrome-c
DA	Dopamine
DAG	Diacylglycerol
DDR	DNA damage response
DED	Death effector domain
DFI	Disease-free interval
DFS	Disease-free survival
DHFR	Dihydrofolate reductase
DIABLO	Direct inhibitor of apoptosis protein (IAP)-binding protein with low isoelectric point
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DSB	Double-strand breaks
E2	Estradiol
EAOC	Endometriosis-associated ovarian cancer
EGFR	Epidermal Growth Factor Receptor
Endo G	Endonuclease G
EOC	Epithelial ovarian cancer
EPT	Estrogen-progestin therapy
ERE	Estrogen response element
ET	Estrogen therapy
FA	Fanconi Anemia
FAK	Focal Adhesion Kinase
FIGO	International Federation of Gynecology and Obstetrics
GPCR	G-protein coupled receptor
GRE	Glucocorticoid Response Element
GSH	Glutathione
GSTs	GSH transferases
GS-X pump	Glutathione S-conjugates pump
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HFD	High fat diet
HGSOC	High grade serous ovarian carcinomas
HMG	High-mobility group
HR	Homologous recombination
HSP	Heat Shock Proteins
IAPs	Inhibitor of apoptosis proteins
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IHC	Immunohistochemistry
IL	Interleukin
IP3	Inositol-1,4,5 triphosphate
IVF	In-vitro fertilization
JNK	c-jun amino-terminal kinase
LGSOC	Low-grade serous ovarian carcinomas
LS	Lynch syndrome
MAPK	Mitogen-activated protein kinase
MC	Mucinous carcinomas
MCL-1	Myeloid cell leukemia sequence 1
MHT	Menopausal hormone therapy
MMP	Matrix metalloproteinase
MMR	Mismatch repair
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic acid
MRP	Multidrug-resistance protein
MSI	Microsatellite instability

NEP	Neutral endopeptidase
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NN	Neuromedin N
NSCLC	Non-small cell lung cancer
NTS	Neurotensin
NTSR1,2,3	Neurotensin receptor 1,2,3
OC	Ovarian cancer
OCs	Oral contraceptives
OR	Odd ratio
OS	Overall survival
p53	Tumor protein 53
p73	Tumor protein 73
PARP	poly(ADP-ribose) polymerase
PCNA	Proliferating cell nuclear antigen
PCs	Proprotein convertases family
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PFS	Progression-free survival
PI	Propidium iodide
PIDD	p53-induced protein with death domain
PINK1	PTEN-induced putative kinase 1
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKD1	Protein kinase D1
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Pegylated liposomal doxorubicin
PNRC	Perinuclear recycling compartment
POL β	DNA polymerase β
RB	Retinoblastoma
RFC	Replication factor C
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Relative risk
RT-PCR	Reverse transcription polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Smac	Second mitochondria-derived activator of caspases
SMDs	Structured membrane microdomains
SSB	Single-strand break

TBP	TATA binding protein
TGF- β	Transforming growth factor- β
TGN	Trans-Golgi network
TIC	Tubal intraepithelial carcinoma
TIL	Tumor infiltrating lymphocytes
TM	Transmembrane domains
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRH	Thyrotropin-releasing hormone
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
XIAP	X-linked mammalian inhibitor of apoptosis protein

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General Introduction

Chapter 1. Ovarian cancer

1.1 Epidemiology and clinical expectations

Ovarian cancer (OC) is one of the most severe gynecological malignancies known in women. Ninety percent of malignant tumors originates from epithelium, but transformation may occur also in the ovarian stroma or germ cells. There are 238,700 estimated new cases in the developed countries and developing countries in 2012, accounting for 4 per cent of all new cases of cancer in women worldwide. The estimated death for ovarian cancer is 151,900 and ovarian cancer is the eighth most common cause of cancer death in female worldwide (Ferlay et al., 2015). The incidence of ovarian cancer is unevenly distributed in the world, with the highest rates in Europe and Northern America, and lowest rates in Western Africa (Ferlay et al., 2015). Generally, ovarian cancer is diagnosed at advanced stage because this disease has often no apparent symptoms at the early stages. In these cases, the European mean age-standardized 5-year survival was low, only 37.6 % (95 % CI 37.1-38.0) based on populations studied between 1999-2007 (De Angelis et al., 2014).

In France, with an estimated 4,615 new cases in 2012 (95% CI, 4095-5136), OC represented 2.9% of all cancers in women, and was the 8th most common type of cancer in women. With 3,140 deaths in 2012, OC represented 5% of all cancer-related deaths among women, making it the 5th cause of death among women. Between 1989 and 2012, the net survival at 1, 5 and 10 years after diagnosis was respectively 76%, 42% and 33%. Corresponding age standardized net survival was 74%, 40%, and 31% (Tretarre et al., 2015).

1.2 Histological types of ovarian cancer

Most tumors of the ovary can be categorized into three types according to tumor origin-epithelial cell tumors, stromal cell tumors and germ cell tumors. Figure 1 shows the type of ovarian cancer from the different origins.

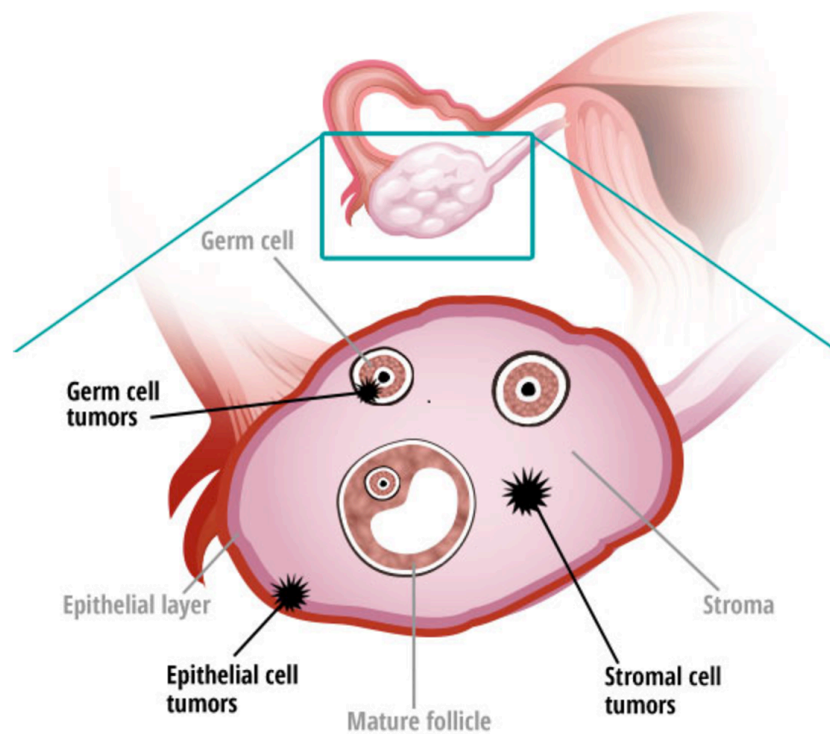


Figure 1. Types of ovarian cancer.

My OC journey. Type and subtype.

<https://www.myocjourney.com/oc-journey/understanding-diagnosis.html>. Accessed 15 Nov, 2016

1.2.1 Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) starts in the surface epithelium of ovary. About 90% tumors of the ovary are from epithelial origin. EOC tumors are further classified into different subtypes based on morphological appearance according to WHO histopathological standards, including serous, endometrioid, clear cell, mucinous and undifferentiated tumors. Additional classification, based on molecular genetics, divides

EOC into two broad categories designated types I and II (Kurman and Shih le, 2011; Vang et al., 2009). Type I tumors, that are relatively stable genetically, include low-grade serous, low-grade endometrioid, clear cell and mucinous carcinomas. Type II consists of high-grade serous, high-grade endometrioid and undifferentiated/mixed tumors, all genetically highly unstable. Table 1 summarizes the characteristics of the histological subtypes of ovarian cancer.

Table 1. Characteristics of the main histological subtypes of ovarian cancer (extracted from (Hollis and Gourley, 2016)).

	HGS	Endometrioid	Clear cell	Mucinous	LGS
Approximate proportion of OC cases	70%	10%	10%	<5%	<5%
Overall prognosis	Poor	Favourable	Intermediate	Intermediate	Intermediate
Tissue of origin / precursor lesion	Distal fallopian epithelium	Endometriosis	Endometriosis	Poorly defined	Serous borderline tumor
Intrinsic chemosensitivity	High	High	Low	Low	Low
Associated hereditary syndromes	Germline <i>BRCA1/2</i>	Lynch syndrome	Lynch syndrome		
Typical stage at diagnosis	80% advanced stage	50% early stage	60% early stage	80% early stage	Typically advanced stage
Frequent molecular abnormalities	Chromosome instability <i>BRCA1</i> , <i>BRCA2</i> <i>TP53</i> , <i>NF1</i> , <i>RBI</i> <i>CCNE1</i> <i>amp</i> .	<i>PTEN</i> , <i>PIK3CA</i> , <i>ARID1A</i> , <i>CTNNB1</i>	<i>PTEN</i> , <i>PIK3CA</i> , <i>ARID1A</i> , chr20q13.2, <i>amp</i>	<i>KRAS</i> , <i>HER2 amp</i>	<i>KRAS</i> , <i>BRAF</i>
Early stage: FIGO stage I or II; advanced stage: FIGO stage III-IV; amp: amplification					

1.2.1.1 Serous ovarian carcinomas

The serous ovarian carcinomas are classified into low- and high- grade lesions. These two subtypes are clinically and molecularly distinct diseases. High grade serous ovarian carcinomas (HGSOC) were traditionally considered as arising from the ovarian epithelium and its inclusion. More recently, the observation of tubal intraepithelial carcinoma (TIC) in the distal fimbriated end of the fallopian tube in women are carriers of BRCA mutations, suggesting that these TICs are probable precursor of advanced HGSOC. Implantation of tubal-type epithelium into the ovary or inclusion cysts may explain the origin of those HGSOC lacking TICs. The relative proportion of HGSOC of ovarian and tubal derivation is unknown. However, extensive examination of the fallopian tubes in HGSOC (ovarian, tubal, or pelvic) shows involvement of the endosalpinx in 70% and TIC in approximately 50% of the cases (Kindelberger et al., 2007).

Low-grade serous ovarian carcinomas (LGSOC) are thought to originate in inclusion cysts and to progress via a slow stepwise transformation of a borderline lesion into a low-grade invasive carcinoma.

HGSOC represents for around 68% of EOC cases (Kobel et al., 2010). *TP53* mutations are the earliest event associated with HGSOC transformation, and p53 dysfunction is ubiquitous for this subtype. Mutations in the *TP53* gene have been shown in 96% of these tumors, and in the remaining cases p53 dysfunction is caused by post-translational mechanisms (Ahmed et al., 2010; Cancer Genome Atlas Research, 2011). Another common features in HGSOC subtype is BRCA pathway mutations, found in 51% cases in a cohort of 489 HGSOC cases (Cancer Genome Atlas Research, 2011). In this study of 489 HGSOCs, *BRCA1/2* germline mutations were 17% and *BRCA* somatic mutations were 3%. 11% of total cases, *BRCA1* was epigenetically silenced. Other alterations were seen in *EMSY* (a BRCA2-interacting transcriptional repressor, amplified or mutated in 8%), *RAD51C* (a component in the homologous recombination repair, hypermethylated in 3%), 2% mutation of *ATM* (ataxia-telangiectasia mutated, the upstream DNA damage response kinase) or *ATR*

(ATM- and Rad3-Related), and *PTEN* (deleted or mutated in 7%) (Cancer Genome Atlas Research, 2011). The term BRCAness has gained acceptance as descriptive of the defective homologous recombination (HR) DNA repair system in sporadic cancers (Turner et al., 2004). Due to the loss of HR, cancer cells with BRCAness feature probably use DNA repair processes such as non-homologous end joining (NHEJ) that result in DNA mutations and gross chromosomal abnormalities (Lord and Ashworth, 2016). This is in line with women carrying *BRCA*-mutations have increased risk to develop EOC, particularly HGSOC (Lakhani et al., 2004; Mavaddat et al., 2012). The understanding of BRCAness has driven the development of therapeutic approaches to cancer that target HR defects, notably poly(ADP-ribose) polymerase (PARP) inhibitors which has been one of the common used targeted therapies. The other alterations in HGSOC are retinoblastoma (RB) protein pathway (p16^{INK4a}/RB), phosphatidylinoside 3-kinases (PI3K)/Akt and Notch signaling cascades (Cancer Genome Atlas Research, 2011).

LGSOC accounts for 3-4% of all EOC cases (Kobel et al., 2010). Common mutations are those of *KRAS*, *BRAF* and *ERBB2* oncogenes (Singer et al., 2003; Vang et al., 2009), all upstream of the mitogen-activated protein kinase (MAPK), resulting in activation of MAPK signaling and proliferation (Hsu et al., 2004). Two thirds of LGSOC harbor these mutations which can also be found in serous borderline tumors, i.e. tumors with semi-malignant potential (Bonome et al., 2005). They seem to share common molecular features with LGSOC and 60% of LGSOCs often contain areas with borderline malignant potential (Malpica et al., 2004), suggesting that borderline serous tumors might transform into LGSOC. TP53 mutations are uncommon and they have lower levels of chromosomal instability than HGSOC (Lim and Oliva, 2013; Vang et al., 2009).

1.2.1.2 Endometrioid carcinomas

Approximately 11% of EOCs are endometrioid carcinomas (EC) (Kobel et al., 2010). Frequent mutations are those in *CTNNB1*, encoding β -catenin (Palacios and Gamallo, 1998), *PTEN* (Obata et al., 1998), *KRAS* (Cuatrecasas et al., 1997), *ARID1A* and in

the gene coding for the catalytic subunit of class I PI3Ks, *PIK3C* (Samartzis et al., 2013; Wiegand et al., 2010; Yamamoto et al., 2011). Most of endometrioid carcinomas develop slowly from transformation of endometriotic lesions, hence endometriosis increases the risk of developing EC (McCluggage et al., 2002; Nezhat et al., 2008; Pearce et al., 2012).

1.2.1.3 Clear cell carcinomas

Clear cell carcinomas (CCC) account for 12% of all EOC cases (Kobel et al., 2010). For unknown reasons, this subtype has a higher prevalence in Japan, where 15-25% of EOCs are CCC (Yoshikawa et al., 2000). Like the endometrioid tumors, the majority of clear cell cases originate from endometriosis (Lim and Oliva, 2013). Almost 50 % of clear cell EOCs exhibit mutated *ARID1A* (Jones et al., 2010; Wiegand et al., 2010), and *PIK3CA* mutations have been found in up to 40 % of cases (Anglesio et al., 2011; Kuo et al., 2009). *C-MET* amplifications have also been described (Yamashita et al., 2013). Clear cell EOCs are almost invariably *TP53* wild-type and have a low level of chromosomal instability (Anglesio et al., 2011).

1.2.1.4 Mucinous carcinomas

Mucinous carcinomas (MC) arise slowly from mucinous borderline lesions, accounting for 3-4% of EOCs (Kobel et al., 2010). *KRAS* mutation is common and an early event in the evolution of these tumors (Gemignani et al., 2003; McCluggage, 2011). *ERBB2* overexpression or amplification is seen in 15-20% (Anglesio et al., 2013; Gilks, 2010).

1.2.2 Stromal cell tumors and Germ cell tumors

Stromal cell tumors are rare. They begin in the tissues that hold the ovaries in place and in those tissues that produce the hormones estrogen and progesterone. About 70% of stromal tumors are found in the earliest stage (stage I), when the cancer is limited to the inside of one or both ovaries.

Germ cell tumors begin in the cells destined to become ova, or eggs. Most of these

tumors are benign, but a small number are cancerous. Germ cell ovarian cancer occurs most commonly in young women in their teens and twenties.

1.3 Risk factors and protective factors

The main risk factors for ovarian cancer are age and certain hereditary mutations, but gynecological conditions and treatments, reproductive history as well as life style factors are also responsible for developing the disease (Lockwood-Rayermann et al., 2009). On the contrary, parity, breastfeeding, oral contraception as well as gynecologic surgery like tubal ligation and hysterectomy may reduce the risk of developing ovarian cancer.

1.3.1 Genetics

It is believed that approximately 10 % of ovarian carcinomas are due to hereditary susceptibility, and germline mutations in *BRCA1* or *BRCA2* account for 90% of cases. Women with *BRCA1* mutations have a 30-40% risk of developing ovarian cancer before the age of 70. The corresponding risk for *BRCA2* mutation carriers is 10-20% (Antoniou et al., 2003; Arts-de Jong et al., 2016; Levy-Lahad and Friedman, 2007; Pal et al., 2005; Risch et al., 2001). *BRCA*-mutations particularly increase the risk of high-grade serous carcinomas. In recent years, other genes cooperated with *BRCA1/2* for the double-strand breaks repair are also responsible for hereditary ovarian cancer, such as *ATM*, *CHEK2*, *RAD51*, *BRIP1*, and *PALB2* (Toss et al., 2015).

Mutation of several tumor suppressor genes are detected in ovarian cancer. *TP53* gene is mutated in 50–80% of high-grade invasive ovarian carcinomas, but rarely in other ovarian cancer subtypes or borderline serous tumors whereas *PTEN* mutations are mostly found in endometrioid endometrial carcinomas and clear cell carcinomas (Lynch et al., 2009; Toss et al., 2015).

Additionally, female carriers with Lynch syndrome (LS) have great predisposition to colon cancer and endometrial cancer. LS is caused by the pathogenic mutation in four DNA mismatch repair genes, including *MLH1*, *MSH2*, *MSH6*, or *PMS2*, in which most frequent mutations were *MLH1* (38%) and *MSH2* (47%). Carriers of Lynch syndrome

have a 6-12% risk of ovarian cancer. Ovarian cancer linked to Lynch syndrome typically develops at a mean age of 45 years as early-stage tumors of mixed type (mucinous/endometrioid/clear cell carcinomas, 33%), endometrioid (25%) and serous carcinoma (22%) histologic subtypes (Helder-Woolderink et al., 2016). In a cohort study, most Lynch syndrome-associated ovarian cancer cases presented early (85% at stage I/II vs. 15% at stage III/IV, $p < 0.001$) and overall survival was excellent (80% 5-year survival), however, patients with advanced disease had a poor prognosis (40% 5-year survival) (Ryan et al., 2017).

Several familial syndromes associated with OC, account for less than 1% of total hereditary cases, such as the Gorlin's syndrome, osteochondromatosis or Ollier's syndrome and the Peutz-Jeghers syndrome (Russo et al., 2009). The corresponding genes are *PTCH1*, *STK11* and *EXTs*.

1.3.2 Endometriosis

Endometriosis is considered as a risk of developing endometrioid or clear cell ovarian cancer, which often arise within the endometriotic lesions. In the meta-analysis of case-control and cohort studies including 444,225 women, endometriosis was strongly associated with the increased risk of ovarian cancer, and endometriosis-associated ovarian cancer (EAOC) showed favorable characteristics including early-stage disease, low-grade disease and a specific histology such as endometrioid or clear cell carcinoma (Kim et al., 2014). Moreover, in another pooled analysis of case-control studies, consisting of 7911 women with invasive epithelial ovarian cancer and 1907 with borderline ovarian cancer and 13,226 controls, self-reported endometriosis was associated with a significantly increased risk of clear-cell (odds ratio (OR) 3.05, 95% CI 2.43–3.84, $p < 0.0001$), low-grade (OR 2.11, 1.39–3.20, $p < 0.0001$), and endometrioid invasive ovarian cancers (OR 2.04, 1.67–2.48, $p < 0.0001$). However, no association was noted between endometriosis and risk of mucinous (OR 1.02, 0.69–1.50, $p = 0.93$) or high-grade serous invasive ovarian cancer (OR 1.13, 0.97–1.32, $p = 0.13$), or borderline tumors (Pearce et al., 2012).

1.3.3 Fertility drugs

Infertility appears to increase the incidence of ovarian cancer, while the potential additional risk associated with the use of fertility drugs is highly doubted (Risch et al., 1994; Rizzuto et al., 2013). Multiple studies showed that fertility drug used in nulligravid women and subfertile women treated with in-vitro fertilization (IVF) might carry an increased risk of borderline ovarian tumors but no convincing evidence of an increased risk of invasive ovarian cancer with fertility drug treatment (Ness et al., 2002; Tomao et al., 2014).

1.3.4 Menopausal hormone therapy (MHT)

Estrogen therapy (ET) or estrogen-progestin therapy (EPT), two main treatments for menopausal symptoms, increase the risk of ovarian cancer. A meta-analysis, based on 9 cohort studies and 3 case-control studies, showed that ET (pooled hazard ratio (HR)/relative risk (RR)=1.37, 95% CI: 1.19 to 1.58) and EPT (pooled HR/RR=1.27, 95% CI: 1.18 to 1.36, $P<0.001$), and ET+EPT (ET followed by EPT, pooled HR/RR=1.55, 95% CI: 1.05 to 2.30, $P=0.027$) were associated with an increased risk of ovarian cancer. However, these hormone therapies were associated with an increased risk of serous ovarian cancer (pooled HR/RR=1.46, 95% CI=1.28-1.67, $P<0.001$), not in clear cell, endometrioid, or mucinous ovarian cancer (Shi et al., 2016).

1.3.5 Talcum powder

Perineal use of talc powder (containing possible carcinogenic particles like asbestos) has been reported to increase the risk of EOC. Accordingly, in a pooled analysis of altogether 8,525 ovarian cancer cases and 9,859 controls, ever use of genital powder was associated with increased risk of EOC (OR 1.24, 95% CI 1.15-1.33) (Terry et al., 2013). The increased risk was approximately the same for the serous, endometrioid and clear cell subtypes, while no significant association was found for development of mucinous tumors. Other studies also provided evidence that perineal talc use may modestly increase the risk of invasive serous ovarian cancer (Gertig et al., 2000).

1.3.6 Obesity

Various studies have reported that obese women (those with a body mass index of at least 30) have a higher risk of developing ovarian cancer. A case-control study of 775 ovarian cancer cases and 846 controls were used to examine the association with body mass index (BMI). There was a significant increased risk of ovarian cancer with increasing BMI, with women in the top 15% of the BMI range having an odds ratio (OR) of 1.9 (95% confidence interval (CI), 1.3-2.6) compared with those in the middle 30% (Purdie et al., 2001). Based on a meta-analysis of data from 47 studies comprising 25,157 women with OC and 81,311 women without OC, the relative risk for ovarian cancer differed according to use of MHT. Thus, the relative risk of ovarian cancer per 5 kg/m² increase in BMI was 1.10 (95% CI 1.07- 1.13) in never users of MHT and 0.95 (95% CI 0.92-0.99) in ever users of MHT (Collaborative Group on Epidemiological Studies of Ovarian, 2012). Women with increased BMI among MHT never users have higher risk of developing this disease. Obesity appears to increase risk of the less common histological subtypes of ovarian cancer, but it does not increase risk of high-grade invasive serous cancers. Besides, reducing BMI is unlikely to prevent the majority of ovarian cancer deaths (Olsen et al., 2013). More recently, the relationship of obesity and ovarian cancer survival was studied on original data from 21 studies, which included 12,390 women with ovarian carcinoma. It appears that higher BMI is associated with adverse survival among the majority of women with ovarian cancer (Nagle et al., 2015).

1.3.7 Smoking

In 2009, the International Agency for Research on Cancer added mucinous ovarian tumors to their list of tobacco-related cancers. A pooled analysis of 21 case-control studies (19,066 controls, 11,972 invasive and 2,752 borderline cases) demonstrated that current cigarette smoking increased the risk of invasive mucinous (odd ratio (OR) =1.31; 95% CI: 1.03-1.65) and borderline mucinous ovarian tumors (OR=1.83; 95 % CI: 1.39-2.41), while former smoking increased the risk of borderline serous ovarian tumors (OR=1.30; 95% CI: 1.12-1.50) (Faber et al., 2013). But no convincing

associations between smoking and risk of invasive serous and endometrioid ovarian cancer were observed.

1.3.8 Protective factors

Parity, breastfeeding, oral contraception as well as gynecologic surgery like tubal ligation and hysterectomy may decrease the risk of developing ovarian cancer. A population-based case-control study showed that parity was inversely associated with risk overall and by tumor behavior and type, with a stronger OR (95% CI) for Type I (0.09 (0.04-0.24) for ≥ 3 full-term births vs. nulliparity) vs. Type II (0.66 (0.43-1.02)) invasive cancers; the OR (95% CI) for borderline tumors was 0.41 (0.22–0.77) (Koushik et al., 2017). Among parous women, per year of combined oral contraceptive use exclusively before the first full-term pregnancy was associated with a 9% risk reduction (95% CI=0.86-0.96) for high-grade serous and endometrioid/clear cell EOC (Cook et al., 2017). More recently, the researchers found that the age adjusted ovarian cancer mortality rate fell in the young women aged 20-49 worldwide. The main reason for the favorable trends is the use of oral contraceptives (OCs), particularly, in the USA and countries of the EU where OCs were introduced earlier (Malvezzi et al., 2016).

Women who underwent tubal ligation and hysterectomy for valid medical reasons have been found to be reversely associated with ovarian cancer. A meta-analysis showed that the summary relative risk (RR) for women with vs. without tubal ligation was 0.70 (95%CI: 0.64, 0.75) and the summary RR for women with vs. without hysterectomy was 0.74 (95%CI: 0.65, 0.84). Furthermore, simple hysterectomy and hysterectomy with unilateral oophorectomy were associated with a similar decrease in risk (summery RR = 0.62, 95%CI: 0.49-0.79 and 0.60, 95%CI: 0.47-0.78, respectively) (Rice et al., 2012). However, in another recent meta-analysis included a total of 40,609 cases of epithelial ovarian cancer and 368,452 controls in 38 publications. The result suggested that tubal ligation was associated with a decreased risk of epithelial ovarian cancer (OR = 0.70, 95% CI = 0.60-0.81) in USA, Asia, Europe and Australia, while hysterectomy show no relationship with epithelial ovarian cancer (OR = 0.97, 95% CI = 0.81-1.14) (Wang et al., 2016). Additional research is needed to confirm whether

tubal ligation and hysterectomy can reduce the chance of developing ovarian cancer risk and their protective capacity differs by individual, surgical, and tumor subtypes and other characteristics.

1.4 Clinical staging

The majority of ovarian cancers are diagnosed at the advanced stage (stage III-IV), particularly for HGSOCs. Table 2 shows the FIGO staging of ovarian cancer (Prat and Oncology, 2015). The late stage discovery is due to non-specific symptoms, such as abdominal swelling, urinary and bowel dysfunction, loss of appetite or feeling full quickly, pain and fatigue (Bankhead et al., 2005; Matsuo et al., 2011). Whereas the non-high-grade serous subtypes are always detected at early stage and thus have better prognosis due to the slower progression (Kobel et al., 2010).

Table 2. 2014 FIGO ovarian, fallopian tube, and peritoneal cancer staging system (Prat and Oncology, 2015)

<u>Stage I</u>	Tumor confined to ovaries or fallopian tube(s)
IA	Tumor limited to one ovary/fallopian tube (capsule intact), no tumor on ovarian/fallopian tube surface, no malignant cells in ascites/peritoneal washings
IB	Tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IC	Tumor limited to one or both ovaries or fallopian tubes, with any of the following:
IC1	Surgical spill
IC2	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
IC3	Malignant cells in the ascites or peritoneal washings
<u>Stage II</u>	Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer
IIA	Extension and/or implants on uterus and/or fallopian tubes and/or ovaries
IIB	Extension to other pelvic intraperitoneal tissues
<u>Stage III</u>	Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes
IIIA	Positive retroperitoneal lymph nodes and/or microscopic extrapelvic peritoneal metastases
IIIA1	Positive retroperitoneal lymph nodes only (cytologically or histologically proven):
IIIA1(i)	Metastasis up to 10 mm in greatest dimension
IIIA1(ii)	Metastasis more than 10 mm in greatest dimension
IIIA2	Microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes
IIIB	Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
IIIC	Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
<u>Stage IV</u>	Distant metastasis excluding peritoneal metastases
IVA	Pleural effusion with positive cytology
IVB	Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)

1.5 Treatment for ovarian cancer

1.5.1 Standard treatment

Primary debulking surgery followed by adjuvant chemotherapy are the standard treatment for ovarian cancer. The debulking surgery typically includes removal of the ovaries, uterus, fallopian tubes, omentum and all visible tumor lesions. The goal of the surgical debulking is to remove gross metastases to a residue no greater than 1 cm². When the diameter of the residual tumor is no more than 1 cm, survival tends to be longer. Debulky tumor masses facilitate to receive adequate levels of chemotherapy and thus influence on the chemosensitivity of residual disease (DiSaia and Tewari, 2001).

Only patients with stages IA and IB, grade 1 cancers can be treated without adjuvant chemotherapy. The remaining early-stage patients and advanced-stage ovarian cancer patients (stage III and IV) are managed with adjuvant chemotherapy to gain better outcomes. The standard chemotherapy for advanced ovarian cancer is the combination of platinum and taxane (Raja et al., 2012; Thigpen et al., 2011).

1.5.2 Development for first-line chemotherapy

In the late 1980s, cisplatin was established as the primary first-line chemotherapy for advanced ovarian cancer patients based on several studies comparing cyclophosphamide and doxorubicin with and without cisplatin (Bhoola and Hoskins, 2006). Then in the late 1980s and early 1990s, paclitaxel was introduced to the treatment of ovarian cancer because of the resistance to platinum. Many trials, including GOG (Gynecological Oncology Group) 111 and OV-10 (National Cancer Institute of Canada Clinical Trials Group) showed the paclitaxel and cisplatin arm had a higher overall response rate (77% vs 66%; $p=0.02$) as compared to cyclophosphamide and cisplatin arm. Thus, paclitaxel in combination with a platinum agent has emerged as the new standard of care for the treatment of advanced ovarian cancer (DiSaia and Tewari, 2001). But with the increased the progression-free survival (PFS) and overall survival (OS), the potential neurotoxicity associated with paclitaxel

and cisplatin became a new issue. Carboplatin was identified as a less toxic drug than cisplatin and became widely in the treatment of cancers. The GOG 158 trial and AGO (*Arbeitsgemeinschaft Gynaekologische Onkologie*) OVAR-3 trial showed that carboplatin/paclitaxel can achieved comparable efficacy to cisplatin/paclitaxel regimen but was associated lower frequency of gastrointestinal and neurologic toxicity than the cisplatin/paclitaxel regimen (du Bois et al., 2003; Neijt et al., 2000). Besides, patients treated with carboplatin/paclitaxel showed better quality of life that cisplatin/paclitaxel regimen (du Bois et al., 2003). Figure 2 shows the progression of therapy for advanced EOC.

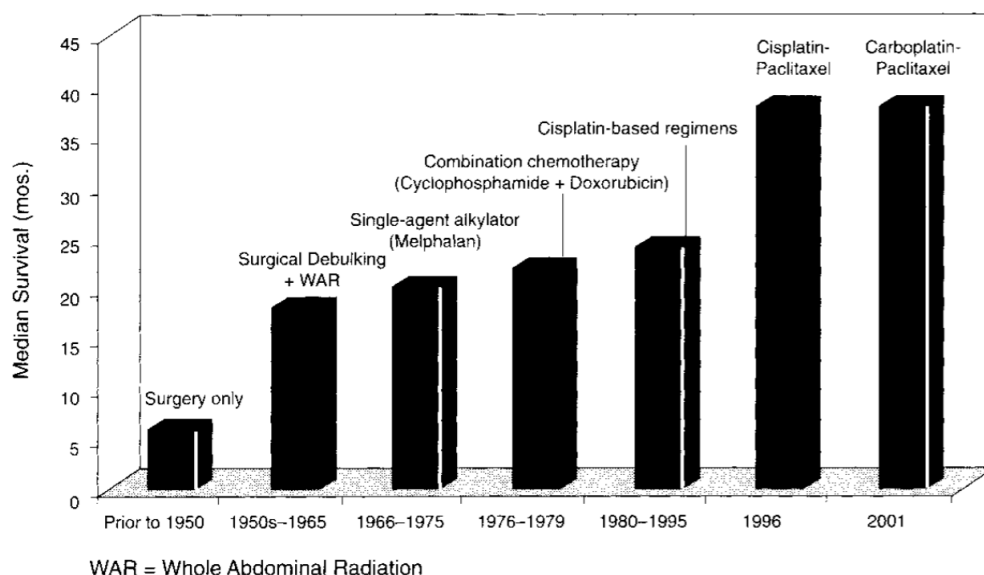


Figure 2. Progression of therapy for advanced epithelial ovarian cancer during the 20th century (extracted from (DiSaia and Tewari, 2001)).

Current first-line chemotherapy is carboplatin (area under the curve, AUC 5-7.5 over 1h)-paclitaxel (175 mg/m² over 3h) administered intravenously at every 3 weeks for 6 cycles. A weekly paclitaxel schedule combined with carboplatin every 3 weeks might be an option for the first-line treatment of ovarian cancer patients. In a Japanese phase 3 trial (JGOG 3016), patients with stage II–IV ovarian cancer were randomly assigned to receive six cycles conventional treatment (carboplatin area under the curve (AUC), the plasma concentration/time curve) 6 mg/mL per min and paclitaxel 180 mg/m² on

day 1) or six cycles dose-dense treatment (carboplatin AUC 6 mg/mL per min on day 1 and paclitaxel 80 mg/m² on days 1, 8, and 15). Median PFS was significantly longer in the dose-dense treatment group (28.2 months, 95% CI 22.3-33.8) than in the conventional treatment group (17.5 months, 95% CI 15.7-21.7); Median OS was 100.5 months (95% CI 65.2- ∞) in the dose-dense treatment group and 62.2 months (52.1-82.6) in the conventional treatment group (Katsumata et al., 2013).

In another phase 3 study from 67 institutions in Italy and France (MITO-7), 822 women with FIGO stage IC-IV ovarian cancer were enrolled in this study and were randomly allocated in a 1:1 ratio to receive either carboplatin (AUC 6 mg/mL per min) plus paclitaxel (175 mg/m²) every 3 weeks for six cycles or carboplatin (AUC 2 mg/mL per min) plus paclitaxel (60 mg/m²) every week for 18 weeks. Patients with dose-dense regimen showed an increased PFS (median 18.3 months, 95% CI 15.2–20.2 vs 17.3 95% CI 16.8-20.9). The quality of life is assessed by the Functional Assessment of Cancer Therapy Ovarian Trial Outcome Index “FACT-O/TOI” score. FACT-O/TOI scores of dose-dense regimen kept stable after week 1 whereas FACT-O/TOI scores of standard regimen worsened at every cycle (Pignata et al., 2014). This dose-dense treatment prolongs progression-free survival and overall survival for patients with advanced ovarian cancer.

Chemotherapy can also be administered intraperitoneally. This route increased dose intensity delivered to the site of disease, but the penetration through tumor tissue is limited and toxicity is high. Thus most patients still receive iv systemic therapy (Raja et al., 2012).

1.5.3 Neoadjuvant chemotherapy/Interval debulking surgery

A neoadjuvant chemotherapy followed by interval debulking surgery is still controversial. Because the EORTC (European Organisation for Research and Treatment of Cancer) trial concluded that interval debulking surgery by an experienced gynaecological oncologist led to improvement in some patients who had not had initial optimal debulking surgery (poor medical condition, inexperienced surgeon, etc) while

the GOG (Gynaecological Cancer Group) trial showed no advantage to patients whose primary surgery included maximal surgical effort to remove disease by a gynaecological oncologist (Vergote et al., 2011).

1.5.4 Treatment for recurrent ovarian cancer

The current first-line chemotherapy yields response rates of more than 80% and results in a complete response rate of 40-60% in advanced ovarian cancer. However, more than 90% of these patients relapse after 2 years (Agarwal and Kaye, 2003). Recurrent patients in most cases becomes incurable so the goals of treatment for recurrent ovarian cancer are to prolong survival, to control disease-related symptoms, to minimize treatment-related symptoms and to maintain or improve quality of life (Jelovac and Armstrong, 2011). Recurrent patients are commonly characterized as platinum sensitive or platinum resistant.

1.5.4.1 Treatment for platinum sensitive recurrent disease

Platinum-sensitive recurrence is defined by the relapse after 6 or more months after the complete initial chemotherapy. These patients commonly respond to platinum retreatment with increasing response rates. Secondary cytoreductive surgery can be considered for patients who recur after an at least 6-month disease-free interval (DFI). When selecting patients for secondary cytoreduction, the most significant preoperative factors are the DFI, success of a prior cytoreductive effort and the number of recurrent sites (Chi et al., 2006; Jelovac and Armstrong, 2011). Once secondary cytoreductive surgery is attempted, the most important factor for improved survival is optimal cytoreduction (diameter less than 1 cm) (Tebes et al., 2007).

Platinum-based combination therapy has become the standard of care for patients relapsing more than 6 months after initial therapy. Based on 3 randomized trials, the main treatments are the combination of carboplatin with paclitaxel, gemcitabine or pegylated liposomal doxorubicin (PLD). The first study is ICON4/AGO-OVAR-2.2 trial, comparing platinum single agent (mostly carboplatin) with paclitaxel plus platinum (mostly paclitaxel plus carboplatin). It showed an improvement in response rate (54%

vs 66%), time to disease progression (9 months vs 12 months), 1-year PFS (40% vs 50%), median survival (24 months vs 29 months), and 2-year survival (50% vs 57%) for the combination arm as compared to single agent (Parmar et al., 2003). The second study from the intergroup trial of the AGO-OVAR, the NCIC CTG, and the EORTC GCG compared carboplatin plus gemcitabine, an antimetabolite, with carboplatin alone. Median PFS was 8.6 months (95% CI, 7.9 to 9.7 months) for gemcitabine plus carboplatin and 5.8 months (95% CI, 5.2 to 7.1 months) for carboplatin. Response rate was 47.2% (95% CI, 39.9% to 54.5%) for gemcitabine plus carboplatin and 30.9% (95% CI, 24.1% to 37.7%) for carboplatin ($P=0.0016$). Gemcitabine plus carboplatin significantly improves PFS and response rate without worsening quality of life for patients with platinum-sensitive recurrent ovarian cancer (Pfisterer et al., 2006). More recently, CALYPSO study, comparing the carboplatin/PLD and carboplatin/paclitaxel combinations, showed the carboplatin/PLD more effective (median PFS 11.3 vs 9.4 months), but to exhibit distinctly different toxicity profiles. Patients receiving the PLD/carboplatin arm experience less alopecia and neuropathy, but instead of more thrombocytopenia, mucositis and hand-foot syndrome. (Pujade-Lauraine et al., 2010).

For platinum sensitive recurrent patients, the choice of the chemotherapy depends on the individual's previously experienced toxicities and side effects of respective combination.

1.5.4.2 Treatment for platinum resistant recurrent disease

Generally, platinum resistant disease will be treated with sequential single agents rather than combination therapy. Table 3 lists the common used chemotherapy agents for the treatment of platinum resistant recurrent ovarian cancer (Jelovac and Armstrong, 2011).

Table 3. Common chemotherapy regimens in recurrent platinum-resistant ovarian cancer

Topotecan daily × 5 d, every 3 wk
Topotecan wkly on d 1, 8, and 15, every 4 wk
Pegylated liposomal doxorubicin every 4 wk
Gemcitabine on d 1 and 8 every 3 wk OR d 1, 8, and 15 every 4 wk
Etoposide orally 14/21 d or 14-21/28 d
Paclitaxel wkly on d 1, 8, and 15 every 4 wk OR d 1, 8, 15, and 21 every 4 wk
Docetaxel every 3 wk

The weekly topotecan regimen has been reported 9%-20% response rates. The weekly topotecan shows much less myelosuppression and alopecia than the “daily × 5” regimen, and is better tolerated as treatment in more heavily pretreated patients (Abushahin et al., 2008; Gordon et al., 2001; Spannuth et al., 2007). Pegylated liposomal doxorubicin and gemcitabine show similar efficacy with topotecan based on the comparisons in several trials (Ferrandina et al., 2008; Gordon et al., 2001; Muggia et al., 1997). Oral etoposide has significant activity in platinum-refractory ovarian cancer, with documented response rates from 6% to 32%. However, it is not commonly used, possibly because of secondary hematologic malignancies associated with its use (Jelovac and Armstrong, 2011). Weekly paclitaxel and docetaxel are all active in platinum- and paclitaxel-resistant ovarian cancer but in view of significant hematologic toxicity of docetaxel, further study is warranted to ascertain its optimal dose and schedule (Gynecologic Oncology et al., 2006; Rose et al., 2003). Hormonal therapy and anti-angiogenesis agents which will be discussed in the following paragraph can also be used in the treatment for platinum-resistant recurrent ovarian cancer.

1.5.5 Targeted therapy

Anti-angiogenesis agent and PARP (poly ADP ribose polymerase) inhibitors have been shown active in the randomized clinical trials in the treatment for ovarian cancer patients. They can be used alone or in combination with chemotherapy.

1.5.5.1 Anti-angiogenic therapy

Angiogenesis (the formation of new blood vessels) provide nutrients and facilitate tumor cell growth and metastasis for tumors beyond a certain size. Angiogenesis is crucial for follicle maturation and the development of corpus luteum during normal ovulation, and the levels of angiogenic markers such as vascular endothelial growth factor A (VEGFA) change during the menstrual cycle (Yap et al., 2009). Angiogenesis plays an important role in disease progression and prognosis for epithelial ovarian cancer. Multiple studies have demonstrated a direct association of microvessel density and VEGF in primary tumors with extent of disease and with time to progression and death after initial therapy (Burger, 2007).

Benvacizumab, a monoclonal humanized antibody targeting vascular endothelial growth factor (VEGF), is the first US FDA-approved anti-angiogenic therapy drug. In the GOG 218 trial, the use of bevacizumab during and up to 10 months after carboplatin and paclitaxel chemotherapy prolongs the median PFS by about 4 months in patients with advanced epithelial ovarian cancer (Burger et al., 2011). Furthermore, ICON (International Collaborative Ovarian Neoplasm)-7 trial compared chemotherapy plus Bevacizumab with chemotherapy alone and showed that Bevacizumab improved progression-free survival in women with ovarian cancer. The benefits with respect to both progression-free and overall survival were particularly greater among those at high risk for disease progression (Oza et al., 2015; Perren et al., 2011). In addition, bevacizumab combined with chemotherapy also showed to improve PFS and objective response rate (ORR) for platinum-resistant recurrent ovarian cancer. In the AURELIA trial, after investigators selected chemotherapy (pegylated liposomal doxorubicin, weekly paclitaxel, or topotecan), patients were randomly assigned to single-agent

chemotherapy alone or with bevacizumab (10 mg/kg every 2 weeks or 15 mg/kg every 3 weeks) until progression, unacceptable toxicity, or consent withdrawal. The median PFS was 3.4 months with chemotherapy alone versus 6.7 months with bevacizumab plus chemotherapy. ORR was 11.8% with chemotherapy versus 27.3% with bevacizumab containing treatment ($P = 0.001$) (Pujade-Lauraine et al., 2014). Bevacizumab (BV) has also been shown to increase PFS when given together with carboplatin and gemcitabine (GC) in the platinum-sensitive recurrent ovarian cancer. Median PFS for BV+GC was 12.4 months versus 8.4 months for GC+PL (placebo). And the ORR (78.5% v 57.4%; $P < .0001$) and duration of response (DOR, 10.4 v 7.4 months; HR, 0.534; 95% CI, 0.408 to 0.698) were significantly improved with the addition of BV (Aghajanian et al., 2012).

Overall, bevacizumab is an active agent in the treatment of ovarian cancer but the dosing and timing need to be investigated because of toxicities issues, such as proteinuria and hypertension (Burger, 2007). Other anti-angiogenesis strategies like inhibitors targeting receptors of VEGF and other receptor tyrosine kinases have been broadly developed.

1.5.5.2 PARP inhibitors

PARP inhibitors are agents which interfere with DNA single stranded break repair. Patients with *BRCA1/2* mutation are particularly sensitive to PARP inhibitors. The use of PARP inhibitors in the treatment of ovarian cancer is based on the concept “synthetic lethality”. When *BRCA1/2* is mutated and homologous recombination repair pathway is compromised, cells become more dependent upon DNA single-strand repair pathway. PARP is a key enzyme involved in the repair of DNA single-strand breaks (SSBs) using the base excision repair pathway. PARP inhibition results in the accumulation of DNA SSBs, which might degenerate DNA double-strand breaks (DSBs) at replication forks (Farmer et al., 2005; Yap et al., 2009). But cells with aberrant *BRCA* function are prevented from repairing DNA damage, thus increasing cytotoxicity then cell death. This provides the basis for a novel synthetic lethal approach to cancer therapy (Ashworth, 2008). Olaparib (also named AZD-2281) is

most commonly investigated oral PARP inhibitor in the treatment of ovarian cancer. A phase I study investigated the activity of Olaparib as single agent in patients with BRCA deficient ovarian cancer. Responses were seen in all dose level from 100mg twice daily and above. 21 of 46 (46%) ovarian cancer patients with a BRCA mutation responded to treatment, including 8 of 10 (80%) platinum-sensitive patients, 11 of 25 (44%) (Jelovac and Armstrong, 2011). In a randomized phase II trial, olaparib (400mg twice daily) maintenance therapy benefited patients with platinum-sensitive relapsed serous ovarian cancer. Of patients with a BRCA mutation, median PFS was significantly longer in the olaparib group than in the placebo group (11.2 months vs 4.3 months) (Ledermann et al., 2014). In the BRCA1 immunohistochemistry study, BRCA has been shown to correlate with *BRCA* genetic events, and could be an predictive of the response to PARP inhibitors (Garg et al., 2013).

Chapter 2. Platinum-based antineoplastic drugs

2.1 The development of platinum-based drugs

In the 1960s, the antitumor properties of platinum were discovered after several platinum compounds were reported to inhibit cell division (Rosenberg et al., 1965). Then the *cis*-Pt(II)(NH₃)₂Cl₂ (cisplatin, CDDP), one of the tested compounds, showed antitumor effect in sarcoma 180 and leukemia L1210 mice models (Rosenberg et al., 1969). Cisplatin or *cis*-diamminedichloroplatinum (II) was the first approved platinum-containing anti-cancer drug. Cisplatin is employed in the treatment for genitourinary disease (testicular, ovarian, cervix, bladder), head and neck cancer and small cell lung cancer. Following the application in the clinical treatment, a number of side effects were confirmed, including nausea and vomiting, renal dysfunction, neurotoxicity and ototoxicity (Ho et al., 2016). For the sake of enhanced therapeutic index or reduced toxicity, thousands of analogues were synthesized and tested. About 28 analogues were evaluated in clinical trials, but only carboplatin and oxaliplatin have achieved wide approval (Pasetto et al., 2006). Figure 3 shows the chemical structures of clinically approved and marketed platinum anticancer drugs (Johnstone et al., 2016).

Chart 1. Chemical Structures of Clinically Approved and Marketed Platinum Anticancer Drugs

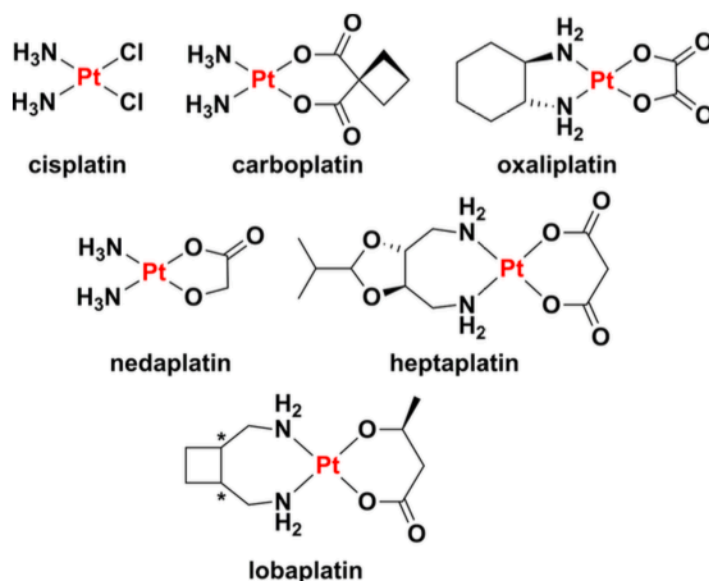


Table 1. Clinically Approved Platinum Anticancer Agents

Generic Name	Research Name	Trade Name	Approval Granted	Scope of Approval
Cisplatin	CDDP	Platinol	1978	Global
Carboplatin	JM8	Paraplatin	1989	Global
Oxaliplatin	I-OHP	Eloxatin	2002	Global
Nedaplatin	254-S	Aqupla アクプラ	1995	Japan
Heptaplatin	SKI 2053R	SunPla 선플라	1999	Korea
Lobaplatin	D-19466	洛铂	2010 ^a	China

Figure 3. The chemical structures of clinically approved and marketed platinum-based anticancer drugs (extracted from (Johnstone et al., 2016)).

2.2 The characteristics of carboplatin

Carboplatin (1,1-cyclobutanedicarboxylato) platinum (II)) is the second generation of platinum-based chemotherapy drug. It is one of the most common used drugs for the treatment of ovarian cancer. Carboplatin is developed by replacing the chloride leaving groups of cisplatin with 1, 1-cyclobutanedicarboxylato ligand, which increases the stability of the leaving groups. Consequently, the ready-to-use infusion solution of

carboplatin is quite stable, with a self-life of 2 years (Di Pasqua et al., 2012). The pharmacokinetics of carboplatin is influenced by its chemical properties, such as higher molecular weight, higher aqueous solubility and lower hydration rate as compared to cisplatin (Los et al., 1991). As a consequence, a higher dose is necessary to achieve comparable anti-cancer effects. Generally, the therapeutic dose of carboplatin is four fold to the dose of cisplatin. Cisplatin appears to be superior to carboplatin on the therapeutic effectiveness (germ cell tumors, bladder cancer, head and neck cancer) whilst for others, like lung cancer and ovarian cancer, the effectiveness is comparable (Lokich and Anderson, 1998). As a derivative of cisplatin, carboplatin has the similar mode of action with cisplatin but is considered to have a reduced neuropathy, nephropathy, nausea and vomiting (Adams et al., 1989). However, the major toxic effect of carboplatin is myelosuppression, characterized by thrombocytopenia (particularly frequent and severe), granulocytopenia, and anemia (Dunton, 2002; Lokich and Anderson, 1998).

2.3 The mechanism of action for platinum-based drugs

Cisplatin and carboplatin are most widely used platinum-based chemotherapeutic drugs. Both of them are non-cell cycle specific alkylating agent. Carboplatin exerts anticancer effect via the similar mechanism as its predecessor, cisplatin. Generally, they function through the formation of DNA lesions followed by the response to DNA damage and the induction of apoptosis. Figure 4 shows the general mode of action of platinum salt-based drugs.

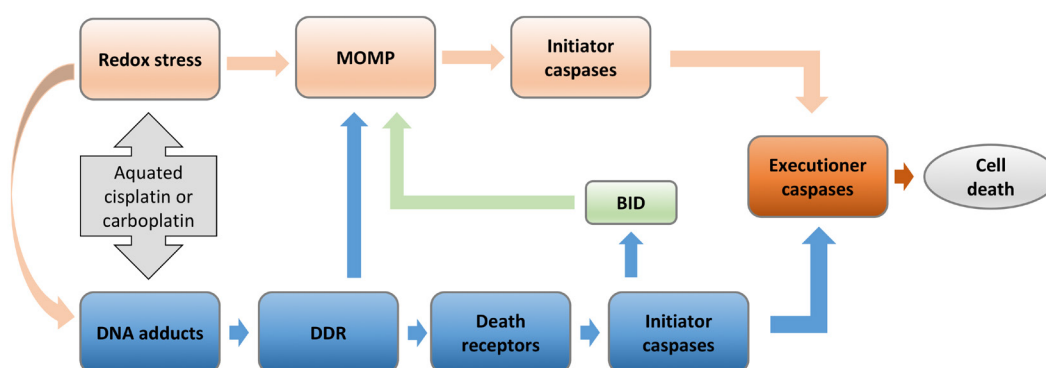


Figure 4. Mode of action of platinum salt-based drugs. Aquaated forms bind to DNA and generate Pt-DNA adducts, followed by the DNA repairing process. DNA damage response is activated if the damage is beyond repair and apoptosis is triggered from both extrinsic and intrinsic pathways. Mitochondrial outer membrane permeabilization (MOMP) is essential for caspase activation and apoptosis. Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8-mediated cleavage of BCL-2 homology 3 (BH3)-interacting domain death agonist (BID; a BH3 domain-only protein), leading to BID activation and MOMP (Tait and Green, 2010). In the cytoplasm, the interaction between cisplatin and glutathione, metallothioneins or mitochondrial proteins results in the depletion of reducing equivalents and/or directly sustains the generation of reactive oxygen species (ROS). ROS can directly trigger MOMP or exacerbate cisplatin-induced DNA damage, thereby playing a dual role in cisplatin cytotoxicity (Galluzzi et al., 2012).

2.3.1 Cellular uptake and intracellular metabolism

The cytotoxic effect induced by platinum complexes is strongly related to the quantity of platinum accumulated in cells. The decreased drug accumulation is one of frequent alterations in platinum-resistant cells. Figure 5 shows the drug uptake for carboplatin.

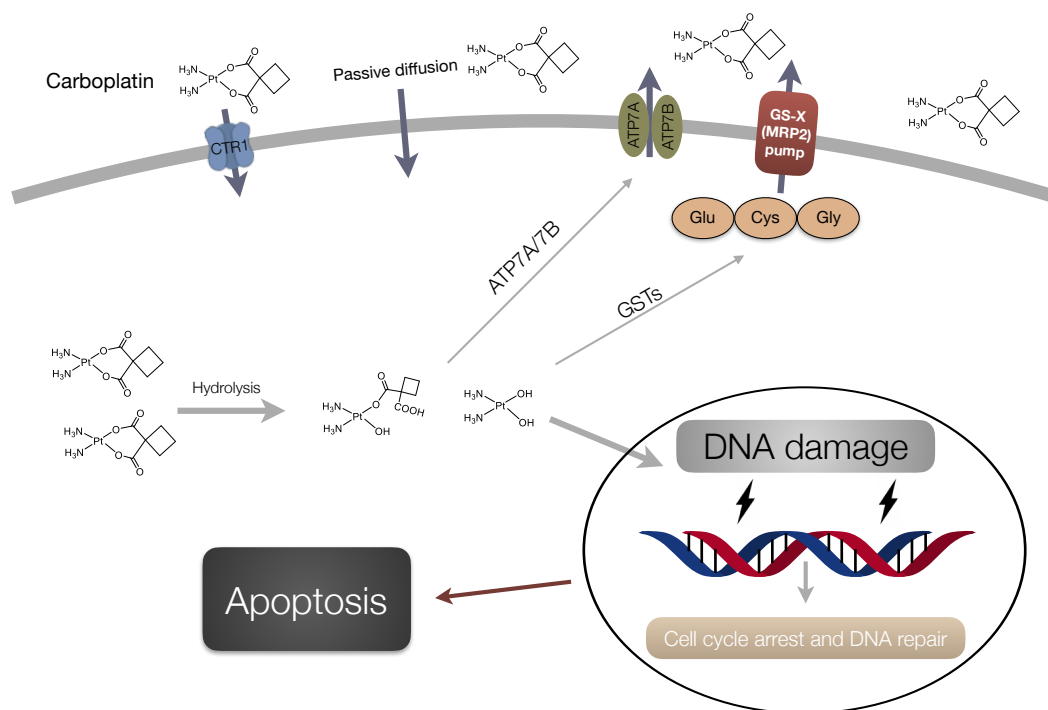


Figure 5. Cellular regulation of drug influx and efflux of carboplatin.

2.3.1.1 Drug influx

To date, platinum-based agents enter into cells through passive diffusion (Ghezzi et al., 2004) as well as a carrier or receptor mediated transport (Pereira-Maia and Garnier-Suillerot, 2003). CTR1 (copper transporter 1, encoded by *SLC31A1*) is the most extensively studied transporters responsible for the platinum influx. The CTR1 is expressed in all tissues and is key player in the copper homeostasis. CTR1 is a 190-amino acid protein of 28 kDa with three transmembrane domains that forms a stable homotrimer in membranes. The transport of copper by CTR1 is energy-independent but is influenced by temperature, pH, and K^+ concentration (Hall et al., 2008; Howell et al., 2010). CTR1 has been found to mediate the cisplatin and its analogues transport into the cell via creation of membrane pore-like homotrimer (Aller and Unger, 2006). Some evidence showed the association between CTR1 and platinum influx. For example, Hozler et al. have reported that enhanced expression of hCTR1 in the

A2780/hCTR1 cells was associated with an increase of platinum accumulation as compared to control A2780/empty vector cells, demonstrating that the exogenously expressed hCTR1 was functional in altering platinum uptake (Holzer et al., 2004). Deletion of yeast *CTR1* gene was associated with the reduction of platinum levels and decreased drug uptake is likely to be responsible for the lower cisplatin adduct level observed in the *ctr1Δ* mutant and consequently the increased survival (Ishida et al., 2002). Mice homozygous and heterozygous knockout cells for *CTR1* showed reduced cisplatin accumulation compared to wild-type cells (Ishida et al., 2002). The absence of CTR1 causes yeast and mammalian cells resistant to copper, cisplatin, carboplatin, and oxaliplatin, whereas overexpression of CTR1 sensitizes cells to toxic effects of these agents (Howell et al., 2010).

The expression of hCTR1 was significantly associated with an improved chemotherapeutic response. YANG et al. analyzed the expression of hCTR1 in specimens from 54 non-small cell lung cancer patients treated with surgical resection and chemotherapy. The median survival time was 15 months in patients with low tumor hCTR1 expression, but >66 months in patients with high tumor hCTR1 expression at the time of follow-up ($P < 0.001$) (Yang et al., 2015). Yoshida and Ishida also showed that the CTR expression in ovarian cancer cells correlates with chemotherapy response rate and histological subtype (Ishida et al., 2010; Yoshida et al., 2013). A meta-analysis of literatures and datasets carried out by Sun et al. suggested that high CTR1 expression was associated with favorable OS, PFS, DFS (disease-free survival) and TR (treatment response) in cancer patients who underwent chemotherapy with acceptable heterogeneity (Sun et al., 2016). Copper transporters, particularly CTR1, play a critical role in the platinum influx and drug sensitivity, indicating its potential use to predict the platinum sensitivity.

2.3.1.2 Drug efflux

2.3.1.2.1 Copper export transporters - ATP7A/7B

In considering the copper transporter CTR1 described above which is responsible

of copper influx, the role of two copper efflux transporters ATP7A and ATP7B on the drug efflux has been studied widely. ATP7A (Menkes's protein) and ATP7B (Wilson disease protein) are copper-transporting P-type ATPase involved in extruding excess of copper. The mutations in *ATP7A* genes cause Menkes disease while Wilson's disease is due to the mutation in the *ATP7B* gene (Harris, 2000). ATP7A is mainly expressed in the intestine, whereas ATP7B locates in liver, kidney and brain. They are homologous in structure, sharing eight transmembrane domains and ~65% amino acid sequence (Hall et al., 2008). ATP7A/7B contains an ATP-binding domain, eight transmembrane domains which are essential for the formation of the copper transportation channel and a cytosolic domain containing six copper binding sites each include a GMTCXXC motif, at N-terminus. Under normal conditions, ATP7A and ATP7B proteins reside in the trans-Golgi network (see Figure 6). When the copper levels elevated, they re-localize in other sites and export excess of copper outside the cells. ATP7A re-localizes in part from the trans-Golgi network to the plasma membrane. In contrast, ATP7B relocates primarily to intracellular vesicular compartments, presumably involved in the secretory export pathway (Samimi et al., 2004). ATP7A appears to regulate the release of Cu ions at the outer membrane, while ATP7B functions to incorporate Cu into apo-ceruloplasmin in either the endoplasmic reticulum or a Golgi compartment. Alternatively ATP7B forces the release of Cu into the bile via the biliary canaliculi (Harris, 2000).

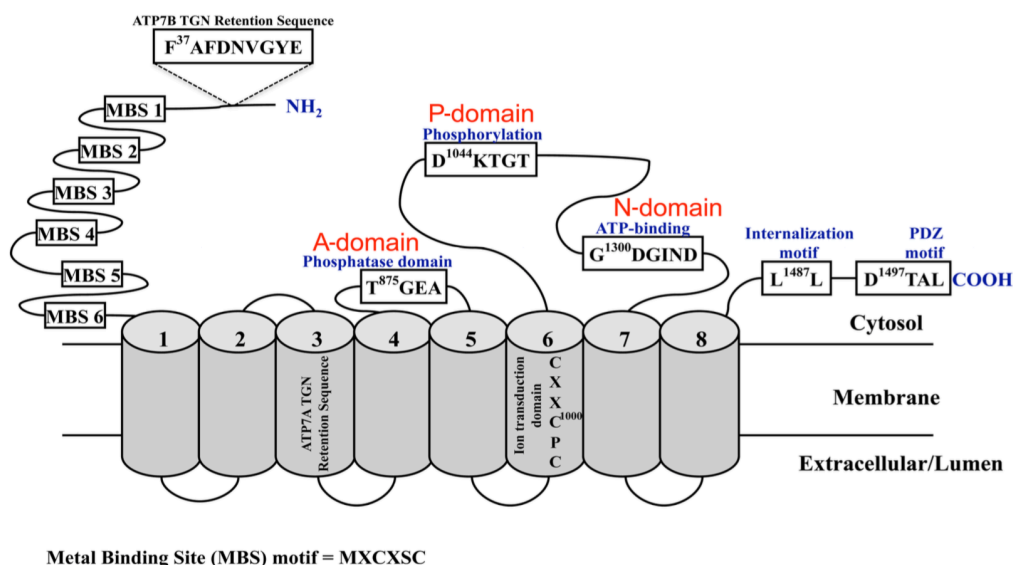


Figure 6. Schematic diagram of the copper-transporting P-type ATPase, ATP7A and ATP7B. Shown are the highly conserved domains: the N-terminal copper-binding domain (MBS 1-6), the phosphatase (A-domain), phosphorylation (P-domain), and ATP-binding (N-domain) domains; and the motifs and sequences required for their localization and trafficking. The cylindrical regions labeled 1-8 represent the transmembrane domains (extracted from (Telianidis et al., 2013)).

Like CTR1, the connection between the platinum accumulation and the expression of ATP7A/7B is demonstrated in many recent studies. ATP7A and ATP7B sequester intracellular Pt and mediate Pt drug efflux *via* a process that involves its transport into vesicles involved in the secretory pathway (Kilari et al., 2016; Safaei et al., 2005a). Komastu et al. showed that ATP7B was overexpressed in cisplatin-resistant prostate carcinoma PC-5 cells but not in the parental PC-3 cells and the revertant PC-5R cells (Komatsu et al., 2000). Samimi and coworkers showed that increased expression of ATP7A in 2008/MNK ovarian cancer cells (2008 cells transfected with ATP7A expressing vectors) mediates resistance to cisplatin, carboplatin and oxaliplatin (Samimi et al., 2004). Katano et al. showed that higher expression of ATP7A/7B protein was detected in three cisplatin resistant ovarian cancer cell lines as compared to cisplatin sensitive parental cell lines (Katano et al., 2002). Similarly, in esophageal squamous cancer cells, ATP7A expression in cisplatin-resistant cell sublines (EC109/cisplatin) was much higher than cisplatin-sensitive cell lines (EC109) at both

mRNA and protein levels. Moreover, ATP7A targeted small interfering RNA duplex added into EC109/cisplatin (cisplatin-resistant cells) cells partially reversed cisplatin-resistance by 37.09 % and hence enhance cell apoptosis (Li et al., 2016b).

In the same study of YANG as mentioned above, the expression of ATP7A/7B was significantly associated with an improved chemotherapeutic response in non-small cells lung cancer patients. The median survival time was found to be 45 months in patients with low ATP7B expression in tumors, whereas it was 20 months in patients with high ATP7B expression in tumors, although the data were not statistically significant ($P=0.162$). Similarly, the median survival time was 20 months in patients with high tumor ATP7A expression, but >66 months in patients with low tumor ATP7A at the time of the follow-up ($P<0.001$) (Yang et al., 2015). In both non-small cell lung cancer xenografts exposed to cisplatin and colorectal cancer patients treated with oxaliplatin, increased levels of ATP7B were associated with Pt resistance (Kilari et al., 2016). Enrichment of cells expressing ATP7A in tumor during cisplatin or carboplatin treatment of ovarian cancers is associated with poor survival (Samimi et al., 2003). Although the expression of copper exporters contributes to the resistance to platinum-based drugs, alterations of its sub-cellular localization are still unclear. The study of Kalayda et al. suggests that changes in the sub-cellular localization of copper efflux transporters may facilitate the sequestration of cisplatin in the vesicular structures of A2780/cis cells (cisplatin-resistant cells). This may prevent drug binding to genomic DNA and thereby contribute to cisplatin resistance (Kalayda et al., 2008). In A2780 cell line, the fluorescent signals corresponding to ATP7A and ATP7B are limited to the perinuclear regions of the cells. On the contrary, in A2780/cis cells the ATP7A and ATP7B proteins are distributed away from the perinuclear region to more peripherally located vesicles in the cytosol. Moreover, the lysosomal compartment in ovarian cisplatin-resistant cells was reduced to just 40% of that in the parental cisplatin-sensitive cells. Enhanced exosomal export was also accompanied by higher exosomal levels of cisplatin export transporters MRP2, ATP7A, and ATP7B (Safaei et al., 2005b). These alterations in the localization of copper transporters ATP7A/7B are associated

with drug resistance.

2.3.1.2.2 GS-X pump

Glutathione S-conjugates (GS-X) pump mediates a detoxification pathway to eliminate the xenobiotics from cells. The release of glutathione S-conjugates from cells is an ATP-dependent process mediated by integral membrane glycoproteins belonging to the recently discovered multidrug-resistance protein (MRP) family (Keppler, 1999). The metabolism and detoxification of xenobiotics consist of three stages: phase I (oxidation), II (conjugation) and III (elimination). In phase I, xenobiotics and endogenous substances are oxidized by cytochrome P450 or Flavin mixed-function oxidase, and phase II deals with the conjugation of phase I products with GSH, glucuronic acid or sulfate mediated by the action of GSH transferases (GSTs). In phase III, the conjugates are transported out of the cytoplasm to the extracellular space or into intracellular compartments (Ishikawa, 1992; Ishikawa et al., 1997).

Some reports suggest that MRP1, MRP2, MRP3 and MRP5 mediate to some extent the platinum drug resistance by increasing drug export (Galluzzi et al., 2012; Stewart, 2007; Young et al., 2001). In particular, MRP2 is intensively studied and is mainly responsible for the increased drug efflux and platinum resistance. MRP2 protein also called canalicular multispecific organic anion transporter 1 (cMOAT) or ATP-binding cassette sub-family C member 2 (ABCC2) are encoded by the *ABCC2* gene. The MRP2 is expressed in the apical membranes of canalicular cells in the liver (Surowiak et al., 2006). The GS-X pump in tumor cells is closely linked to platinum resistance. The increased expression of MRP2 mRNA and protein levels was observed in the ovarian cancer A2780/cis cells (Januchowski et al., 2016; Taniguchi et al., 1996). The overexpression of MRP2 is associated with decreased formation of Pt-DNA adducts and decreased G2 arrest in melanoma cells resistant to platinum (Liedert et al., 2003). More reports reinforced the viewpoint that MRP2 expression levels might correlate with the chemoresponse to platinum-based drugs and clinical outcome (Korita et al., 2010; Surowiak et al., 2006; Yamasaki et al., 2011).

2.3.2 Binding to DNA or other targets

Before the interaction with DNA, all platinating agents are activated by the aquation in the chloride or carbonate buffer. The aquation rate of carboplatin is 2–4 orders of magnitude lower than that of cisplatin. However, only a 4–20 times higher dose of carboplatin is needed to obtain similar cytotoxicity as cisplatin both *in vitro* and *in vivo* (Wang et al., 2012). Carboplatin is activated by aquation spontaneously which undergoes hydrolysis of 1,1-cyclobutanedicarboxylate, becoming positively charged (de Sousa et al., 2014). The aquated form interacts with intracellular nucleophilic molecules including DNA, RNA and protein. It is generally acknowledged that DNA is the preferential and primary target for platinum-based agents (Wang and Lippard, 2005).

When bind to DNA, platinating agents favor the N7 position of purine based of guanosine (G) and adenosine (A) (Rabik and Dolan, 2007). Figure 7 shows the DNA adducts generated by platinating agent. The DNA adducts comprises monoadducts, intrastrand crosslinks (1,2-d(GpG), 1,2-d(ApG), 1,3-d(GpXpGp)), interstrand crosslinks (G-G) and DNA-protein crosslinks. In the condition of cisplatin, the intrastrand crosslink accounts for 80%-90% of total adducts, including 1,2-d(GpG) intrastrand crosslinks (60-65% of all adducts), 1,2-d(ApG) intrastrand crosslinks (20-25%) and 1,3-d(GpXpGp) intrastrand crosslinks (5-10%) respectively (Enoiu et al., 2012; Kelland, 2007). On the other hand, around 2% of adducts is G-G interstrand crosslinks which induce more steric changes on the DNA (Kelland, 2007; Rabik and Dolan, 2007). Some authors described the interstrand crosslink more cytotoxic, because it inhibits the process of DNA replication causing changes that generate errors in replication, with the accumulation of cells in G2/M phase and the induction of apoptosis (de Sousa et al., 2014; Deans and West, 2011; Enoi et al., 2012).

In the case of carboplatin, same adducts can be formed but they show different ratio. The percentages of the carboplatin-DNA adduct after 7-12 h post-incubation in CHO cells, 1,2-d(GpG) (30%), 1,2-d(ApG) (16%), 1,3-d(GpXpGp) (40%), and Pt-G monoadduct (14%), were different from cisplatin-adducts. After 12 h post-incubation,

the number of interstrand cross-links (determined by alkaline elution) were about 3-4% of the total amount of adducts (Blommaert et al., 1995).

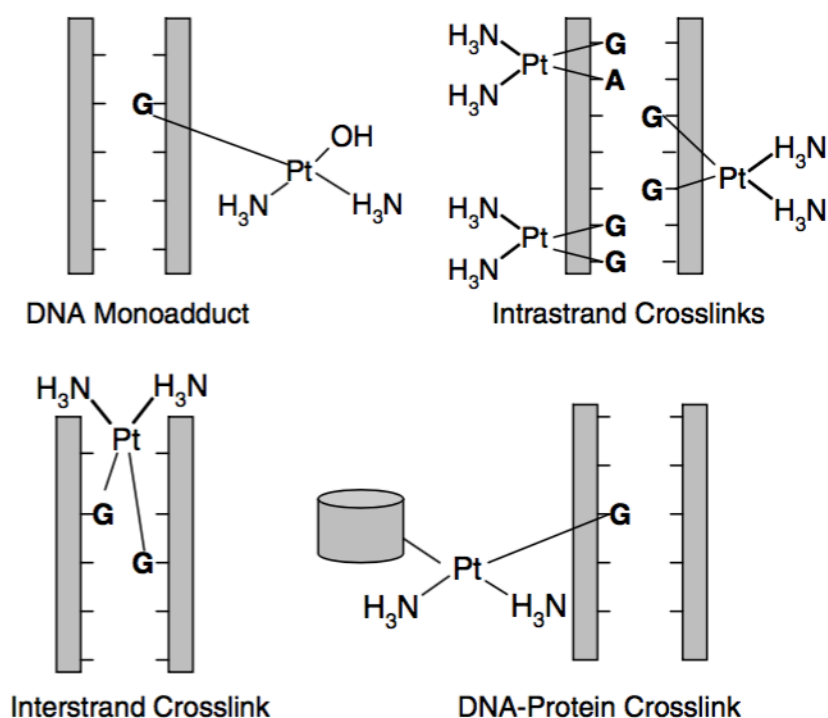


Figure 7. Different types of DNA-protein and DNA-DNA adducts. Platinating agents react with DNA to form monoadducts, intrastrand crosslinks (1,2-d(GpG), 1,2-d(ApG), 1,3- d(GpXpGp)), interstrand crosslinks (G-G), and DNA-protein crosslinks (extracted from (Rabik and Dolan, 2007)).

2.3.3 Cellular processing of DNA lesions

The crosslink distorts the DNA duplex structure. Cisplatin and carboplatin intrastrand crosslinks bend the double helix by 32-35° toward the major groove. Both 1,2-d(GpG) and 1,2-d(ApG) intrastrand crosslinks unwind DNA by 13°, while the 1,3-d(GpXpG) intrastrand lesion unwinds DNA by 34°. Interstrand lesions induce even more steric changes in DNA bending of the double helix toward the minor groove by 20-40°, and extensive DNA unwinding of up to 80° (Jamieson and Lippard, 1999; Rabik and Dolan, 2007). The contortion impedes DNA replication and transcription. The

recognition and repair of carboplatin-induced DNA lesions are complicated and performed by multiple pathways. The fate of the cells depends on the outcome of DNA repair.

2.3.3.1 The involvement of high-mobility group (HMG) box

The DNA damage is recognized by over 20 individual candidate proteins, which binds to distorted DNA induced by platinum salts. The recognition proteins include the hMSH2 or hMutSa component of the mismatch repair (MMR) complex, the nonhistone chromosomal high-mobility group1 and 2 (HMG1 and HMG2) proteins, the human RNA polymerase I transcription 'upstream binding factor' (hUBF), and the transcriptional factor 'TATA binding protein' (TBP) (Siddik, 2003). The phosphatidyl inositol 3-kinase-like kinases ATM (ataxia-telangiectasia mutated), and ATR (ATM- and Rad3-related) are also important sensors for the DNA double strand breaks and stalled DNA replication forks, respectively (Marechal and Zou, 2013; Roos and Kaina, 2013). Mostly the recognition proteins are likely to transduce the DNA damage signals to downstream repair factors. However, the high-mobility group (HMG) box family is an exception. High mobility group (HMG) proteins recognize and bind to DNA at the 1,2-d(GpG) intrastrand crosslinks (Rabik and Dolan, 2007). In breast cancer cells MCF-7, HMG1 overexpression correlates with cisplatin and carboplatin sensitivity (He et al., 2000). The HMG domain consists of an L-shaped arrangement of three α helices with two independent DNA binding surfaces. Binding of a single HMG domain to DNA results in a distortion of the path of DNA through as much as 130° (Jordan and Carmo-Fonseca, 2000). Binding of HMG proteins block the nucleotide excision repair (NER) and transcription factors from repairing DNA lesions, working as a 'shield' or 'camouflage' (Zorbas and Keppler, 2005) for the DNA adducts. Without repairing the DNA, the cells eventually undergo apoptosis.

2.3.3.2 NER repair pathway

The nucleotide excision repair (NER) is an ATP-dependent multiprotein complex that recognizes the bulky DNA adducts induced by intrastrand cross-links and

subsequently removes the segment of DNA that contains the lesion (Ahmad, 2010). The gap that remains is then filled by DNA polymerases using the undamaged single stranded DNA. The 1,2 intrastrand crosslinks are repaired less efficiently than the more distorting 1,3 intrastrand crosslinks, in accordance with the hypothesis that the 1,2 intrastrand crosslinks are more cytotoxic lesion (Ahmad, 2010; Rabik and Dolan, 2007).

There are several proteins participating in NER repair. XPA-G are seven proteins deriving from xeroderma pigmentosum are major proteins involved in NER pathway. ERCC1 is also important excision repair protein and forms the structure-specific endonuclease ERCC-XPF enzyme complex with XPF. NER expression level is associated with the sensitivity to platinum drugs. Testicular cancer, generally very responsive to cisplatin, has low levels of XPA and ERCC1-XPF, providing further correlative evidence for the importance of NER in cisplatin resistance (Rabik and Dolan, 2007).

Increased expression of genes involved in NER is one of the reasons for platinum salts resistance. In non-small-cell lung cancer (NSCLC) patients, ERCC1 mRNA overexpression has been related to poor response and survival in cisplatin treatment (Rosell et al., 2003). Overexpression of ERCC1 is associated with liver fibrogenesis and cancer, indicating that ERCC1 could be related to the resistance of HCC to chemotherapeutics (Fautrel et al., 2005). The expression of the ERCC1 gene was elevated in the cisplatin-resistant ovarian cancer cells, while the levels of expression of ERCC1 in cisplatin-hypersensitive, repair-deficient cells are 30 to 50-fold lower than in inherently resistant cells. In primary ovarian tumors, levels of XPB transcripts were significantly higher in tumors resistant to cisplatin than in tumor samples from patients who responded well to platinum treatment. High ERCC1 expression determined by IHC staining is associated with platinum-resistance in epithelial ovarian cancer (EOC) patients (Du et al., 2016). The cytotoxicity induced by cisplatin was enhanced by 2 to 8 fold in ovarian cancer cell line A2780 by disturbing the NER pathway using ERCC1 antisense RNA (Selvakumaran et al., 2003). These findings propose the inhibition of NER pathway as an adjuvant targeting therapy or an alternative to overcome

chemoresistance.

McNeil et al. showed that two inhibitors against the ERCC1-XPF endonuclease specifically are able to inhibit NER and resensitize melanoma cells to cisplatin and reduce the ERCC1-XPF heterodimer levels in ovarian cancer cells (McNeil et al., 2015). More recently, small molecules inhibitors (SMIs) targeting the protein-protein interaction domain of ERCC1-XPF can reduce the expression of the heterodimer as well as inhibited NER activity. These SMIs against ERCC1-XPF were shown to potentiate cisplatin cytotoxicity and inhibit DNA repair in lung cancer cells. More interestingly, one of compounds named NSC 16168 potentiated cisplatin antitumor activity in a lung cancer xenograft model (Arora et al., 2016).

2.3.3.3 Mismatch repair

The mismatch repair (MMR) system is important for the platinum-induced cytotoxicity because MMR proteins participate the recognition and resolution process (Galluzzi et al., 2012). DNA lesions are recognized by two major heterodimers, MSH2-MSH6 (MutS α) or MSH2-MSH3 (MutS β). Subsequently, MutL α (a heterodimer formed by MLH1 and PSM2) binds to the MutS-DNA complex and activates MutH. Afterward, the strand excision happens in a proliferating cell nuclear antigen (PCNA)-, replication factor C (RFC)-, and ATP-dependent process (Xiao et al., 2014). Rather than a major component to repair the lesions, MMR functions more likely as a detector of different types of adducts. An acknowledged viewpoint is that MMR proteins attempt to repair platinum-adducts, fail, and hence activate the apoptotic signal (Galluzzi et al., 2012; Siddik, 2003). Loss of MMR proteins causes the destabilization of the genome, particularly microsatellite instability (MSI) and reduced apoptosis. As described in previous Chapter, carriers of Lynch syndrome which possesses mutations the hereditary non-polyposis colorectal cancer DNA mismatch repair genes (*MSH2*, *MLH1*, *PMS1* and *PMS2*) have higher risk of ovarian cancer (Aarnio et al., 1999; Guillotin and Martin, 2014; Song et al., 2014). Increasing studies have shown that *MLH1* and *MSH2* are often mutated or under-expressed in the context of resistance to cisplatin and carboplatin, but the loss of mismatch repair does not cause resistance to oxaliplatin or

satraplatin (JM216; bis- acetatoammine- dichlorocyclohexylamine platinum (IV)) (Ahmad, 2010). In the absence of MMR, the drug-induced lesions are not recognized, therefore ATM (Ataxia telangiectasia mutated)/ATR (Ataxia telangiectasia and Rad3-related protein) cannot be recruited and a cellular response is not triggered; thus suggesting that the loss of MMR confers resistance to many chemotherapies (Guillotin and Martin, 2014).

Due to the high predisposition for MMR deficiency to cancer progression and drug resistance, a number of studies investigated the therapy targeting MMR deficiency. One approach is the synthetic lethality targeting MMR deficiency. Inhibition of specific polymerases, including dihydrofolate reductase (DHFR), DNA polymerase β (POL β) and DNA polymerase γ (POL γ), as well as PTEN-induced putative kinase 1 (PINK1), is synthetically lethal with MMR deficiency by causing accumulation of oxidative DNA damage (Xiao et al., 2014). Methotrexate, a DHFR inhibitor, is currently under a phase II trial for MSH2 deficient colorectal cancer (MESH, NCT00952016) (Guillotin and Martin, 2014). More recently, colorectal cancer patients with MMR deficiency showed a better response to pembrolizumab, a PD-1 (programmed cell death protein 1) inhibitor than patients without MMR deficiency (Lynch and Murphy, 2016). The well accepted hypothesis is that MMR-deficient tumors contain greater numbers of tumor infiltrating lymphocytes (TIL) and more neoantigen production generated by frameshift mutations acquired from microsatellite instability (Sloan et al., 2016). Forty-one colorectal cancer patients were recruited in a phase II trial to determine the activity of pembrolizumab (PD-1 inhibitor) in patients with intact or deficient MMR. Patients with deficient MMR had improved response rates compared to those with proficient MMR (78% vs. 40%) and immune-related progression free survival rates were also improved in those with deficient MMR colorectal cancer (11% vs. 0%). Whole-exome sequencing revealed that mismatch repair-deficient tumors carried more somatic mutations than mismatch repair-proficient tumors (1782 vs. 73; $P=0.007$) and high somatic mutation loads were associated with prolonged progression-free survival ($P=0.02$) (Le et al., 2015). Sloan and coworkers showed the increased PD-L1

expression in MMR-deficient endometrial tumors compared MMR-intact, suggesting a potential target and a better predictor of response for PD-1/PD-L1 blockade treatment in endometrial carcinomas (Sloan et al., 2016).

2.3.3.4 BRCA1/2 and homologous recombination

The unrepaired lesion caused by interstrand crosslinks in the replicative phase obstructs DNA replication, leading to replication fork collapse and subsequently, resulting in the formation of double strand breaks (DSBs) (Clingen et al., 2008) (crosslinks shown in Figure 7). DSBs are recognized by proteins that contain both signaling (kinase) and repair activity. The most-important players are ATM and the homologous ATR proteins. Both are activated by DSBs, followed by the phosphorylation of various downstream substrates such as CHEK1 and CHEK2, histone 2AX (H2AX) and BRCA1 (Roos and Kaina, 2006). These proteins regulate cell cycle arrest, repair and the induction of apoptosis. The failure in the repair of DSBs will eventually induce apoptosis. The two major pathways for DSBs repair are homologous recombination (HR) and non-homologous end joining (NHEJ). NHEJ is a common and fast but error-prone pathway by which directly joins together DNA ends without a specific requirement for sequence homology (Neal and Meek, 2011). In contrast, HR is an accurate and error-free recombination event between homologous DNA sequences, using an undamaged DNA template to repair the break (Mao et al., 2008). The choice between NHEJ and HR is regulated by multiple factors. Evidence show that HR is likely to be employed in cells during S and G2 phases of the cell cycle (Neal and Meek, 2011). Fanconi Anemia (FA) and BRCA proteins are critical components involved in DNA double-strand break repair by homologous recombination (HR) repair pathway (Kennedy et al., 2004). As described in the previous chapter, either germline mutations or somatic mutations on BRCA genes are great risks for ovarian cancer. Approximately 50% of high grade serous ovarian cancers exhibit genetic or epigenetic alterations in the FA/BRCA pathway (Cancer Genome Atlas Research, 2011; Konstantinopoulos et al., 2015). Notably, the presence of a germline or somatic mutation in a homologous recombination related gene has been shown to

be extremely sensitive to platinum-based drugs and poly (ADP ribose) polymerase (PARP) inhibitors which is on the basis of synthetic lethal strategy (Konstantinopoulos et al., 2015). The presence of germline and somatic homologous recombination mutations was highly predictive of primary platinum sensitivity ($P=0.0002$) and improved overall survival ($P=0.0006$) (Pennington et al., 2014). However, it has been shown that cisplatin resistance can develop in initially cisplatin-sensitive tumors because of secondary mutations that compensate for BRCA1/2 deficiency and restore the BRCA1/2 function (Lord and Ashworth, 2013). Secondary mutations restoring the *BRCA2* open reading frame seemed to be a dominant mechanism of resistance to platinum compounds and PARP inhibitors in breast and pancreatic cancer cell lines with the *BRCA2* 6174delT frameshift mutation. These findings were extended to cell lines derived from a patient with platinum-resistant ovarian cancer and to platinum-resistant BRCA1- and BRCA2-associated ovarian tumors (Bouwman and Jonkers, 2014).

2.3.4 Apoptosis

The DNA damage response (DDR) is a complicated process. Once sensing the damage, signal is transduction to downstream effectors, either to maintain genomic stability or to elicit cell death if the damage is beyond repair. Platinum-based drugs induce two different modes of cell death: apoptosis and necrosis (Wang and Lippard, 2005). Apoptosis is distinguished from necrosis by the features of cell shrinkage, blebbing of the cell surface, chromatin condensation and DNA fragmentation dependent on the energy supplied by ATP (Lieberthal et al., 1996). Necrosis induced by DNA-damaging anticancer agents was due to the activation of PARP (poly (ADP-ribose) polymerase). PARP is required for DNA repair and its inhibitors are shown to achieve synthetic lethality in BRCA-pathway-deficient tumors (Annunziata, 2011). However, excessive DNA damage caused induces hyperactivation of PARP, which cleaves the glycolytic coenzyme NAD^+ and transfers ADP ribose moieties (ADPR) to carboxyl groups of nuclear proteins, thereby causing NAD^+ /ATP depletion and resulting in necrosis (Fuertes et al., 2003). The choice of the cell death mode depends

on multiple factors, such as the concentration of drugs, the availability of energy, the metabolic condition or the failure of apoptotic pathways. At high doses, platinating agents could damage molecules involved in cellular energy supply and also proteins directly or indirectly involved in the apoptotic process (i.e., p53, Bax, Bcl-2, and caspases), leading to necrotic cell death (Gonzalez et al., 2001). Lieberthal and Levive showed that mouse proximal epithelial tubular cells underwent necrotic cell death over a few hours after treatment with a high concentration of cisplatin (800 μ M), whereas cells underwent apoptosis following exposure to a lower concentration (8 μ M) of the drug and over several days (Lieberthal et al., 1996). Thus, platinum based agents initially induce apoptosis but necrosis could take over either directly or as a consequence of defective apoptosis with dependence on the damage level and ATP status (Fuentes et al., 2003).

2.3.4.1 The typical apoptotic pathway

DNA damage mostly activates the extrinsic death receptor-mediated pathway and/or the intrinsic mitochondrial pathway (see Figure 8). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors results in assemblage of DIS-complex (death-inducing signaling complex) consisting of FADD (Fas-associated protein with death domain) and pro-caspase-8 and -10 which leads to the activation of the downstream effector caspases such as caspase-3 (Fulda and Debatin, 2006; Roos and Kaina, 2013). At the same time, the mitochondria induce apoptosis by the release of several proteins such as cytochrome-c (cyt-c), apoptosis-inducing factor (AIF), Smac (second mitochondria-derived activator of caspases)/DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low isoelectric point), Omi/HtrA2 or endonuclease G (Endo G). The release of cyt-c triggers the activation of caspase-3 through the formation of the cyt-c/Apaf-1/caspase-9-containing apoptosome complex whilst Smac/DIABLO neutralizes the inhibitory effects to the IAPs (Inhibitor of apoptosis proteins), resulting in promoting caspase activation (Indran et al., 2011). On the other hand, Endo G and AIF may act in a caspase-

independent manner to execute cell death (Cregan et al., 2004; Kim et al., 2008).

Crosstalk between the extrinsic and intrinsic pathways occurs through caspase 8-mediated cleavage of BCL-2 homology 3 (BH3)-interacting domain death agonist (BID; a BH3 domain-only protein), leading to BID activation and mitochondrial outer membrane permeabilization (MOMP) (Tait and Green, 2010). MOMP is essential for caspase activation and apoptosis. I will explicate MOMP and its governor in the paragraph below.

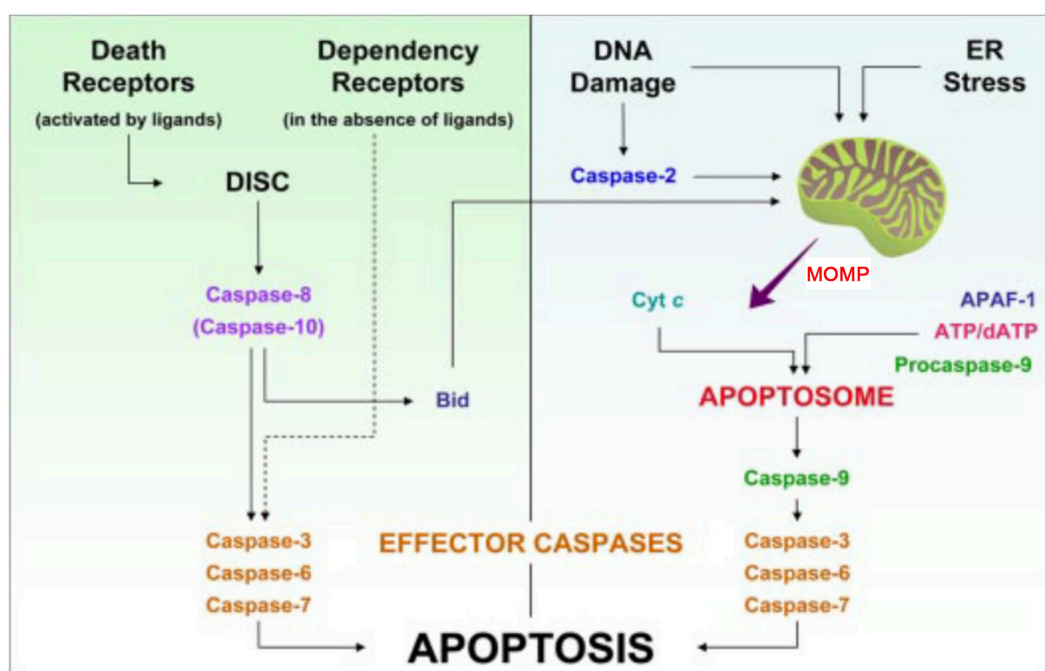


Figure 8. Schematic diagram of extrinsic and intrinsic apoptosis (extracted from(S. and V., 2013)). *Left:* extrinsic pathway. *Right:* intrinsic pathway.

2.3.4.2 Key factors involved in the apoptosis induced by platinum-based drugs

2.3.4.2.1 Role of p53 and p73

p53 plays a key role in the regulation of cell cycle arrest and induction of apoptosis. DSBs activate ATM and ATR which phosphorylate CHEK1, CHEK2 and p53. Upon phosphorylation, p53 becomes stabilized by the prevention of ubiquitination by MDM2 (murine double minute 2 homolog, also known as E3 ubiquitin-protein ligase Mdm2)

and blocks proliferation by upregulation of p21, which triggers G1/S arrest (Roos and Kaina, 2013). It is believed that low levels of DSBs only drive the transcription of the p21 gene, finally causing cell-cycle arrest. With high levels of DSBs, however, p53 accumulates above a particular threshold and, thus, can activate pro-apoptotic genes such as *BAX* (BCL2-associated X protein), *PUMA* (p53 upregulated modulator of apoptosis), *FAS receptor*, *Apaf-1* and *Pidd* (p53-induced protein with death domain) (Roos and Kaina, 2006). Figure 9 shows the p53-mediated apoptotic processes in response to DNA damage.

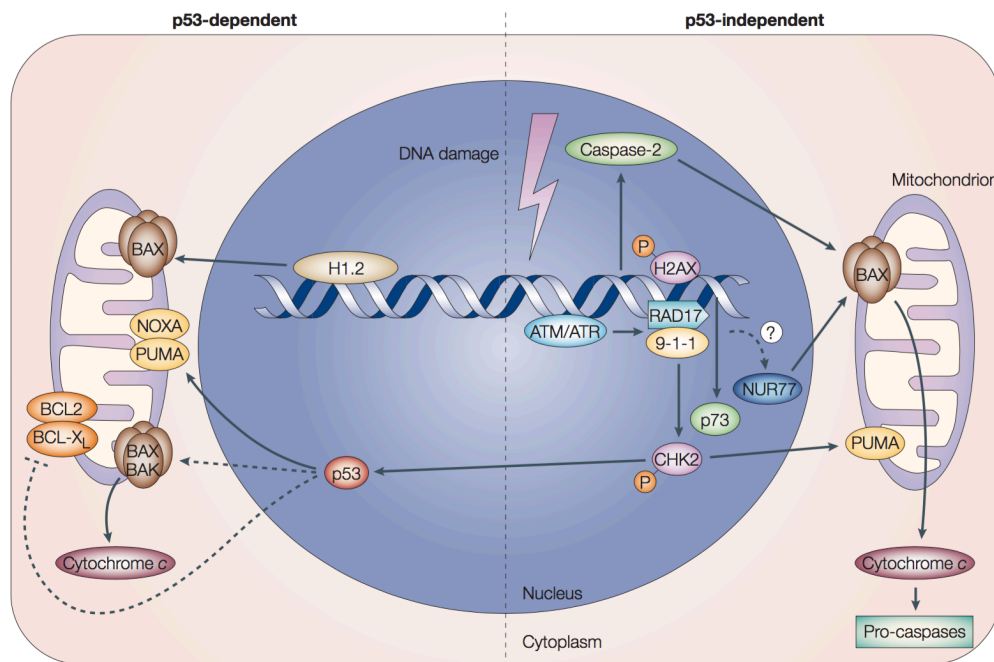


Figure 9. p53-dependent and p53-independent processes in response to DNA damage (extracted from (Zhivotovsky and Kroemer, 2004)). p53-mediated apoptosis might require the transcriptional activation of several genes involved in the extrinsic pathway or the mitochondria (*NOXA* or *PUMA*) to induce apoptosis. Alternatively, extranuclear p53 protein can regulate BCL-2 family mediated by transcription-independent mechanisms and direct physical interactions between p53 and members of the BCL-2 family (dashed arrows). Alternatively, DNA damage can trigger apoptosis by p53-independent routes that might involve caspase-2, NUR77 and p73. Whether the pathway that links DNA damage to apoptosis is p53-dependent or p53-independent, the activation of the mitochondria-mediated pathway is essential for programmed cell death. In the center of the diagram, a series of proteins that are involved in the formation of DNA-damage foci (ATM (ataxia telangiectasia mutated)/ATR (ATM- and Rad3-related), RAD17, gamma H2AX, 9-1-1

and CHEK2 (checkpoint kinase-2)) are linked with both DNA-repair and apoptotic machineries.

In fact, the inactivation of *TP53* occurs in approximately half of human cancers (Hientz et al., 2016). Due to the critical role of p53 in the regulation of cell cycle and apoptosis, p53 deficiency is associated with poor response to platinum-based drugs. Ovarian cancer patients harboring wild-type *TP53* reportedly have a higher probability to benefit from cisplatin-based chemotherapy than patients with *TP53* mutations. Moreover, testicular germ cell tumors, which are particularly sensitive to cisplatin, are one of the few cancers in which *TP53* is rarely inactivated (Galluzzi et al., 2012). However, it has been reported that the apoptosis can be triggered by p53-independent pathway which might involve p73, caspase-2 and p38 MAPKs (Zhivotovsky and Kroemer, 2004). p73 was identified as a p53 homolog, and exhibits structural similarities with p53. Similar to p53, p73 can transactivate p21, and induce cell cycle arrest and apoptosis when overexpressed (Jost et al., 1997). The nuclear translocation of endogenous p73 α in response to DNA damage, which is highly suggestive of a functional pro-apoptotic role for p73 α even in the absence of p53 (Oniscu et al., 2004). In response to DNA damage, ATM/ATR activate CHEK1 and CHEK2, which in turn activate a transcription factor, E2F1 (Urist et al., 2004). E2F1-mediated transactivation of *p73* gene give rise to an increased level of p73 protein. p73-induced apoptosis was shown to be mediated by transcriptional upregulation of *PUMA* (p53 upregulated modulator of apoptosis), which in turn provokes BAX mitochondrial translocation and cyt-c release (Roos and Kaina, 2006). p73 was also shown to activate the p53 target gene that encodes a Bcl-2 homology 3 (BH3)-only member, *NOXA* (Flinterman et al., 2005), which interacts with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9 (Oda et al., 2000; Ploner et al., 2008). The role of caspase-2 and p38 MAPKs in the p53-independent apoptosis will be discussed in the following paragraphs.

2.3.4.2.2 Caspases and inhibitors of apoptosis proteins (IAPs)

The caspases involved in the apoptosis are classified into two groups: the initiator caspases (including caspase-2, -8, -9 and -10) and the executioner caspases (consisting of caspases-3, -6 and -7) (Kitazumi and Tsukahara, 2011). Initiator caspases possess long prodomains that contain one of the two characteristic protein-protein interaction motifs: the death effector domain (DED) or the caspase recruitment domain (CARD) and are involved in interacting with the upstream adapter molecules. The effector caspases with short prodomains perform downstream execution steps of apoptosis by cleaving multiple cellular substrates and are typically processed and activated by upstream caspases (Li and Yuan, 2008). The morphological hallmarks of apoptosis result, including DNA fragmentation and membrane blebbing (Mcllwain et al., 2013). Figure 10 shows the structure of caspases related to apoptosis.

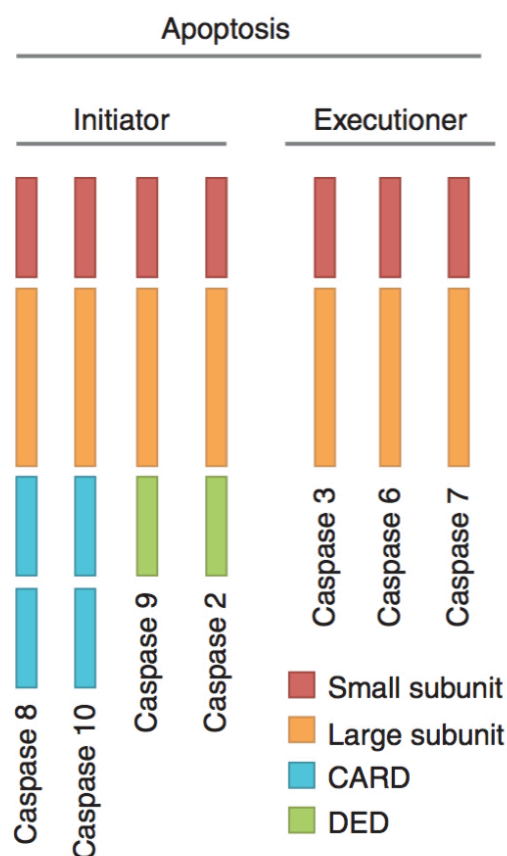


Figure 10. The structure of human caspases related to apoptosis (adapted from (Mcllwain et al., 2013)).

The IAPs are important regulators of apoptosis due to the fact that they inhibit both the intrinsic and extrinsic pathways. Eight human IAP proteins have now been identified although XIAP (X-linked mammalian inhibitor of apoptosis protein) and survivin remain the better-known members (Elmore, 2007). Increased levels of XIAP which is phosphorylated by AKT and thereby stabilized by inhibiting both its auto-ubiquitylation and cisplatin-induced ubiquitylation activities are associated with decreased cisplatin-stimulated caspase-3 activity and apoptosis (Wang and Lippard, 2005). Increased levels of survivin which is frequently upregulated in response to cisplatin by phosphoinositide-3-kinase (PI3K)/AKT1-dependent mechanisms, inversely correlate with cisplatin responsiveness and favorable clinical outcome in gastric esophageal and ovarian cancer and NSCLC patients (Galluzzi et al., 2012).

Caspase-2 appears to be a unique protease sharing the features of both initiator- and effector- caspases, which is associated with DNA damage response, cell cycle regulation and apoptosis (Figure 9) (Aksenova et al., 2016; Puccini et al., 2013). Procaspase-2 consists of a prodomain containing a CARD domain as well as catalytic subunits p19 and p12. Similar to other initiator caspases, caspase-2 activation initially occurs by dimerization, and auto-processing then occurs between the small and large subunits of the catalytic domain yielding a fully active enzyme. Further processing results in removal of the N-terminal CARD, generating a fully mature tetramer (Puccini et al., 2013). Procaspase-2 is the only pro-caspase present constitutively in the nucleus and nuclear import of pro-caspase-2 is regulated by two nuclear localization signals (NLS) in the prodomain (Zhivotovsky and Orrenius, 2005). In response to DNA damage, a protein scaffold known as the PIDDosome, which contains p53-induced protein with death domain (PIDD, also known as LRDD) and an adaptor protein, RIP (receptor-interacting protein) -associated ICH-1/CED-3 homologous protein with death domain (RAIDD, also known as CRADD), has been shown to recruit and activate caspase-2 (Kumar, 2009). The initial stage p53-mediated apoptosis induced by DNA damage occurs by a PIDD- and caspase-2-dependent mechanism (Baptiste-Okoh et al., 2008a). Previous studies have shown that caspase-2 acted the upstream of MOMP

and is responsible for cleavage of Bid (Gao et al., 2005; Guo et al., 2002; Wagner et al., 2004). However, other studies have found that caspase-2 acts downstream of Bax and even caspase-3 and may serve as a substrate of caspase-3, indicating that caspase 2 may play roles both as an initiator caspase and also as a participant in a caspase 3-dependent apoptotic amplification loop (Baptiste-Okoh et al., 2008b; Paroni et al., 2001).

Caspase-2 also participates in the p53-independent pathway. A zebrafish study found that caspase-2 is required in a CHEK1-suppressed apoptotic pathway in p53-deficient cells, indicating that CHEK1 acts as a suppressor of caspase-2 (Sidi et al., 2008). This pathway is dependent on the ATM kinase, which upon activation by DNA damage phosphorylates the PIDD protein, triggering the assemblage of PIDDosome complex, leading to caspase-2 activation (Puccini et al., 2013). Moreover, caspase-2 appears to promote the mitochondria apoptotic pathway either by activating the pro-apoptotic BCL-2 family proteins thereby causing the release of cyt-c from mitochondria or by promoting the release of cyt-c independently of the BCL-2 family proteins via the interaction with anionic phospholipids, especially cardiolipin (Lassus et al., 2002; Roos and Kaina, 2006).

2.3.4.2.3 Sustained JNK and p38 kinase activation

The c-jun amino-terminal kinases (JNKs) and p38 MAPKs respond to environmental stress and chemotherapeutic drugs (see Figure 11). JNKs have a well-documented functional redundancy to phosphorylate their cognate and noncognate substrates, which include c-Jun, JunD, ATF2, c-Myc, p53 and other proteins like BCL-2, BCL-xL, Bad and Bim (Chen, 2012; Verma and Datta, 2012). A large body of evidence showed that sustained activation of JNK and p38 kinase may trigger apoptosis by transcriptional regulation. Sustained activation of JNK and p38 kinase by cisplatin treatment is accompanied by sustained upregulation of AP-1 (activator protein-1). The elevated AP-1 activity stimulates cisplatin-induced apoptosis through transcriptionally activation of gene that encodes *FasL* (Roos and Kaina, 2006), subsequently promoting the apoptosis in a Fas-induced cell death pathway. Mansouri et al. proposed that the

JNK > c-Jun > FasL > Fas pathway plays a major role in mediating cisplatin-induced apoptosis in ovarian cancer cells, and that the duration of JNK activation is critical in determining whether cells survive or undergo apoptosis. They found that JNK and p38 pathways were activated differentially in response to cisplatin, with the cisplatin-sensitive cells (cisplatin-sensitive human ovarian carcinoma cell line-2008) showing prolonged activation (8-12 h), and the cisplatin-resistant cells (2008 resistant sub-clone (2008C13)) showing only transient activation (1-3 h) of JNK and p38 (Mansouri et al., 2003). This difference possibly accounts for the resistance formation. Similarly, Li et al. showed that a prolonged JNK activation was maintained for at least 12 hours in cisplatin-sensitive human ovarian cancer A2780 cells, and only a transient activation (3 hours) was detected in cisplatin-resistant A2780/cis cells in response to cisplatin treatment. More interestingly, the selective stimulation of the JNK pathway by lipofectamine-mediated delivery of recombinant JNK1 reversed chemoresistance in A2780/cis cells, suggesting the potential role of JNK pathway in the chemoresistance (Li et al., 2005).

It has been documented that JNKs is also associated with pro-apoptosis by the regulation of Bcl-2 family members. JNK activated by environmental stress either transcriptionally induces gene expression of *Bim* or phosphorylates and releases Bim, which triggers Bax/Bak-dependent apoptosis (Lei and Davis, 2003). Another potential target of JNK signaling under the stress induced by UV radiation is Bid, also a proapoptotic BH3-only member of the Bcl-2 group, which is proteolytically activated to generate a fragment that translocates to the mitochondria and induces cyt-c release (Tournier et al., 2000).

To date, the role of JNK pathway in apoptosis is still controversial because it is also reported that JNK signaling also contributes to cell survival through inhibiting the phosphorylation of pro-apoptotic proteins (Yan et al., 2016). It is most likely that JNK activation modulates the apoptotic process in a cell type and stimulus dependent manner (Liu and Lin, 2005).

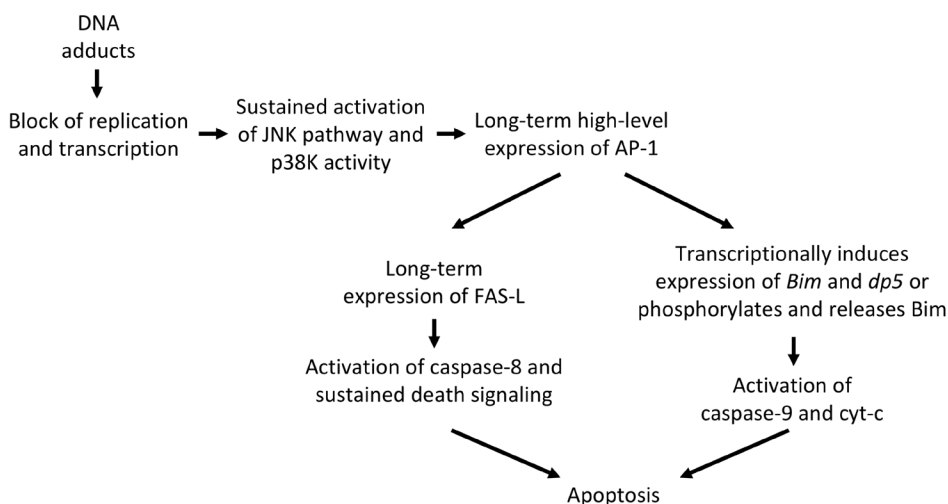


Figure 11. The role of sustained JNK and p38 kinase activation in the apoptosis induced by platinum-based agents (adapted from (Roos and Kaina, 2006)).

2.3.4.2.4 Bcl-2 family and Mitochondrial outer membrane permeabilization (MOMP)

Mitochondrial outer membrane permeabilization (MOMP) is considered a 'point of no return' as this event is responsible for engaging the apoptotic cascade in numerous cell death pathways (Chipuk et al., 2006). MOMP is directly governed by the BCL-2 family proteins and the fate of cells depends on the balance between anti-apoptotic factors and pro-apoptotic factors. B-cell lymphoma-2 (BCL-2)-family comprises three subfamilies that contain one to four BCL-2 homology (BH) domains (see Figure 12). The anti-apoptotic subfamily comprises proteins that contain four BH domains. Most members of this subfamily also contain transmembrane domains (TM) and are therefore typically associated with membranes. The pro-apoptotic BAX-like subfamily lacks BH4 domains and promotes apoptosis by forming pores in mitochondrial outer membranes. The BH3-only subfamily is a structurally diverse group of proteins that only display homology within the small BH3 motif (Taylor et al., 2008). BH3-only proteins (Bid, Bim, Bad), which are induced transcriptionally or post-translationally by cytotoxic stress signals, carry out their pro-apoptotic function either by neutralization of the pro-survival BCL-2 family proteins (Bcl-2, Bcl-xL) or by direct activation of the

pro-apoptotic effectors BAX and BAK (Czabotar et al., 2014).

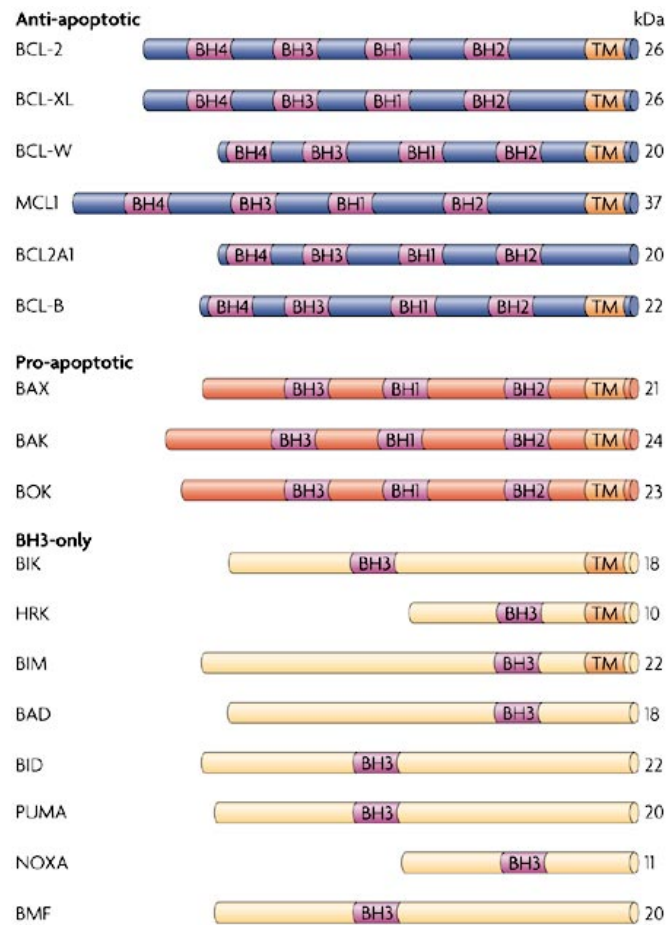


Figure 12. The structure of Bcl-2 family (Taylor et al., 2008)

BCL-2 family plays a critical role in the apoptosis induced by platinum-based drugs. Increasing evidence shows that the dysregulation of BCL-2 family is associated with drug resistance. The elevated levels of the anti-apoptotic counterparts including BCL-2, BCL-xL and MCL-1 (myeloid cell leukemia sequence 1) reportedly correlate with cisplatin resistance and tumor recurrence in multiple clinical scenarios, including head and neck cancer, ovarian cancer and NSCLC (Galluzzi et al., 2012). Beale and coworkers observed a significant inverse correlation between BCL-2 levels and cisplatin sensitivity in 12 parental ovarian cell lines (Beale et al., 2000). PENG et al. investigated the association of BCL2-938C>A (rs2279115) and BAX-248G>A (rs4645878) promoter region SNPs and the clinical responses and outcomes of 235

non-small cell lung cancer (NSCLC) patients treated with platinum-based chemotherapy. They provided evidence that BCL2-938C>A (The BCL2-938C>A A allele was associated with an increase in BCL-2 expression) and BAX-248G>A (The BAX-248G>A A allele was associated with a decrease in BAX expression) were associated with poor progression-free survival and poor response to chemotherapy respectively (Peng et al., 2015).

The stimulation of MOMP could be a promising targeting therapy. One of the most advanced and best-characterized is ABT-737 (BH3 mimetic inhibitor), which predominately binds to BCL-2 and BCL-xL and BCL-w then induces BAX/BAK-dependent killing (van Delft et al., 2006). The ongoing clinical trials are evaluating the combination of cisplatin with ABT-737 and its derivative ABT-263 for the treatment of several neoplasms (Galluzzi et al., 2012). Moreover, ABT-737 reversed the chemoresistance of cancer cells against conventional anticancer agents (Fulda et al., 2010; Oltersdorf et al., 2005). In ovarian cancer cells, apoptosis induction was strongly reinforced when ABT-737 was combined with carboplatin by inducing Noxa and Bim or counteracting MCL-1 (Simonin et al., 2013). Taken together, this strategy of combining ABT-737 with platinum compounds appears to be an attractive option for improving response or reversing resistance to platinum-based agents.

2.4 The rationale for my study

In order to study the contribution of NTS/NTSR1 complex to platinum-based drugs, it is necessary to understand the characteristic and mechanism of platinum-based drugs. Then we verified every process with which the NTS/NTSR1 complex may associate, mainly on the drug uptake, DNA damage response and apoptosis. The details will be discussed in the following chapters.

Chapter 3. The NTS/NTSR1 complex

3.1 Neurotensin

3.1.1 General introduction on the peptide and its metabolism

3.1.1.1 General presentation on neurotensin

Neurotensin (NTS) is a linear tridecapeptide of 1673 Da fortuitously isolated from bovine hypothalamus extracts during purification of substance P (Carraway and Leeman, 1973). NTS was subsequently found in the intestinal extracts of bovines and humans because of the development of the technique of radioimmunoassay (Hammer et al., 1980; Kitabgi et al., 1976). NTS was described as the neuromodulator in the central nervous system (CNS) and as a digestive hormone in the intestines and associated organs.

The human sequence, identical with bovine, dog and rat, presents the following amino acids sequence: pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-COOH (Hammer et al., 1980). The carboxyl terminal hexapeptide (8-13) sequence which is highly conserved among most vertebrates (Rostene and Alexander, 1997), holds the biological and pharmacological properties of the whole peptide (Kitabgi et al., 1985) (See Table 4). It is also the portion of the sequence the most sensitive to enzymatic degradation, which rapidly inactivate the peptide in the bloodstream.

Table 4. Primary structure of neurotensin in vertebrates (adapted from (Rostene and Alexander, 1997)).

Species	Sequence ^a												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Human, cow, dog, rat	pGlu	Leu	Tyr	Glu	Asn	Lys	Pro	Arg	Arg	Pro	Tyr	Ile	Leu
Guinea pig	pGlu	Leu	Tyr	Glu	Asn	Lys	<i>Ser</i>	Arg	Arg	Pro	Tyr	Ile	Leu
Possum	pGlu	Leu	<i>His</i>	<i>Val</i>	Asn	Lys	<i>Ala</i>	Arg	Arg	<i>Val</i>	Tyr	Ile	Leu
Chicken	pGlu	Leu	<i>His</i>	<i>Val</i>	Asn	Lys	<i>Ala</i>	Arg	Arg	Pro	Tyr	Ile	Leu
Alligator	pGlu	Leu	<i>His</i>	<i>Val</i>	Asn	Lys	<i>Ala</i>	Arg	Arg	Pro	Tyr	Ile	Leu
Frog	pGlu	<i>Ser</i>	<i>His</i>	<i>Ile</i>	<i>Ser</i>	Lys	<i>Ala</i>	Arg	Arg	Pro	Tyr	Ile	Leu

^a Includes amino acid sequences predicted from cloned DNA. Italicized residues are changes from human sequence.

3.1.1.2 Biosynthesis and maturation

Like most neuropeptides, NTS is released from a precursor polypeptide of 170 amino acids (AA) (169 AA for rat), which is highly conserved between species. Maturation of pro-NTS leads to the release of two related peptides NTS and neuromedin N (NN) (Lys-Ile-Pro-Tyr-Ile-Leu-OH). Both peptides share a common carboxyl terminal tetrapeptide and similar pharmacological activities (Kitabgi et al., 1992a). NTS and NN peptides are localized in tandem near the carboxyl terminal of the precursor. They are flanked and separated by three dibasic sequences KR (Lys-Arg) (Dobner et al., 1987). These dibasic sequences constitute the consensus recognition sites for cleavage by the endoproteases belonging to the proprotein convertases family (PCs) (Feliciangeli and Kitabgi, 2002; Seidah and Prat, 2012). The three KR sites undergo different tissue-specific endoproteolytic maturation, which generate various combinations of biological products including NTS, NN, and their long fragment, designated as "large NTS" and "large NN" (cleavage C-terminal only) (See Figure 13).

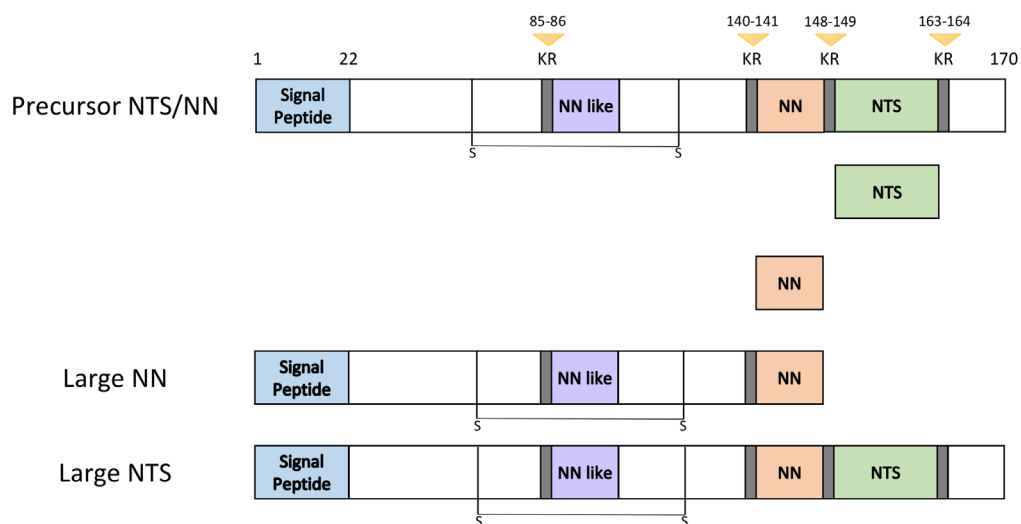


Figure 13. Schematic representation of the structure of NTS/NN precursor and its processing products. Pro-NTS/NN is 170 residues long and starts with a 22-residue signal peptide. The NN and NTS sequences are located in tandem near the C-terminus

of the precursor. The four Lys-Arg (KR) dibasic processing sites are represented on the top. The disulfide bridge between Cys⁶¹ and Cys¹¹⁰ is also represented.

Table 5 shows the cleavage sites and products in different tissues. In brain, the NTS/NN precursor is processed by PC2 to produce mainly NTS and NN (Carraway and Mitra, 1990; Kitabgi, 2006b; Kitabgi et al., 1992b), while in the intestinal tissues, the pro NTS/NN is mainly processed to NTS and a large peptide ending at the C-terminal side with NN sequence, the large NN (Carraway and Mitra, 1990). In the adrenal chromaffin cells, the precursor is processed by PC5-A to generate NTS, as the major products, the large NN, and a large peptide ending with the NTS sequence, "large NTS" (Barbero et al., 1998).

Table 5. The cleavage sites and the major products of the precursor NTS/NN. The extent of cleavage at sites 1, 2, 3, and 4 of pro-NTS/NN is indicated by the number of asterisk (*) as follows: ***major; **intermediate and *minor. The major processing products formed upon cleavage are indicated by pound sign (#) whose number is indicative of the relative amounts of products in each system (adapted from (Kitabgi, 2006b)).

Tissues	Cleavage site				Major products			
	1	2	3	4	NTS	NN	Large NTS	Large NN
brain	*	***	***	***	###	###	##	##
intestine		*	***	***	###	##	#	###
adrenals		*	**	***	###	##	###	###

3.1.1.3 Secretion and plasma level of NTS

In the periphery, the NTS/NN precursor is synthesized and matured in endocrine N cells located in the intestinal mucosa, specifically in jejuno-ileum of all mammals. (Helmstaedter et al., 1977; Kitabgi and Freychet, 1978; Sundler et al., 1977). The peptide is stored in the cytoplasmic secretory granules, clustered along the basolateral portions close to proximity to capillaries (Ferris et al., 1991). The NTS and related peptides are directly released in the portal circulation from these storage sites followed by various stimuli. Among these stimuli, the most powerful is the presence of fatty acids in the intestinal lumen. In human, massive ingestion of fatty acids leads to a significant increase of NTS in plasma, ranging from 2 pM to 26 pM, 15-60 minutes after a meal with high content of fat (Drewe et al., 2008; Ferris et al., 1991; Theodorsson-Norheim and Rosell, 1983). The plasma level of NTS remains high for at least 10 hours after the ingestion of a meal (Gullo et al., 1998). Other factors, such as bombesin or substance P, stimulate more moderately the release of NTS. In rat, these gastrointestinal hormones induced a transient peak of NTS in the portal circulation of conscious rats (Rokaeus et al., 1982). On the other hand, luminal acid or hyperosmolar solutions may reach the enteroendocrine cells to stimulate the release of NTS in rats and the release of NTS to the circulation or to the intestinal lumen may further inhibit gastric acid secretion (Rudholm et al., 2009; Schubert, 2008; Wallin et al., 1995).

3.1.1.4 Catabolism and elimination

Once released by peripheral secreting cells, NTS is subject to proteolytic inactivation by peptidases which terminate the transmission of the messages very quickly. All peptidases target the 8-13 sequence which carries the biological activity of NTS. The three main sites for the peptidases are Arg⁸-Arg⁹, Pro¹⁰-Tyr¹¹ and Tyr¹¹-Ile¹². Using specific inhibitors of metallopeptidase, it has been shown that the cleavages of peptide bonds Arg⁸-Arg⁹ and Tyr¹¹-Ile¹² exclusively result from the activity of metallo-endopeptidase EP 24.15 and neutral endopeptidase (NEP) EP 24.11 respectively, while the cleavage of the Pro¹⁰-Tyr¹¹ peptide bond requires the combined action of enzymes EP 24.11 and EP 24.16. Other exopeptidases also participate in the further

degradation of the breakdown products generated by these endopeptidases. For example, the NT (1–10) fragment produced by both EP 24.11 and 24.16 can be further degraded into NT (1–8) by angiotensin-converting enzyme (ACE) while the NT (9–13) fragment generated by EP 24.15 yielded NT (11–13) through action of a post-proline dipeptidyl aminopeptidase, and the latter fragment was converted to Tyr by aminopeptidase (Checler et al., 1986; Kitabgi, 2006a) (See Figure 14). However, the NTS inactivation profiles differ from one tissue to another mainly because of dissimilarities in enzymatic equipment of each production site.

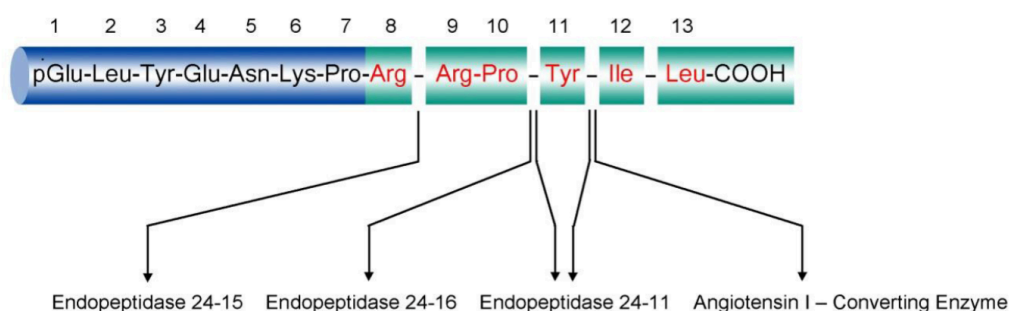


Figure 14. Peptidases involved in the proteolytic inactivation of neurotensin. Green: NTS (8-13) fragment. Arrows point to the peptide bonds cleaved by the peptidase (Extracted from the thesis of Zherui WU in 2015).

NTS is released into the portal circulation from the intestinal mucosa. The liver plays a major role in NTS clearance by the release of a peptidase into the circulation (Brook et al., 1987). In rats, the rate of NTS is high in the portal hepatic-circulation (40 fmol/ml), but not in the hepatic vein and the abdominal aorta. Intestinal release NTS is not correlated with increase in the rate in the general circulation. This hepatic metabolism thus limits the action of the peptide to the small intestine, the liver and the intervening circulation (6 fmol/ml) (Ferris et al., 1991). In addition, kidneys also play an important role in the elimination of the peptide (Shulkes et al., 1984). The clearance of endogenous NTS and its metabolites are particularly greatly reduced in human with chronic renal failure (CRF) or in nephrectomized rats. Isolated kidney perfusion studies show that the NTS metabolism in this bodies mainly the result of filtering mechanisms

and reabsorption (Gillatt et al., 1990; Shulkes et al., 1984). In anesthetized dogs, neurotensin is rapidly catabolized in a vascularly perfused isolated ileal segments with an estimated half-life between 2 and 6 minutes (Barelli et al., 1994); and in humans, it is estimated at about 4 minutes (Blackburn et al., 1980).

3.1.1.5 Potential biological activity of matured and degraded products of NTS

The rapid degradation of the NTS limits the studies of biological processes associated with its long-term effects. To overcome this problem, stable synthetic agonist has been developed. JMV 449, a pseudopeptide analogue of the NTS fragment (8-13) changed. Replacement of both Arg 8 and Arg 9 with lysine and replacement of peptide bond Lys8-Lys9 by CH₂NH did not change the pharmacological profile as compared to the carboxyl terminal hexapeptide (NTS 8-13) and show the same affinity for neurotensin receptors. JMV 449 was slightly more potent than neurotensin on the activities of hypothermic and analgesic in the mice (Dubuc et al., 1992). The binding capacity of JMV 449 is about three times more potent than NTS to mouse brain neurotensin receptors (Lugrin et al., 1991). In addition, the JMV 449 has the same affinity as the NTS (8-13) which makes him an ideal candidate in chronic treatments *in vitro* and *in vivo*.

Pharmacokinetic data demonstrate that the rapid degradation of NTS and low general blood circulation restricts its effects at the production site. Some effects on the distant organs require prolonged action. However, the long forms "large NTS" and "large NN" and the amino terminal metabolites from the degradation of the peptide, NTS (8-13) generally have higher plasma stability and are detectable in the peripheral blood circulation at a higher ratio (Friry et al., 2002). The long molecular forms of the NTS and NN are released into the hepatic portal circulation, concomitantly with the NTS and NN, after exposure to oleic acid-taurocholate (Carraway et al., 1992). Recent data have shown that large NTS and large NN are capable to bind and activate NTSR1, although with an affinity 5 to 10 times lower than the NTS or NN, but they are enable to produce of (IP3) in *in vitro* models (Friry et al., 2002). Besides, large NTS and large NN exhibit more resistant to degradation, in view of the role of NTS/NTSR1 complex

in the tumor progression, they are worthy of being studied distinctly.

3.1.2 The structure and regulation on the gene of neurotensin

3.1.2.1 The gene of neurotensin

The human gene NTS was localized on chromosome 12 and mapped at the band q21 by fluorescence *in situ* hybridization (Bean et al., 1992; Marondel et al., 1996). The NTS/NN gene encompasses 10.2 kb and comprises three introns and four exons, the fourth of which encodes both NTS and NN (Kislauskis et al., 1988). Exon 1 contains only the sequence encoding the signal peptide (AA 1-22), responsible for directing the peptide to the regulated secretory site. Exons 2, 3 and 4 respectively encode for fragments 23-44, 45-119 and 120-169 AA precursor. This characteristic of the gene precludes alternative splicing of the NTS/NN primary transcript to generate an mRNA encoding either NTS or NN alone (Rostene and Alexander, 1997) (See Figure 15).

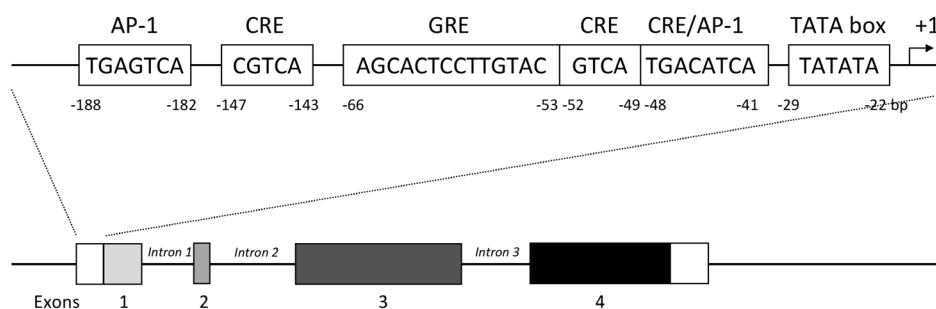


Figure 15. Schematic representation of structure of the promoter (top) and gene (bottom).

3.1.2.2 The regulation of NTS/NN promoter activity

Expression of the gene encoding NTS/NN is mostly restricted to the neuron and the endocrine N cells in small intestine. In rat and human NTS/NN promoter, the key regulatory elements were characterized on the proximal 216 bp upstream of the transcription site (Bean et al., 1992; Kislauskis et al., 1988; Kislauskis and Dobner,

1990). This promoter contains a TATA box, and several regulatory sequences such as AP-1 (Activator Protein 1), GRE (Glucocorticoid Response Element), and a proximal site CRE/AP-1 (TGACATCA pattern) which binds a complex of proteins including the factors c-Jun, JunD and CRE-binding (cAMP response element-binding, CREB), ATF-1 and ATF-2 (Evers et al., 1995a; Wang et al., 2011b) (See Figure 15). All the elements stimulated by various stimuli cooperate together and lead to the concerted transcriptional response of NTS/NN gene to control its tissue-specific expression in the body.

Among the identified regulatory factors, the Ras and Src proteins involved in intestinal differentiation, play a significant role in the regulation of the promoter of the NTS. Since CaCo2-ras cells (human colon cancer line), stably transfection of Ha-Ras, express high levels of human NTS/NN gene. In this cell line the NTS increased gene expression is regulated at transcriptional level. This phenomenon is accompanied by cellular morphological differentiation to a small bowel phenotype (Evers et al., 1995b). In the same vein, Src kinase activates the human NTS/NN promoter through a proximal AP-1/CRE domain, since transfection of Src results in near 8-fold increase of NTS/NN promoter activity while mutation on AP-1/CRE results in sharp decrease of Src-mediated NTS/NN induction through a Raf-dependent pathway (Banker et al., 1997).

Estradiol (E2) also enhances the expression of the NTS in several brain regions expressing ER α receptor in the female rat in despite of the absence of estrogen response element (ERE) in the NTS/NN gene promoter (Alexander, 1993; Alexander and Leeman, 1994). Some evidence showed that estrogen modulated the transcription of NTS/NN gene through activation of the cAMP/PKA pathway. Estradiol induces the accumulation of cAMP, and promotes the phosphorylation of the cAMP response element-binding protein (CREB) in these brain regions. Additionally, estrogen action can be blocked by protein kinase A (PKA) inhibitor, while overexpression of the endogenous PKA peptide inhibitor (heat-stable inhibitor of cAMP-dependent protein kinase) also blocks the effects of estrogen on NTS/NN transcription (Aronica et al., 1994; Watters and Dorsa, 1998).

3.2 The receptors of neurotensin

3.2.1 Highlighting three binding sites of neurotensin

Two classes of NTS binding sites were initially identified using (³H)-NTS and ¹²⁵I-Tyr₃-NTS radio-ligand binding technique. The high affinity site, NTSR1 (K_d = 0.15-0.5 nM), is sensitive to Na⁺ ions and GTP, which decrease the affinity of the NTS. The low affinity site, NTSR2 (K_d = 5-7 nM), is less sensitive to Na⁺ ions and insensitive to GTP. The NTS binding to the low affinity sites can be selectively blocked by levocabastine, an antihistamine-1 drug (Vincent, 1995). These receptors both belong to the superfamily of receptors with seven transmembrane domains coupled with heterotrimeric G proteins.

The third subtype of receptor, NTSR3, exhibits an affinity for NTS between 0.1 to 0.5 nM. (Mazella et al., 1988). The sequencing of this receptor in human revealed an entire homology with the protein gp95/Sortilin. The open reading frame of the NTS/gp95/sortilin cDNA encodes a protein of 833 amino acids containing an N-terminal signal peptide, a putative cleavage site for furin, a long luminal domain, a single transmembrane domain and a short cytoplasmic tail (Mazella et al., 1998; Vincent et al., 1999). NTSR3 is described as a sorting protein between cell surface and intracellular compartments (Mazella, 2001). Table 6 summarizes the characteristics of the three types of neurotensin receptors.

The actions of NTS are mainly mediated through these three receptors. NTSR1 is widely found in CNS, periphery, and in a large variety of solid tumors. NTSR1 is considered as a predominant receptor mediating the effects of NTS specifically on cellular proliferation, migration, and invasion (Kim et al., 2015). The low affinity NTSR2 was shown a localized distribution and its expression has been detected in prostate cancer and B cells (Swift et al., 2010) from chronic lymphocytic leukemia (Saada et al., 2012). In contrast to NTSR1 and NTSR2, NTSR3/sortilin which is not specific to NTS binds various neurotrophic factors and neuropeptides (Nykjaer and Willnow, 2012). The studies on NTSR2 and NTSR3 related to tumor pathology are still too preliminary and lack of supports from literatures. We will focus our discussion only on the periphery

physiology of NTSR1.

Table 6. Summary of characteristics of neurotensin receptor subtypes (adapted from (Kleczkowska and Lipkowski, 2013)).

Characteristic	NTSR1	NTSR2	NTSR3
Size	50-60 kDa	Mouse 2-45 kDa	100 kDa
Classification	G-protein coupled 7-transmembrane spanning regions	G-protein coupled 7-transmembrane spanning regions	gp95/Sortilin Type I amino acid receptor single transmembrane-spanning region
Agonists	<ul style="list-style-type: none"> ■ NTS ($K_d=0.1-0.3$ nM) ■ NN ■ Xenopsin ■ CleasJMV449 	<ul style="list-style-type: none"> ■ NTS ($K_d=3-10$ nM) ■ NN ■ Xenopsin ■ SR48692 ■ SR142948A ■ Levocabastine 	<ul style="list-style-type: none"> ■ NTS ($K_d=0.1-0.3$ nM) ■ Receptor associated protein (RAP) ■ Cleaved sortilin propeptide
Antagonists	<ul style="list-style-type: none"> ■ SR48692 ■ SR142948A ■ D-Trp¹¹-neurotensin 	<ul style="list-style-type: none"> ■ Levocabastine^a 	<ul style="list-style-type: none"> ■ Uncleaved sortilin propeptide ■ SR48692 and SR142948A do not block NT binding in COS-7 cell extracts but block NT effects in cancer cell lines

^a Levocabastine exerts a species-dependent agonist (mouse) or antagonist (human) profile (St-Gelais et al., 2006).

3.2.2 Molecular and biochemical characterization of high affinity NTSR1

3.2.2.1 The structure of NTSR1

The protein sequence of human NTSR1 isolated from colon cancer cell line, HT-29 (Vita et al., 1993) consists of 418 amino acid (46kDa) and possess 84% sequence homology with rat sequence (424 AA, 47 kDa). NTSR1 belongs to the superfamily G-protein coupled receptors. NTSR1 contains seven hydrophobic transmembrane

helices (TM), three extracellular loops (EL), three intracellular loops (IL), a short intracellular carboxyl terminal end and a long extracellular N-terminal end (Tanaka et al., 1990) (See Figure 16).

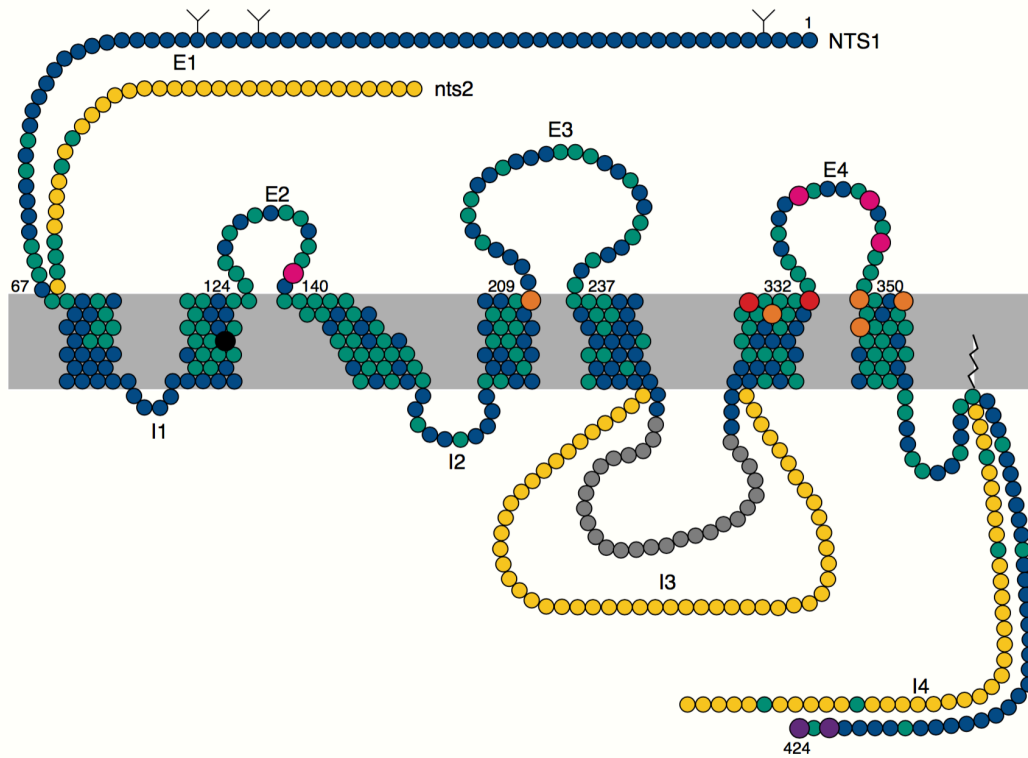


Figure 16. The schematic diagram of neurotensin receptor, NTSR1 (blue). The invariant residues between the NTSR1 and ntsr2 receptors are colored in green. Regions of the ntsr2 receptor that strongly differ from NTSR1 (E1, I3 and I4) are indicated in yellow. The N-terminal (1), C-terminal (424) and the first residues of each extracellular segments of the NTSR1 receptor are numbered. The Na⁺-binding site (Asp113) of NTSR1 is shown in black. Residues involved in the binding of NTS (pink), SR48692 (orange) or both (red) are indicated. Residues crucial for NTSR1 internalization are in violet. The region of the third intracellular loop (I3) that is essential for coupling to phospholipase C is in grey. Glycosylation sites (Y) are highlighted (Extracted from (Vincent et al., 1999). E represents extracellular loop and I represents intracellular loop).

The regions and amino acids involved in ligand binding, signal transduction and receptor internalization were determined by site-directed mutagenesis experiment. The binding of NTS to NTSR1 implicates several amino acids, Arg³²⁷ on TM6 and Trp³³⁹, Phe³⁴⁴ and Tyr³⁴⁷ on extracellular loop 3 (EL3). Mutation on these residues

results in the partially or completely loss of NTS binding (Barroso et al., 2000; Botto et al., 1997; Labbe-Jullie et al., 1998; Pang et al., 1996; Richard et al., 2001). Additionally, three glycosylation sites (extracellular) are reported as essential for the NTSR1 functions. X-ray crystallography studies showed the structure of NTSR1 bound to peptide NTS (White et al., 2012) (See Figure 17). The binding pocket is open on the extracellular surface of NTSR1 with a movement to the internal regions of TM2 and TM6, which is obstructed by a β -hairpin in extracellular loop 2 (EL2) at proximal N-terminal.

The binding of NTS to the extracellular surface of NTSR1 induces the conformational changes of intracellular domains, allowing them to interact with related G proteins. The intracellular loop IL3 has been identified to interact and activate the Gq signalization pathway (Kitabgi, 2002) and the phospholipase A2 (PLA2). While the proximal portion of the intracellular C-terminal end activates Gs and Gi/o (Gailly et al., 2000). The two intracellular domains are connected to extracellular loop by TM6 and TM7, suggesting the binding of NTS to NTSR1 leads to the conformational changes on IL3 and intracellular carboxyl terminal following the movements on adjacent TM6 and TM7.

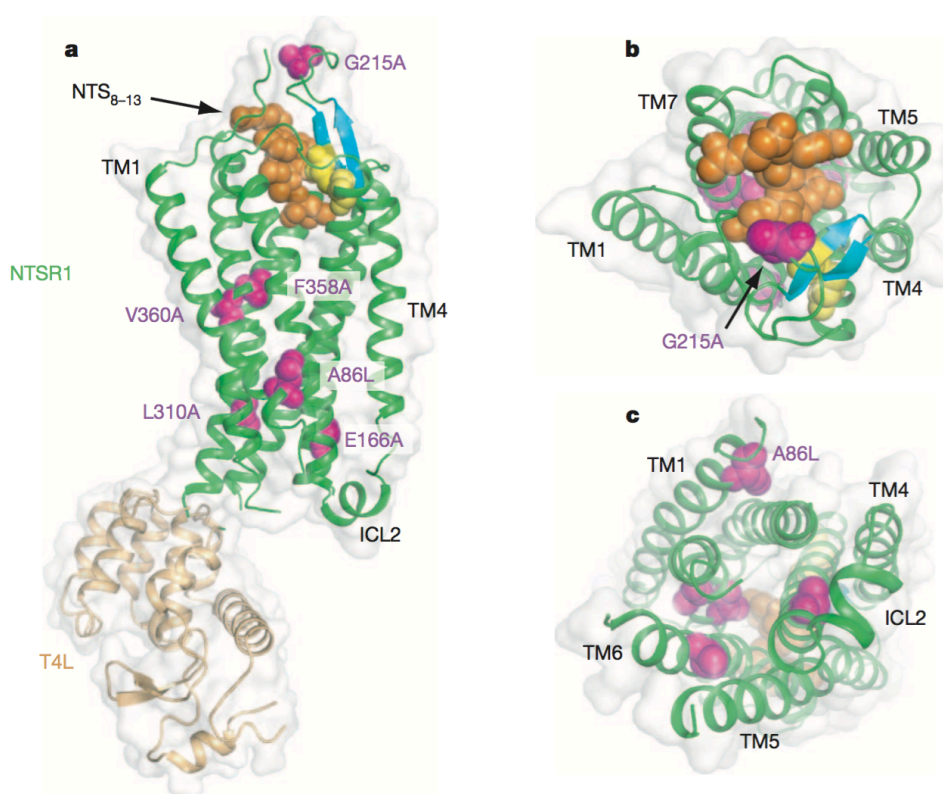


Figure 17. Overview of the NTSR1 structure bound to peptide NTS (8-13) (extracted from (White et al., 2012)). a–c, Cartoon representation of NTSR1-GW5-T4L (NTSR1-GW5 is a thermostable mutant of NTSR1 containing six stabilizing mutation on A86L, E166A, G215A, L310A, F358A and V360A and T4 lysozyme (T4L) was engineered into intracellular loop 3 (ICL3) to improve the probability of obtaining well-diffracting crystals); side view (a), extracellular view (b), intracellular view (c). Space-filling models are used to depict the agonist NTS (8-13) (orange), the side chains of thermostabilizing mutations (purple) and the disulphide bond (yellow) between the conserved residues C142 and C225. Also shown are the β -hairpin in extracellular loop 2 (blue–green) and the π -helix in intracellular loop 2 (ICL2). T4L has been omitted from the intracellular view for clarity.

SR 48692 is a non-peptide antagonist of NTSR1 and compared to NTS, SR 48692 has a lower affinity for NTSR2 than NTSR1 (Gully et al., 1993). This compound potently inhibited ^{125}I -neurotensin binding to high-affinity neurotensin receptor in mouse, rat and human brain (Bozou et al., 1989; Tanaka et al., 1990; Zsuzger et al., 1992). SR 48692 antagonized the effect of NTS on K^+ -evoked dopamine release and NTS-induced Ca^{2+} response (Gully et al., 1993). Interestingly, SR 48692 and NTS bind to a distinct,

but partially overlapping binding site in the receptor (Labbe-Jullie et al., 1998). TM6 and TM7 contain most of the residues that important for the binding of both NTS and SR 48692, close to each other, which might explain the observation that SR 48692 behaves as a competitive antagonist of NTS binding and NTS-induced effects.

3.2.2.2 The gene and promoter

The human gene *NTSR1*, located on the long arm of chromosome 20 (20q13), spans more than 10kb (Laurent et al., 1994). It contains three introns, four exons, and a tetranucleotide repeat polymorphism microsatellite in the 3'-flanking region. The microsatellite polymorphism, locating at less than 3 kb from the poly(A) site, has at least 23 alleles (Le et al., 1997). The initiation site is located at 438 bp from the methionine initiation codon. The 5' regulatory region of *NTSR1* gene consists of 1300 bp, lacking the typical TATA and CAAT box but contains multiple potential Sp1 binding sites which may play a major role in the activation of transcription, as well as a CACCC box which is a crucial cis-regulatory element for the Me₂SO (dimethyl sulfate) induced expression (Le et al., 1997; Tavares et al., 1999).

3.2.2.3 Distribution of *NTSR1* in CNS and periphery

3.2.2.3.1 In the CNS

The *NTSR1* is located mainly in the substantia nigra, ventral tegmental area, amygdala nucleus, striatum and in the cingulate cortex, entorhinal and paraolfactory (Rostene and Alexander, 1997; Sarrieau et al., 1985) (See Figure 18). In the human spinal cord, neurotensin receptors are distributed in the gray matter and the higher density of neurotensin receptors is in lamina II of the dorsal horn (Faull et al., 1989), indicating the role of neurotensin in modulating sensory-motor functions in the human spinal cord.

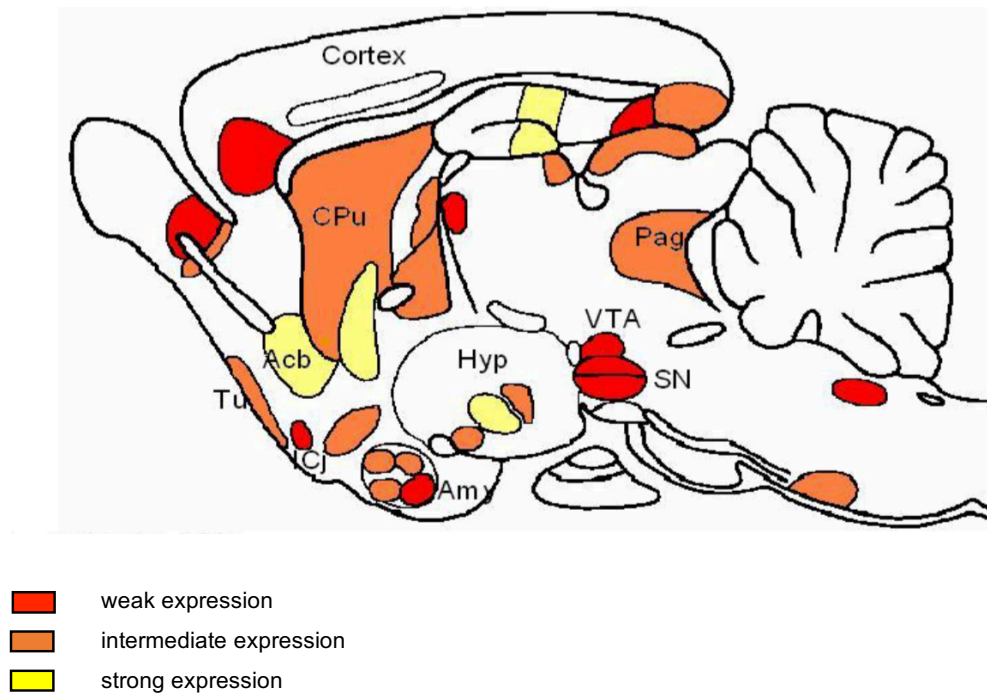


Figure 18. Distribution of NTSR1 in the brain (Sarrieu et al., 1985).

3.2.2.3.2 In the periphery

Neurotensin receptors are present at relatively low concentration in the area where the peptide is found, mainly in the digestive canal especially in the distal part of the jejunum (Kitabgi and Freychet, 1978; Kitabgi et al., 1979). Other sites have been identified, including mast cells, endothelial cells (Schaeffer et al., 1995). Additionally, the NTS binding sites have been determined in rat adrenal gland and the uterus (Goedert et al., 1984; Pettibone and Totaro, 1987), suggesting a direct role of NTS in some cardiovascular effects (Schaeffer et al., 1995) as well as hyperglycemia and hypercholesterolemia (Mitra and Carraway, 1995).

In human, mRNA encoding NTSR1 was initially identified by Northern Blot in the small intestine and in blood mononuclear cells (Vita et al., 1993). More recently, RT-PCR analysis indicated the absence of NTSR1 mRNA in a majority of human tissues including prostate, heart, thymus, ovary and mammary gland (Elek et al., 2000),

whereas it was detected in pancreas, duodenum, colon, spleen and liver of rat tissue (Mendez et al., 1997). However, PCR technique does not determine if it is expressed in epithelial cells of the tissue or in other cell types.

At the subcellular level, NTSR1 is predominantly localized at the membrane level. However, nuclear pools have been demonstrated both within the nucleus and in the nuclear envelope (Toy-Miou-Leong et al., 2004a). The long-term stimulation by NTS seems to induce the accumulation of endocytosed receptor in the peri-nuclear compartment.

3.2.3 Regulation of the membrane expression of NTSR1 and its intracellular trafficking

The interaction between a ligand and a GPCR receptor results in the transmission of extracellular signal to various intracellular mediators. However, to avoid overstimulating target cell, expression of membrane receptor is strictly regulated by respective ligand. Generally, the transmission signal is accompanied by a rapid cellular desensitization including decoupling G protein, followed by sequestration of activated receptor in intracellular compartments causing the transient disappearance of active membrane sites. After a various period of latency, cells undergo a resensitization to the signal by recycling or de novo synthesis of the receptor. In the case of NTSR1 receptor, the regulation depends on the conditions of exposure to the agonist.

3.2.3.1 Cellular desensitization and NTSR1 internalization

Cell desensitization phenomenon is characterized as a marked decrease or loss of responsiveness to the ligand at the effective dose. It functions as a warning for responsiveness to continuous or repeated stimulation. This regulation of NTSR1 has been demonstrated in several cell lines. According to the study in HT-29 cell line, neurotensin-stimulated IP3 production and Ca²⁺ mobilization is desensitized by neurotensin and other agents (Turner et al., 1990). Similarly, pre-incubation of an isolated rat colon segment with 5 nM NTS for 20 minutes, resulted in a significant decrease of the maximal effect of neurotensin while the cellular response to the peptide

is completely abolished with 10 nM NTS, then extensive washout of the tissue recover the response in a time-dependent manner (Mule et al., 1995).

Desensitization results from two mechanisms, like most GPCRs. A major mechanism is phosphorylation of the GPCR followed by the uncoupling the receptor from the G protein. Firstly, decoupling of the receptor and G protein put an end to cell signaling, followed by the internalization of residual receptors which occupy the membrane binding sites to the peptides. The phosphorylation takes place on the specific residues at intracellular loops and carboxyl terminus of NTSR1. In this later, Ser415, Ser417 and Tyr416 are essential for uncoupling G protein while Thr422 and Tyr424 are crucial for the receptor internalization (Chabry et al., 1995; Hermans et al., 1996; Oakley et al., 2001). Phosphorylation of the receptor promotes the binding to the arrestin family proteins, which physically prohibits further coupling to G proteins and targets them to clathrin-coated pits for endocytosis (Kohout and Lefkowitz, 2003; Luttrell and Lefkowitz, 2002). The agents blocking the formation of vesicles, such as hypertonic sucrose, potassium depletion and cytosol acidification are capable to prevent NTSR1 internalization by hindrance of clathrin-coated pits (Vandenbulcke et al., 2000). Furthermore, the complex of β -arrestines-NTSR1 are stable and persistent after the internalization into endocytic vesicles, which slows down the intracellular trafficking of NTSR1 and influence the cell resensitization (Oakley et al., 2001).

3.2.3.2 Intracellular trafficking of NTS-NTSR1 after internalization and resensitization

F. Vandenbulcke et al., had shown the intracellular trafficking of the NTS/NTSR1 complex after internalization of NTSR1. In COS-7 cells transfected with NTSR1 receptor, the ligand remains concentrated in cell periphery, associated with its receptor in the first 5 minutes incubated with NTS. In the following 30 minutes, both ligand and receptor are detected in the early endosomes because of the colocalization with FITC-transferin, acridine orange (low pH indicator) and rab 5A, which are markers for early endosomes. After 30 minutes, fluo-NTS associated with its receptor is shuffled from early endosomes to a compartment where rab 7 colocalizes with syntaxin 6, suggesting that internalized NTSR1 transit through late endosomes and may have a

connection with the TGN (trans-Golgi network). However, studies after prolonged incubation with fluo-NT (45-60 minutes) may implicate the TGN in the further regulation of internalized NTSR1 (Vandenbulcke et al., 2000). Whereas internalized ligand accumulates in the TGN, receptors are targeted to lysosomes in which they are presumably degraded by acid hydrolases rather than recycled (Donato di Paola et al., 1993; Hermans et al., 1997; Turner et al., 1990).

In consequence, cell resensitization to neurotensinergic signal appears several hours after the cessation of the stimulation by agonist and leads to *de novo* synthesis of NTSR1. However, other evidence show that chronic treated with saturated dose of agonist alter trafficking and induce recycling of NTSR1 to the membrane after a transient accumulation in the perinuclear recycling compartment (PNRC) in several cell lines (CHO, CHP212, and N1E-115) (Najimi et al., 1998; Nguyen et al., 2002; Souza et al., 1997; Toy-Miou-Leong et al., 2004b). More recent study shows that the activity of endothelin-converting enzyme (ECE-1) and β -arrestins are crucial for recycling NTSR1 and improving the degradation of NTS (Law et al., 2012). Additionally, the receptor gene transcription is stimulated if the agonist exposure is very intense, persistent or chronic. In doing so, some new synthesized receptors restore the sensitivity and others are stored in sub-membrane pools (Najimi et al., 1998).

Therefore, a prolonged exposure induces cell adaptation, leading to recycling of the receptor to the membrane and constant responsiveness to neurotensinergic signal (See Figure 19). This results in the maintenance of the activation of signals associated with NTSR1 (Ca^{2+} mobilization and MAPK activation) and target genes of NTS, such as tyrosine hydroxylase (TH), the metalloproteinase MMP-9 or Bcl-2 (anti-apoptotic Bcl-2 family member) (Najimi et al., 2002b; Somai et al., 2002; Souza and Forgez, 2006). These genes and its activation generates or amplify the oncogenic effects. In this context, we hypothesized the potential role of complex NTS/NTSR1 in the tumor progression.

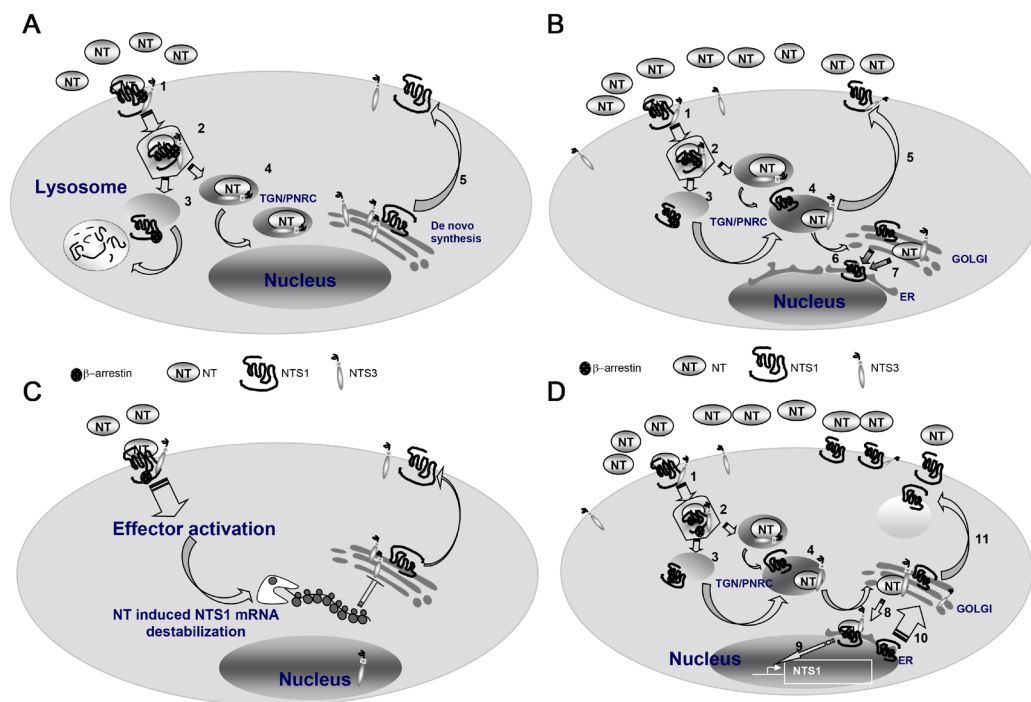


Figure 19. Schematic representation of NTSR1 regulation upon acute and chronic agonist exposure. NT represents neurotensin; NTS1 represents neurotensin receptor 1; NTS3 represents neurotensin receptor 3. (A) and (B): acute exposure; (C) and (D): chronic exposure.

(A) Upon acute agonist exposure NTSR1 is internalized with NTS and NTSR3, and then while NTSR1 is targeted to lysosomal vesicles, NTS and NTSR3 are directed to the TGN/PNRC. Cell resensitization occurs from de novo synthesis receptor a few hours after agonist removal. (B) Acute agonist exposure induced post-transcriptional NTSR1 mRNA destabilization limiting the number of de novo synthesized receptor. (C) Upon chronic and high agonist concentration, NTSR1 is also targeted to the TGN/PNRC compartment, and the nuclear membrane. The NTSR1 traffic through the TGN to reach the nucleus remains hypothetical. NTSR1 accumulated in the PNCR is recycled to the plasma membrane. Permanent cell sensitization to NTS ensues. (D) Signaling within NTSR1 trafficking caused by persistent and high dose of agonist induced NTSR1 gene activation. The consequences included an increase of de novo receptor synthesis, contributing to cell resensitization and NTSR1 accumulation in sub-membrane pools. It is hypothesized that NTSR1 gene activation could be obtained by activation of nuclear NTSR1 by endocytosed NTS transported with NTSR3 (extracted from (Souaze and Forgez, 2006)).

3.3 Physiological effects mediated by NTS/NTSR1

NTS belongs to the neuropeptide family and its location in both CNS and periphery reflects the dual functions of the neuromodulator and endocrine factor. NTS has been implicated in the pathophysiology of several CNS disorders such as, schizophrenia, drug abuse, autism, Parkinson's disease (PD), pain, central control of blood pressure, eating disorders. Meanwhile, it is also associated with cancer and inflammation in the periphery (Boules et al., 2013).

3.3.1 Central functions of NTS

NTS is considered as a modulator to several neurotransmitter systems including dopaminergic, serotonergic, GABAergic, glutamatergic and cholinergic systems. NTS administration intracerebroventricularly or intracisternally are mostly related to locomotor activity, barbiturate sedation, hypothermia, muscle relaxation and analgesia in rats or mice.

1. NTS shares some properties of neuroleptic agents such as decreased locomotor activity and antagonism of amphetamine-induced locomotion (Nemeroff et al., 1977; Van Wimersma Greidanus et al., 1982);

2. NTS induces hypothermia by the interaction of brain dopamine (DA) system and thyrotropin-releasing hormone (TRH) antagonize the hypothermia induced by NTS (Nemeroff et al., 1980; Nemeroff et al., 1977);

3. NTS produces an antinociceptive effect resulting from the interaction with the opioid peptide system (Mazella and Vincent, 2006; Nemeroff et al., 1979);

4. NTS decreases food intake in a dose-related fashion in food-deprived rats (Luttinger et al., 1982b);

5. NTS administration potentiated ethanol and pentobarbital-induced sleep in mice while the tripeptide TRH shows opposite effect (Luttinger et al., 1982a);

6. Neurotensin produces muscle relaxation in the Julou-Courvoisier traction test, which is totally abolished by the i.c. administration of TRH (Osbaahr et al., 1979);

7. NTS reversely regulates maternal aggression, linking NTS to a social behavior (Gammie et al., 2009);

6. NTS central injection sensitizes to the locomotor stimulant effect of amphetamine (Blackburn et al., 2004; Rouibi and Rompre, 2014).

3.3.2 Periphery effects of NTS

In the periphery, the NTS/NTSR1 complex essentially exerts its effects in the gastrointestinal tract and associated glands (pancreas, liver) as well as in the cardiovascular system. It also plays an important role in the inflammatory response.

3.3.2.1 In the gastrointestinal tract

NTS exerts a physiological role in improving the absorption of nutrients by acting on several aspects of digestive function: inhibiting gastric acid secretion, gastrointestinal motility (Degolier et al., 1997) and promoting the translocation of fatty acids from the intestinal lumen to the lymph circulation (Armstrong et al., 1986). More recently, high level of neurotensin was linked to increased fat absorption and obesity and blocking NTSR1 activation decreased intestinal fatty acid absorption and body weight gain in wild-type mice fed a HFD (high fat diet) without altering food intake (Li et al., 2016a).

Neurotensin can stimulate pancreatic enzyme secretion (Harada et al., 1986), biliary secretion (Gui et al., 2000) and also stimulate growth of the exocrine pancreas of the rat (Wood et al., 1988). Neurotensin produced dose-related increases in enzyme output in bile-pancreatic juice of conscious rats when administered acutely, and significant increases in pancreatic weight, DNA, and protein content after chronic administration of the same doses (Wood et al., 1988). Besides, neurotensin has been found to produce hyperglycemia (Carraway et al., 1976) and hypercholesterolemia (Peric-Golia et al., 1979) after iv injection into rats.

In terms of the effects on intestinal smooth muscles, NTS relaxes the rat ileum (Kitabgi and Freychet, 1978) and duodenum (Carraway and Leeman, 1973), contracts

rat proximal colon (Mule et al., 1995) and the guinea-pig ileum (Kitabgi and Freychet, 1978) and colon (Kitabgi and Vincent, 1981).

3.3.2.2 In the cardiovascular system

NTS is involved in a variety of cardiovascular actions including effects on heart rate, myocardial contractility, systemic blood pressure, coronary vascular tone, venous smooth muscle tone, and regional blood flow in gastrointestinal tract, cutaneous and adipose tissue. NTS could trigger cardiovascular reflexes by stimulating primary visceral afferents synaptically connected with preganglionic sympathetic neurons at the spinal cord (Osadchii, 2015). Table 7 shows an overview of the cardiovascular effects of NTS:

Table 7. An overview of experimental studies on cardiovascular responses to neurotensin

NTS action	Species	Administration mode	References
Heart rate acceleration	Rat	Intravenous bolus;	Kerouac et al., 1981;
		Intravenous infusion	Rioux et al., 1982a
	Guinea-pig	Intravenous infusion	Bachelard et al., 1985
Myocardial contraction	Rat	Infusion in perfused heart	Rioux et al., 1984
	Guinea-pig	Intravenous infusion	Bachelard et al., 1987
Blood pressure modulation	Rat	Intravenous infusion	Chahl and Walker, 1981;
			Quirion et al., 1980d;
			Gully et al., 1996;
			K. Kaczyńska, M. Szereda- Przestaszewska, 2012
	Dog	Intravenous infusion	Bauer et al., 1995; Ertl et al., 1993
Coronary vasoconstriction	Guinea-pig	Intravenous infusion	Bachelard et al., 1987;
			Kerouac et al., 1981; Nisato et al., 1994
	Rat	Infusion in perfused heart	Quirion et al., 1980c; Rioux et al., 1984
Coronary vasodilation	Guinea-pig	Infusion in perfused heart	Bachelard et al., 1986
Gastrointestinal blood flow regulation	Dog	Intravenous infusion	Bauer et al., 1995; Ertl et al., 1993
	Human	Intravenous infusion	Fletcher et al., 1985
	Rat	Intravenous infusion	Onarheim et al., 1982
	Cat	Intra-arteria infusion	Harper et al., 1984
	Dog	Intravenous infusion	Konturek et al., 1983

Venous smooth muscle contraction	Rat	Injection in isolated portal vein	Helle et al., 1980; Rioux et al., 1980
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3.3.2.3 The effects on immunomodulation

Recent in vivo evidence supports the view that NTS may be involved in acute and chronic inflammatory disorders, mainly in the intestinal mucosa and associated organs such as the colon, pancreas, stomach, liver, and adrenal gland. The stimulation of neurotensinergic system activates immune cells, such as lymphocytes, macrophages and mast cells. Also, neurotensin has been shown to stimulate several steps of the phagocytic processes including adherence to substrate, chemotaxis, ingestion of inert particles and production of superoxide anion in resting murine peritoneal macrophages (Katsanos et al., 2008). In addition, a pro-inflammatory role of NTS has been described during colonic acute inflammatory response to *Clostridium Difficile* toxin A which mediates pseudomembranous colitis in humans via several aspects, such as colonic secretion, epithelial cell damage, neutrophil infiltration, and colonic mast cell degranulation (Castagliuolo et al., 1999; Madan and Petri, 2012).

3.3.2.4 The effects on tissue repair

In chronic intestinal pathology, NTS stimulates wound repair via direct epithelial cell migration induced through the upregulating of COX-2 and PGE₂ release, which was determined in the models of chronic mucosal ulcerations induced in experimental mice (Brun et al., 2005).

In the CNS, NTSR2 expression is up-regulated during astrocytic reaction and surrounds the lesions sites in sections of stab-wounded rat brains, suggesting that NTSR2 may play a role in regulating glial response to injury (Nouel et al., 1999). In addition, NTS interacted with NTSR1 protects adult mice brain from focal ischemia through the decrease of caspase-3 activity and the increase Bcl-2 expression which suggests an anti-apoptotic effect of NTS (Choi et al., 2012).

3.4 NTS/NTSR1 complex and cancer

The physiological properties including trophic, pro-inflammatory and anti-apoptotic of neurotensinergic system suggest its potential contribution to tumor progression when the neurotensinergic system is deregulated.

3.4.1 The aberrant expression of NTS/NTSR1 in tumors

Dysregulation of the NTS has been observed in many cancers such as colonic adenocarcinoma (Ulich et al., 1983), small cell lung cancer (Moody et al., 1985), medullary thyroid carcinoma (Baca and Schmidt-Gayk, 1981), fibrolamellar hepatocellular carcinoma (Ehrenfried et al., 1994), pancreatic cancer (Wang et al., 2011a) and breast cancer (Dupouy et al., 2009). Compared to the overexpression in tumors, neurotensin is not or low expressed in the corresponding normal tissues.

NTSR1 is also upregulated in a large variety of tumors. Dysregulation of NTSR1 was first observed in medulloblastomas, neuronal neoplasm of the central nervous system (Giangaspero et al., 1985), then in meningiomas (Mailleux et al., 1990; Przedborski et al., 1991) and astrocytic tumor (Camby et al., 1996) and more recently in neuroendocrine tumors (Kim et al., 2015) and gliomas (Ouyang et al., 2015). Not only in tumors of CNS, aberrant expression of NTSR1 are also detected in a majority of solid tumor tissues and cancer cell lines but not in normal tissues or at a very low level, for example breast cancer (Dupouy et al., 2009; Souaze et al., 2006), colon cancer (Gui et al., 2008; Maoret et al., 1994), pancreatic cancer (Wang et al., 2011a; Wang et al., 2000), uterine leiomyomas (Rodriguez et al., 2010), prostate cancer (Valerie et al., 2011), head and neck squamous carcinomas (Shimizu et al., 2008) and non-small cell lung cancer (Alifano et al., 2010).

To date there is more and more evidence to support the view that NTS exert numerous oncogenic effects involved in tumor growth and metastatic spread. These effects are mostly mediated by NTSR1, making the NTS/NTSR1 complex an actor in cancer progression (Dupouy et al., 2011).

3.4.2 Oncogenic signaling pathway associated with NTSR1 activation

The signaling pathways involved in the interaction of NTS with NTSR1 have been

studied in a variety of cellular systems, particularly the murine neuroblastoma cell line N1E-115, the human colon adenocarcinoma cell line HT-29, and Chinese hamster ovary (CHO), expressing rat NTSR1, CHO-NTSR1. Figure 20 summarizes the coupling of NTSR1 to heterotrimeric G proteins and the subsequent activation of second messengers and cellular effectors. In all the systems studied, NTS leads to the activation of phospholipase C (PLC) (Najimi et al., 2002a; Wang and Wu, 1996) via the coupling with the subunit $G\alpha_{q/11}$, then followed by the hydrolysis of the membranous phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). These two second messengers induce the stimulation of the protein kinase C (PKC) and the mobilization of intracellular calcium respectively, which are both key effectors conferring the NTS oncogenic properties (Amar et al., 1986; Bozou et al., 1989; Hermans et al., 1992; Snider et al., 1986; Turner et al., 1990). Besides, NTSR1 was shown to be coupled with other heterotrimeric G proteins, like $G\alpha_{i/o}$ and $G\alpha_s$. The activation of $G\alpha_{i/o}$ was mainly described in neuronal cells, whereas activation of $G\alpha_s$ was observed in cells transfected with the NTSR1, suggesting that the coupling occurs when NTSR1 is over-expressed in these cells (Gailly et al., 2000; Yamada et al., 1993).

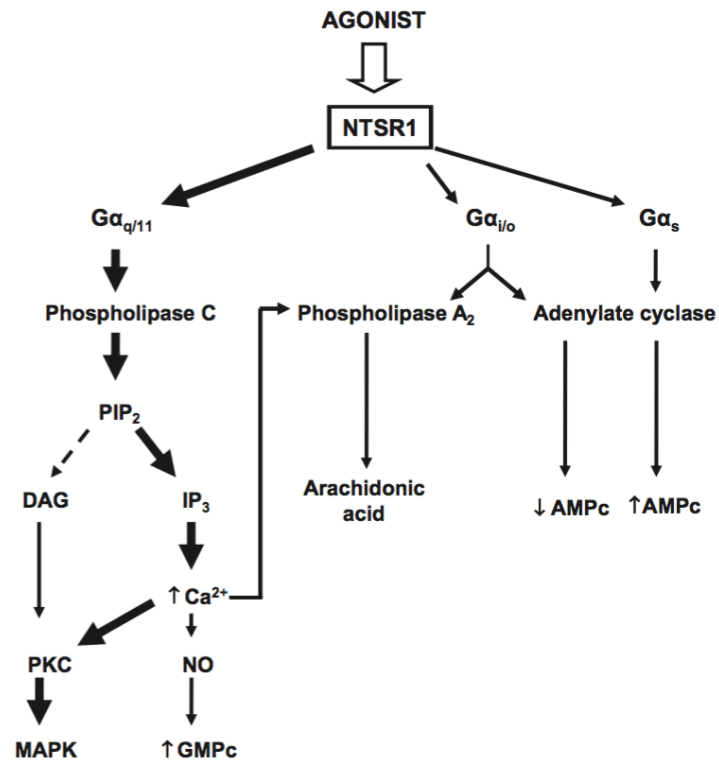


Figure 20. Major effectors activated following NTSR1 stimulation (extracted from (Dupouy et al., 2011)).

Upon the stimulation of NTSR1, several signaling pathway mediate the actions involved in cellular proliferation, survival, migration, and invasion. Three main pathways are highlighted in Figure 21.

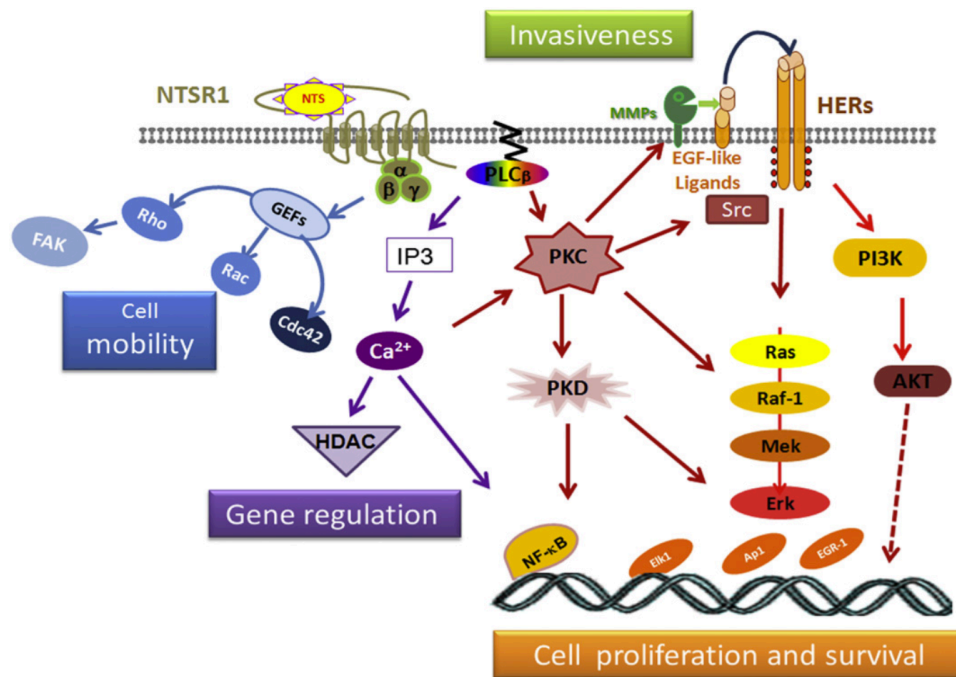


Figure 21. Summary of the signaling pathway by which NTS could contribute to cancer progression. NTS interacts with NTSR1 to activate three major pathways: the blue pathway concerns the small GTPases activation, which are mainly involved in cellular cytoskeleton organization and mobility; the purple pathway concerns the intracellular Ca^{2+} mobilization which is involved in many gene regulations, and the red/yellow pathway which conducts via PKC to ERK cascade activation. The ERK cascade can be activated directly through PKC or PKD activation, or indirectly through the transactivation of EGFR in a PKC-MMP-EGF-like ligand dependent pathway. Once EGFR autocrine regulation is ongoing tumoral process, gravity will depend on its dimer partner (extracted from (Dupouy et al., 2011)).

3.4.2.1 PKC and MAPK cascade

The oncogenic effects induced by NTS are mostly PKC-dependent. Activation of PKC by NTS was demonstrated using broad isotype inhibitor, mostly Gö6976, which preferentially inhibits the conventional PKCs α , β and γ . The use of the specific NTSR1 antagonist, SR48692, blocked the activation of PKC, confirming that NTSR1 mediates these effects. PKC activation can induce the stimulation of mitogen activated protein kinase (MAPK), mostly ERK 1/2. In human colorectal cancer cells HCT116 cells and HT29 cells, neurotensin-induced activation of ERK and stimulation of DNA synthesis

in a PKC-dependent manner (Muller et al., 2011).

Several pathways for the stimulation of MAPK cascade were described, involving PKC-dependent activation of ERK, and protein kinase D (PKD), and transactivation either dependent or independent on the epidermal growth factor receptor (EGFR), which will be described in the next paragraph. In the prostate cancer cell line PC-3, neurotensin stimulated mitogenesis by a PKC-dependent transactivation of EGFR (Hassan et al., 2004). On the contrary, in the pancreatic cancer cell line Panc-1, NTS potently stimulates c-Raf-1-MEK-ERK then induces DNA synthesis without independence of EGFR transactivation (Guha et al., 2003), suggesting that PKC directly stimulates Raf-1 independently of the Ras activation. In the same cell line, S. Guha et al. also showed that neurotensin induces early and transient activation of protein kinase D1 (PKD1) in a PKC-dependent pathway (Guha et al., 2002). PKD was identified as a downstream PKC effector and its involvement has been documented in a variety of cellular processes important to cancer development, including cell growth, apoptosis, motility, and angiogenesis (LaValle et al., 2010). The significant and prolonged duration of ERK1/2 signal induced by NTS, suggested that PKD1 could integrate the signaling events downstream of NTSR1 leading to cell proliferation and act as an amplifier of cancer progression (Kisfalvi et al., 2010).

Recent data has demonstrated that NTSR1 signaling requires intact structured membrane microdomains (SMDs). Y. Heakal and M. Kester showed that the alteration in the microdomains composition interferes with NTSR1-mediated MAPK signaling within microdomains by disrupting the coupling of the receptor to the Gαq/11 subunit in MDA-MB-231 cells (Heakal and Kester, 2009).

NTS-stimulated MAPK will further induce the early growth response gene-1 (Egr-1), also named Krox-24, as well as the AP-1 transcription factor c-Fos and the transcription activator, ETS domain-containing protein (Elk1) at the transcriptional and translational levels. Both mechanisms can be prevented by the NTSR1 antagonist (Ehlers et al., 1998; PoinotChazel et al., 1996; Portier et al., 1998; Zhao et al., 2007).

3.4.2.2 EGFR transactivation

As mentioned above, PKC activation stimulates lateral pathways involving Epidermal Growth Factor Receptor (EGFR) transactivation in some cancer cell lines. This transactivation is mainly mediated by the release of EGFR-like ligands (TGF- α , Hb-EGF, or amphiregulin) which are present as inactive pro-ligands at the cell membrane. These ligands are released by proteolytic cleavage involving proteins of the metalloproteinase family, including ADAMS (A Disintegrin And Metalloprotease) and MMP (Matrix Metalloprotenase) (Kataoka, 2009; Sanderson et al., 2006; Zhao et al., 2004). The ectodomain shedding of these ligands produces soluble functional forms that directly bind to EGF receptors and activate their downstream signaling pathways. Another mechanism of activation of EGFR involves Src protein. NTS induces a time-dependent increase in EGFR Tyr⁸⁴⁵ phosphorylation and phosphorylation of c-Src and signal transducer and activator of transcription 5b (Stat5b) (a downstream effector of Tyr⁸⁴⁵) which is associated with expressions of proliferation, differentiation and survival genes (Amorino et al., 2007). In parallel, a synergistic stimulation of NTS combined to EGF was shown on DNA synthesis resulting from a prolongation of the ERK signal duration (Kisfalvi et al., 2005). These findings suggest a cooperative relation between NTSR1 and the EGFR system, which is exerted either by the enhancement of a common downstream signaling pathway or by a direct transactivation of the EGFR.

3.4.2.3 RhoGTPases and non-receptor kinases FAK and Src

NTS can modulate the activity of the small RhoGTPases Rac1, RhoA and Cdc42, which are partly responsible for the cytoskeleton dynamics known to contribute to the formation of various cytoplasmic extensions like lamellipodia, filopodia, pseudopodia, or invadopodia. This modulation affects the process of cell migration. In the human glioblastoma cell line U373, NTS was associated with the stimulation of the Rac1 and Cdc42 proteins activity but not RhoA this was corroborated with other data obtained on human colonic epithelial cells overexpressing NTSR1 (Servotte et al., 2006; Zhao et al., 2003). Moreover, in small cell lung cancer cells, it was shown that the NTS

increased tyrosine kinase activity and tyrosine phosphorylation of FAK (Focal Adhesion Kinase), a protein contributing to the regulation of protein dynamics at cell–matrix interface, and also involved in adhesion and cell migration (Leyton et al., 2002; Tallett et al., 1996). The NTS/NTSR1 pathway also activates other intracellular tyrosine kinases, among them Src and Etk/Bmx, in a prostatic cancer cell line (Lee et al., 2001). Etk/Bmx is activated by FAK, possibly through Src, and the three activated tyrosine kinases form a signaling complex potentially involved in the alternative NTS-induced trophic effects consecutive to androgen deprivation.

3.4.3 Oncogenic effects of NTS/NTSR1 complex

3.4.3.1 Tumor growth

The contribution of NTS/NTSR1 complex in tumor growth stimulation was reported in several studies by taking advantage of specific NTSR1 antagonists, SR48692 and SR142948A (Gully et al., 1993; Gully et al., 1997). A decrease of at least 50 % in tumor volume and weight was observed in xenografts of colon and small cell lung cancer cells when animals were treated daily with a NTSR1 antagonist (Maoret et al., 1999; Moody et al., 2001). The NTS/NTSR1 complex was positively correlated to the tumor growth and metastasis in breast and lung experimental tumors (Dupouy et al., 2014; Younes et al., 2014).

To corroborate these finding, in vitro experiments showed that NTS stimulates the growth of cancer cells from various origins, as exogenous NTS was found to induce the growth stimulation of pancreas, colon, prostate, and small cell lung cancer cells in culture (Carraway and Plona, 2006). Tritiated thymidin incorporation experiments performed on prostatic and pancreatic cancer cells showed that NTS stimulated the growth of cancer cells and promoted DNA synthesis (Guha et al., 2002; Ishizuka et al., 1993; Seethalakshmi et al., 1997). NTS-induced anti-apoptotic effects were first described, by the team, in the MCF-7 breast adenocarcinoma cell line. These effects were accompanied by a concomitant increase in Bcl-2 protein expression, dependent on the mitogen-activated protein kinase (MAPK) pathway stimulation and EGFR

activation (Somai et al., 2002).

3.4.3.2 Metastasis

Transcriptome analysis performed on Head Neck Squamous Cell Carcinoma (HNSCC) cells exposed to NTS analog confirmed the activation of metastatic gene signatures with the regulation of 10 genes involved in adhesion, migration, invasion and apoptotic processes such as the MMP-1 matrix metalloproteinase and the IL-8 interleukin (Shimizu et al., 2008).

Indeed, in vitro experiments demonstrated that NTS stimulates several cellular effects involved in metastatic process. NTS enhances EGF-induced migration and invasion in breast cancer cells via cell migration assay and collagen invasion assay (Dupouy et al., 2014). In tumoral cells, NTS was shown to modulate the migratory ability of initially adherent cells, like those of glioblastoma (Servotte et al., 2006), colon cancer (Wang et al., 2006), pancreatic ductal adenocarcinoma (Mijatovic et al., 2007), HNSCC (Shimizu et al., 2008), and breast cancer (Souaze et al., 2006). NTS was also shown to induce the acquisition of an invasive cellular phenotype in invasion assays. When prostate adenocarcinoma cells LNCaP were submitted to NTS, a 75% increase of their basal invasive capacity was observed in Matrigel. Further, Neurotensin induces invasiveness in androgen-dependent cells and promotes the invasive potential of androgen-independent cells. These acquired invasive properties were correlated with intrinsically secreted NTS since the effect was abolished by specific NTS silencing (Vias et al., 2007). Similarly, in HNSCC cells expressing NTSR1, NTS agonist increased the number of invasive cells in Matrigel by three to four fold (Shimizu et al., 2008)..

3.4.3.3 Neoangiogenesis

Angiogenesis is a fundamental process for tumor growth by providing nutrients and oxygen to the tumor cells. NTS can promote the formation of new blood vessels through interaction with the uPAR (urokinase-type plasminogen activator receptor), resulting in induction of angiogenesis (Ushiro et al., 1997). The uPA/uPAR system

facilitates extravasation and intravasation of cancer cells by regulating local proteolysis and attachment of the cells to components of the extracellular matrix and is also implicated in proliferation of some tumor cells and migration of tumor and endothelial cells (Andres et al., 2012; Reuning et al., 2003). Indeed, the NTS released in the circulation of tumor is likely to induce the fibrinolytic activity in bovine vascular aortic and human umbilical vein endothelial cells, and enhance the migration of vascular endothelial cells (Ushiro et al., 1997).

Tang et al, has shown that in CD133⁺ liver tumor-initiating cells (TICs, also known as cancer stem cells), neurotensin promotes neoangiogenesis by the concomitant up-regulation of IL-8 and CXCL1 (C-X-C Motif Chemokine Ligand 1) with simultaneous activation of p-ERK1/2 and Raf-1 (Tang et al., 2012). Figure 22 summarizes the various oncogenic effects of NTS/NTSR1 complex.

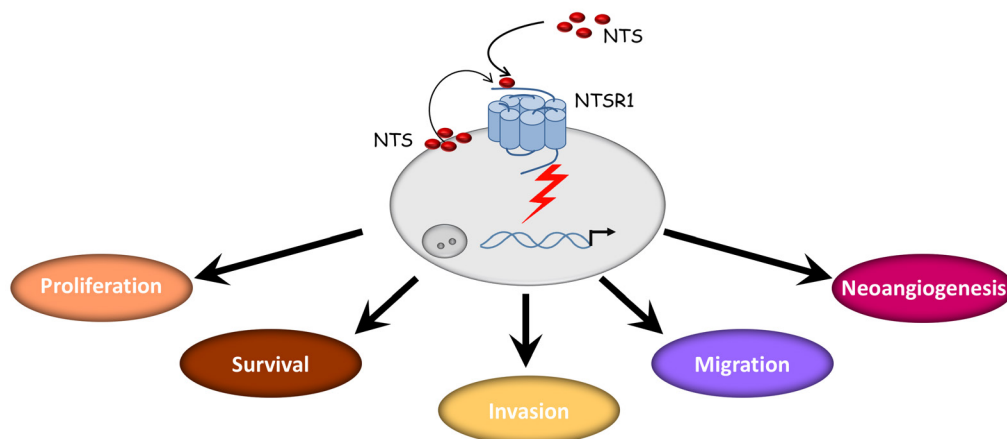


Figure 22. Schematic representation of NTS oncogenic effects (adapted from (Wu et al., 2012)).

Chapter 4

Rationale and aims of the thesis

Chapter 4. Rationale and aims of the thesis

4.1 Rationale of the study

The data presented in the introduction suggested that when NTSR1 is continuously activated by NTS, the neurotensinergic system contributes to the tumor progression, metastasis and poor prognosis. The complex of neurotensin (NTS) and its high affinity receptor 1 (NTSR1) has been shown to promote cancer progression via proliferation, survival, migration, invasion and neoangiogenesis *in vitro* and/or *in vivo*. Moreover, we have shown that the sustained activation of NTS/NTSR1 induces overexpression and activation of growth factor EGFR, HER2 and HER3, which are associated with tumor progression and metastatic process in the lung cancer and breast cancer. Recent work in our team suggested that NTS/NTSR1 promoted tumor metastasis and invasion by activation WNT/ β -catenin signaling pathway in the model of hepatocellular carcinoma (HCC). All these data suggest the NTS/NTSR1 inhibiting agent as a potential strategy for patients carrying NTS+/NTSR1+ tumors. However, to date the situation in ovarian cancer is still not clear. The standard treatment for ovarian cancer is surgery and platinum-based chemotherapy. The majority of patients exhibit a satisfied response initially. However, more than 90% of these patients relapse after 2 years and recurrent patients in most cases becomes incurable due to the development of chemo-resistance. In this context, it will be promising to introduce the agents blocking NTS/NTSR1 pathway into the chemotherapy to improve the drug sensitivity or overcome chemo-resistance. On the other hand, the role of the complex NTS/NTSR1 in the platinum-based chemotherapy has not been considered. Therefore, I studied whether NTS/NTSR1 inhibitors could enhance chemotherapy and the related mechanism. This study contributes to better understanding of unknown molecular mechanisms involved in the synergy effect of the NTSR1 antagonist and overcoming resistance by a non-toxic agent with specificity.

4.2 Aims of the study

4.2.1 Characterize cellular and experimental tools and confirm the influence of NTSR1

antagonist on response to platinum salt-based drug in vitro and in vivo;

4.2.2 Study the expression of NTS and NTSR1 in ovarian cancer patients and the contribution of NTS and/or NTSR1 expression in the survival and drug response;

4.2.3 Evaluate and characterize the mechanism of the improved response when platinum salt-based chemotherapy combined with NTS/NTSR1 inhibitor in ovarian cancer cells.

Chapter 5. Results

Manuscript

Neurotensin receptor 1 antagonist SR48692 improves response to carboplatin by enhancing apoptosis and inhibiting drug efflux in ovarian cancer

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Summary

In order to verify our hypothesis that the blockade of NTS/NTSR1 pathway enhance the response to chemotherapy in ovarian cancer, we employed the SR 48692, a specific antagonist against neurotensin high affinity receptor 1, to inhibit the activation of NTS/NTSR1 pathway. Firstly, we studied the mRNA expression of NTS and NTSR1 in the two candidate cell lines, SKOV3 and A2780 both express NTS but the stable abundant NTSR1 mRNA was only detected in SKOV3. We studied the *in vitro* response to carboplatin with or without SR 48692 by the cell viability assay in SKOV3. Meanwhile, we evaluated the *in vivo* drug response in xenografted mice. Mice injected with A2780 cells formed tumors within 2 weeks, while SKOV3 tumors were visible about 2 months after grafting. For this reason, our studies on experimental tumor were initiated with A2780 cells. Our results demonstrated that in the presence of NTSR1 antagonist, cells or tumors showed an enhanced response to carboplatin. Subsequently, we analyzed the mRNA expression of NTSR1 in the tumors derived from the mice. Instead of the low level of NTSR1 expression found in cultured cells, the expression of NTSR1 was significantly increased in the tumors. This may because the cells formed 3D structure in the tumors. In the 3D environment, paracrine of NTS may stimulate NTSR1 expression as the group previously described (Najimi et al., 1998; Souaze et al., 1997; Toy-Miou-Leong et al., 2004b). We also analyzed the NTS expression in treated tumors. NTS was upregulated by carboplatin. This upregulation decreased in the presence of NTSR1 antagonist. We proposed that the abolition of NTS and NTSR1 improves the response to chemotherapy treatment.

We then analyzed the mechanisms involved in these effects, and studied the apoptosis induced by carboplatin or the combination of carboplatin and SR 48692 by flow cytometry and Hoechst staining. These results consolidated our observation that NTSR1 antagonist enhances the response to carboplatin because SR 48692 stimulated the apoptosis induced by carboplatin in SKOV3 and A2780-R1 (A2780 cells stably transfected with NTSR1 expressing vector). Consequently, we studied the caspase activation and Bcl-2 family members and confirmed that carboplatin in

combination with SR 48692 can overstimulate the caspases, downregulate the anti-apoptotic factors and upregulate the pro-apoptotic factors.

Previous study from our group has shown that under intense and chronic NTS stimulation, the cellular trafficking change, with intensification of the recycling pathway. We hypothesized that these changes may influence the efflux of the drug. Therefore, we presumed that the enhanced response was due to an increased accumulation of carboplatin in the cells and an increase of DNA damage in the nucleus. Consequently, we measured the platinum content in the extract of whole cell and DNA. We found that the SR 48692 decreased the efflux of carboplatin and increase the DNA damage induced by the platinum.

To place our study in a clinical context, the expression of NTS and NTSR1 were researched on 46 ovarian cancer specimens. The detailed statistical analysis of the data collected by immunohistochemistry showed that the expression of NTS and NTSR1 was higher in cancer tumors than in normal ovaries. Additionally, the NTS and NTSR1 mRNA expression was analyzed in a 491 high grade ovarian serous cystadenocarcinoma cohort from the TCGA database. Higher level of NTSR1 was linked to higher stages and with platinum resistant status. These results strengthen our viewpoint that the blockade of NTS/NTSR1 pathway may enhance the response to chemotherapy or sensitize tumor cells resistant to platinum.

Neurotensin receptor 1 antagonist SR48692 improves response to carboplatin by enhancing apoptosis and inhibiting drug efflux in ovarian cancer

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Introduction

Epithelial ovarian cancer (EOC) is the seventh most common cancer and the eight most frequent cause of cancer death among women globally (1). As an example, 22,280 new cases and 14,270 deaths were estimated in the United States for 2016. This represents about 2.4 percent of all cancer deaths. Because this disease has no apparent symptoms, the majority of females with EOC are diagnosed at advanced stages, and the percent surviving at 5 year rate is 46.2% (2). While inherited factors account for 5–15% of all ovarian cancers, the majority are due to genetic defaults linked to BRCA 1/2 mutations (3). Consequently, EOC is one of the most lethal gynecological malignancies in women.

The standard of treatment for advanced ovarian cancer patients consists of primary debulking surgery and platinum-based chemotherapy combined with taxane (4,5). The combination of carboplatin plus paclitaxel, results in a complete response rate in 40-60 % of the cases. However, more than 90% of these patients relapse after 2 years and recurrent patients in most cases becomes incurable due to the development of chemo-resistance (6). EOC remains a real clinical challenge, and despite the development of new therapeutic strategies; there is an urgent need to optimize the current treatment. To this regard, we develop here argument for the use of neurotensin blockage to improve the sensitivity to platinum-based chemotherapy.

Neurotensin (NTS) is a 13-amino-acid peptide acting as a neurotransmitter in the central nervous system and local hormone in gastrointestinal tract (7). NTS activates three subtypes of receptors. NTSR1 and NTSR2, belong to G-protein coupled receptors family, exhibiting high (sub-nanomolar) and low (nanomolar) affinity for NTS, respectively (8). NTSR3 known as gp95/sortilin, is a sorting protein member of the family of Vps10p-domain receptor family (9). The complex of Neurotensin (NTS) and its high affinity receptor 1 (NTSR1) was shown to contribute to cancer progression (10). NTSR1 was found abnormally overexpressed in several types of solid cancers (11), in

association with the dysregulation of the beta-catenin pathway or epigenetic regulation (12-15). Moreover, the presence of NTS/NTSR1 complex enhanced the tumor growth and metastasis process in the colon, breast, lung, pancreatic, glioma, head and neck squamous cell carcinomas (8,16-21). Our group has demonstrated that this cell aggressiveness was due to the establishment of the epidermal growth factor receptors autocrine activation, occurring under the sustained stimulation of NTSR1 (8,13,18).

Recently, it was shown that NTS plasma concentration is increased in rats treated with cisplatin and oxaliplatin for several weeks, causing specific damage of the sciatic nerve, and producing variable effects in motor and behavioral tests (22). Thus, we hypothesized that NTS/NTSR1 complex may contribute to chemotherapy resistance and that the blockage of NTS/NTSR1 may improve the response to platinum salt-based chemotherapy.

Up to now the most studied approach to antagonize NTSR1 activation is the use of the non-peptidic component Meclizine (SR-48692) (23). This drug was developed to counteract the action of NTS on neurotransmitters in the brain (24). Meclizine exhibits a nanomolar affinity for different tissues and cells from various species (23). This compound crosses the blood brain barrier, and was reported to reverse most of intracellular mediator signal linked to NTSR1 activation, such as intracellular Ca^{2+} mobilization, inositol monophosphate; cyclic GMP; cyclic AMP activation (25). This component showed some agonist effect of NTSR2, which may explain its partial or non-response on particular central nervous system effect induced by NTS as hypothermia or analgesia (26,27). Meclizine was also tested on non-selected patient with lung cancer, after a first line of chemotherapy using cisplatin combined with etoposide, but failed to demonstrate an increase in overall survival. Globally, meclizine is very efficient and specific to counteract the physiological and pathological action of NTS mediated by NTSR1.

In this article, we showed that SR 48692, a specific NTSR1 antagonist enhanced the

response to carboplatin in ovarian cancer cells expressing both NTS and NTSR1. SR 48692 improved the effect of carboplatin by increasing the drug efficiency and reducing the survival action of NTS. We also showed the absence of NTS and NTSR1 expression in tissues from normal ovary samples, whereas these markers are present in 75% of EOC. In addition, cisplatin resistance is also correlated with highest expression of NTSR1 mRNA.

Results

NTSR1 antagonist enhances the carboplatin treatment of the ovarian cancer experimental tumor

Expression of NTS and NTSR1 was evaluated in two ovarian adenocarcinoma cell lines, SKOV3 (Epithelial; TP53 *mut*; BRCA1/2 *wt*; Non high grade serous subtype) and A2780 (Epithelial; TP53 *wt*; BRCA1/2 *wt*; Non high grade serous subtype) by RT-PCR. A very strong expression of NTS and a weak expression of NTSR2 were detected in both SKOV3 and A2780 cell lines. In contrast, NTSR1 mRNA was only detected in SKOV3 cells (Figure 1A). Tumors initiated within SKOV3 cells grow very slowly (28). For this reason, we chose to generate experimental tumor from A2780 cells, which exhibit palpable tumors within 10-14 days after cell injection. The experimental group treated with SR48692 used a dose of 1mg/kg/day because this dose has been shown to be efficient for overexpressing NTSR1 hepatocellular carcinomas (29). We treated the experimental group with 4mg/kg carboplatin every two days, 4 times totally. This treatment enabled us to treat the mice at sub-toxic dose of carboplatin, since the LD₅₀ for carboplatin injected i.p for mouse is 115mg/kg. (30).

As shown in figure 1B, the tumor growth rate of the control group was very high, and therefore the experiment was terminated after 15 days. In the carboplatin treated group, the tumors were slightly responsive at the beginning of the treatment, and reached the same tumor volume as the control group at the end of the experiment. SR48692 treatment did not affect the tumor growth, but when used in combination with carboplatin, the tumor growth rate was significantly decreased by 2 fold. The ratio of tumor size to the average of the control tumor size (% T/C) showed that the combine treatment was immediately efficient and remained efficient all along the experiment (Figure 1C).

We observed a strong diminution of tumor size using a very specific NTSR1

antagonist in tumors generated in cells (A 2780) which do not express NTSR1 when cultured on plastic (Figure 1A). We analyzed the NTS and NTSR1 mRNA expression levels in the tumors and observed an unexpectedly high and significant 6 fold increase of NTSR1 mRNA levels as compared to those performed from cultured cells (Figure 2D). In contrast, the levels of NTS mRNA remained similar between the tumor and cell cultured conditions (Figure 2E). A more thorough analysis of mRNA levels from tumors in treated animals revealed that the NTS mRNA levels from tumors with carboplatin treatment were almost 10 fold higher than those from control mice (Figure 2F). This result was confirmed at the protein level, as the labelling intensity for long fragment NTS was much stronger in tumors from mice treated with carboplatin (Figure 1H). The combined treatment with carboplatin and SR48692 abolished this increase both at the RNA and protein levels (Figure 1F and H). The antagonist, SR48692 when used alone had no effect on NTS mRNA levels (Figure 1 H) or on the protein levels (not shown). Lastly, KI 67 labelling was less frequent and weaker in tumors treated with the combination as compared to control or carboplatin treatment alone.

The expression of NTSR1 in experimental ovarian tumors and the induction of NTS, when tumors are exposed to carboplatin, suggest that the occurring autocrine NTS regulation interferes in platinum salt-based therapy.

NTSR1 antagonist improves the carboplatin response by inhibiting cell growth and survival

In vitro studies were performed, in SKOV3 cells and A2780 cells stably transfected with NTSR1 expression vectors. The clone A2780-R1 was selected amongst several alternatives because it had a similar NTSR1 mRNA level as SKOV3 cells. The NTSR1 expression in A2780-R1 was confirmed by RT-PCR (Figure 1A) and immunofluorescence (data not shown).

In SKOV3 cells, carboplatin inhibited cell growth in a dose-dependent manner, as measured with the presto blue assay. The addition of SR48692 to carboplatin enhanced this effect as the IC_{50} for carboplatin was $127.50 \pm 13.75 \mu M$ while the IC_{50} for the combination of carboplatin and SR48692 was $70.19 \pm 6.12 \mu M$ (Figure 2B).

Apoptotic cells were detected by Annexin V/PI dual labeling assay in the flow cytometry performed on SKOV3 and A2780-R1 cells. A higher number of cells were detected in Annexin V positive quadrants (Q2 and Q3) when cells were treated with $5 \mu M$ SR48692 and $150 \mu M$ carboplatin (dose corresponding approximatively to the IC_{50} as determined above), as compared to the carboplatin treatment alone (Figure 2C). The number of apoptotic cells was increased by two fold for the SKOV3 cells, and four fold for the A2780-R1 cells when the combined treatment of carboplatin plus SR48692 treatment was compared with carboplatin alone treatment (Figure 2 D and E).

This finding was confirmed by examining nuclear morphological changes in SKOV3 cells using Hoechst 33258 staining. Cells were scored as apoptotic if their nuclei present chromatin condensation and nuclear beading or fragmentation. The control and SR48692 treated cells showed no morphological cellular or nuclei changes (data not shown). In cells treated with carboplatin for 48h, only several cells showed apoptosis-like changes. While cells treated with the combination carboplatin and SR48692, more cells displayed morphological changes as indicated by arrows (Figure 2F). The apoptotic cells were counted and the apoptotic ratio was calculated by the comparison to the total number of cells in each field. The percentage of apoptotic cells increased from $1.56 \pm 0.10\%$ for carboplatin treated cells to $4.15 \pm 0.25\%$ for the combination treatment (Figure 2G).

NTSR1 antagonist enhances the apoptosis induced by carboplatin through the activation of the caspase-mediated pathway and the apoptotic related protein from the Bcl-2 family.

Carboplatin exerts its cytotoxicity via the formation of DNA lesions, which ultimately results in cell cycle arrest and the induction of cell death through the apoptotic pathway. To elucidate the role of caspases in carboplatin induced apoptosis in SKOV3 cells, we first examined the activation of executioner caspase-3 by western blot analysis. Caspases are synthesized as inactive pro-enzymes and are activated by proteolytic processing in response to apoptotic stimulus. As shown in Figure 3A, when cells were treated with carboplatin, both the pro-enzyme of caspase-3 (32 kDa) and two active caspase-3 sub-units (20 kDa and 17 kDa) were detected after 48h (lane 3) of treatment. Furthermore, caspase 3 activation was more pronounced at 48h treatment (lane 4) when cells were treated with the combined treatment. Stimulation of the other members of the caspase family was measured by a caspase colorimetric protease assay kit. As shown in the Figure 3B, all five caspases (2, 3, 6, 8, and 9) were activated by carboplatin, and in the presence of SR48692, these caspases activation was even significantly enhanced. SR48692 had the effect of over-stimulating both caspases, those which act as initiators (Caspase-2, 8 and 9), and those which act as effectors (caspase-3 and 6). This effect may be attributed to the improvement of carboplatin response when combined with SR48692.

Mitochondrial membrane permeability is regulated by the Bcl-2 protein family. The fate of cells is determined by the balance between anti- and pro-apoptotic members. We studied the mRNA expression of the Bcl-2 family member. As expected Bcl-2 and Bcl-xL, were downregulated, while the mRNA of Bak and BCL2L1 were increased with the combined treatment as compared to carboplatin single treatment (Figure 3C). The Bcl-2 family protein profile, Bcl-2 and Bcl-xL, followed a similar regulation as their respective transcript. An increase in Bak protein expression with the combined treatment was also observed (Figure 3D).

NTSR1 antagonist facilitates platinum accumulation in the nucleus and

increases the formation of DNA double strand breaks

Platinum accumulation in the whole cell extract and nucleus was compared under carboplatin and carboplatin plus SR48692 treatments. In SKOV3 cells and in A2780-R1, the Platinum content of the whole cell was increased by two fold after 6-hour exposure to the combined treatment as compared to the carboplatin (Figure 4A). No increase was observed in A2780 wild type cells. In order to evaluate if SR48692 facilitates drug access to the targeted DNA, platinum-DNA bound was assayed. After a 6-hour treatment, an increase of 2.02 ± 0.23 and 2.61 ± 0.33 fold of platinum-DNA bound was observed in SKOV3 and A2780-R1 cells, respectively (Figure 4B). In A2780 wild type cells, a slight but no statistical increase of platinum content in the nucleus was seen in the presence of SR48692 (Figure 4B).

As a consequence of the platinum accumulation in the nucleus, we detected more DNA damage when cells were treated with the combined treatment. The phospho-Ser₁₃₉ H2AX protein (γ -H2AX) involved in the DNA repair is recruited at the site of DNA double strand breaks. The number, the size (as see in figure 4C left), and the intensity of dots increased in the nucleus of cells treated with carboplatin and SR48692, as compared to carboplatin single treatment. Barely no labelling was detected in control SKOV3 cells and cells treated with SR48692 alone. The intensity of labelling was estimated, γ -H2AX labeling was increased from 124.40 ± 4.51 % for carboplatin treated cells to 184.10 ± 8.41 % for the combine treatment as compared to the control cells (Figure 4C right).

NTSR1 antagonist reduces carboplatin efflux from the cells

The P-type transport ATPase family (ATP7A, ATP7B) and the Multidrug resistance-associated protein 2 (MRP2) have been reported to be associated with the platinum efflux and consequently to platinum salt drug resistance (31). In SKOV3

cellular model, we found that the mRNA expression levels of ATP7A, ATP7B and MRP2 were decreased when cells were treated with the combination of SR48692 and carboplatin as compared to carboplatin single treatment (Figure 5A). The decrease was even more pronounced at the protein level suggesting a decrease of the cellular efflux (Figure 5B). ATP7A shuttles from the Golgi apparatus to the cell membrane when the copper concentration is increased in cells and contributes to the copper efflux (32). We investigated whether the localization of ATP7A was altered when SKOV3 cells were exposed to 500 μ M carboplatin for 1 hour. In the control cells and the cells treated with SR48692, ATP7A accumulated in the trans-Golgi network while the labeling was dispersed in the cytoplasm when cells were exposed to carboplatin (Figure 5C). However, in the cells treated with both carboplatin and SR48692, the labeling of ATP7A was less dispersed in the cytoplasm as compared to cells treated with carboplatin alone (Figure 5C). The quantification of the ATP7A immuno-labelled particles was made according to their size (pixels) and showed that the number of particles was two to three folds higher in cells treated with carboplatin alone as compared to the cells treated with the combination of carboplatin and SR48692. This suggests that the mobilization of ATP7A in the cytoplasm, for the efflux of carboplatin, is antagonized by the NTSR1 antagonist.

NTS and NTSR1 are expressed in ovarian cancer

NTS proform and NTSR1 immunohistochemistry was performed on 46 ovarian cancer specimens. Figure 6A showed a typical positive and negative labelling for both markers in low grade serous carcinomas. The clinical and the histological characteristics are shown in table 1 supplementary data. The patients presented the classical risk factors for ovarian adenocarcinoma: BRCA mutations, nulliparity, endometriosis history, menopause hormone therapy. The predominant pathological subtype detected amongst the ovarian adenocarcinomas was serous differentiation

(66.7%). The majority of the tumors were FIGO stage 3 or 4 (78.8%), and 30.3 of patients were metastatic. This series is representative of patients with ovarian cancer.

In malignant tumors (borderline tumor and adenocarcinoma), the NTSR1 distribution was in most cases homogeneous (Figure 6B). A Hirsch score superior to 50 was considered as moderately positively or strong labeling. With this scoring, 72 % and 74 % of the cases were positively labeled with the NTSR1 and NTS proform antibody, respectively. NTSR1 and NTS cytoplasmic localization was predominant and 67 % of the samples were positive for both NTSR1 and NTS (Figure 6B). NTSR1 and NTS scores were also analyzed in nonmalignant tissues. The highest score for NTSR1 were found in the clear cell carcinomas. NTSR1 or NTS staining was negative or very weak in 9/10 samples. The patients were not the same amongst the positive sample scores for NTS and NTSR1 (Figure 6B).

NTSR1 expression was significantly higher in adenocarcinomas (102.1 ± 54.4) than in borderline tumors (67.7 ± 33.8) and normal ovaries (16 ± 23.5) ($p < 0.001$). NTS expression was also significantly higher in adenocarcinomas (97.3 ± 48.2) than in borderline tumors (81.2 ± 55.6) and normal ovaries (28.5 ± 32.5) ($p < 0.001$) (Figure 6C).

NTS and NTSR1 mRNA expression was also analyzed in a cohort of 491 high-grade ovarian serous cystadenocarcinoma from the TCGA database. Figure 6D shows the distribution of the NTSR1 and NTS Z-score within the cohort. For both markers; this distribution was homogenously dispersed amongst the specimens. FIGO stages were available in 484 patients. 24 (4.9%) tumors were FIGO stage II (3 stage IIa, 4 stage IIb, 17 stage IIc), 381 (78.8%) tumors were FIGO stage III (7 stage IIIa, 21 stage IIIB, 353 stage IIIC), and 79 (16.3%) were stage IV. NTSR1 mRNA expression was significantly increased in higher stages ($p = 0.01$ for IV and IIIC stages vs others).

Platinum status was available in 287 patients; 90 (26.1%) patients were resistant, 197 (57.1%) were sensitive. NTSR1 mRNA higher expression was significantly

associated with platinum resistant status ($p=0.0076$), whereas NTS mRNA expression was not different for the resistant or the sensitive groups (Figure 6E).

Discussion

Surgery is the initial treatment for stage I-IVA epithelial ovarian cancer. It is usually followed by several cycles of chemotherapy using platinum-based drugs (mainly carboplatin) combined or not with other anticancer agents (Paclitaxel, or docetaxel). Drug resistance is a major obstacle for ovarian cancer therapy because most patients relapse because of a resistance or weak sensitivity to the platinum-based treatment. Therefore, improving the response to existing treatment by introducing a non-toxic sensitizing agent might optimize the current chemotherapy.

As the consequence of specific NTSR1 overexpression and activation in tumor cells, the NTS/NTSR1 complex enhances tumor progression by promoting proliferation (8,18,33,34), metastasis (8,18,35,36) as well as survival (37,38). This basic data translates into a worst prognosis in cancers from patients overexpressing NTSR1 (21,35,39). In this context, NTSR1 inhibiting agents, such as a NTSR1 antagonist, are predisposed to improve the cancer outcome. Multiple studies showed that neurotensin receptor 1 antagonist, SR48692, inhibited proliferation of tumor cells *in vitro* as well as *in vivo* in small cell lung cancer (17), pancreatic ductal carcinoma (40), breast cancer (41), colon cancer (16) and malignant melanoma (38). An interesting study showed that the NTS blood concentration increased when rats were chronically treated with cisplatin or oxaliplatin, suggesting that in addition to enhancing cancer progression, NTSR1 activation may also interfere in platinum salt-based drug sensitivity (22).

In this study, we investigated the synergistic effect of a NTSR1 antagonist and platinum salt-based treatment in ovarian cancer cells and xenografted tumors in nude mice. We designed experimental tumors in order to mimic the clinical context; therefore, we waited until each measurement of the tumor reach 7 to 8 mm before starting the treatment. The tumor growth rates were similar and very high, around 30 times within 15 days, for the control, SR 48692 and carboplatin groups, whereas it is only of 10 times in the group treated with the combination carboplatin and the NTSR1

antagonist. This result indicates that blocking NTSR1 activation changes the cellular homeostasis of cancer cells to become more responsive to the chemo-toxic drug.

Several possible mechanisms to boost the sensitization to chemotherapy were investigated. Carboplatin acts through the formation of DNA adduct, followed by apoptosis induction as cellular response to DNA damage. Before reaching the DNA, Carboplatin enters the cell via passive infusion or facilitated by active uptake with a number of transport proteins. Platinum can be extruded from the cells by the GS-X pumps (MRP 1-5) after chelation with glutathione or via the copper efflux system (ATP7A/B) (42). The decrease of cellular platinum accumulation is known to contribute to resistance to platinum-based drugs (43). Here we demonstrate that blocking NTSR1 chronic activation, downregulates the three proteins involved in the carboplatin efflux (MRP2, ATP7A and ATP7B), which maintains the accumulation of carboplatin in the tumor cells, and consequently its transfer to the nucleus. Along with the studies regarding the mechanism of resistance of platinum-based drugs, more evidence to support the view that the overexpression MRP2, ATP7A and ATP7B contributes to the formation of resistance (44-46). In particular, MRP2 (Multidrug resistance-associated protein 2) showed a major role in chemo-protection by elimination of drug conjugates with glutathione. (44,47,48). In our cellular model, SR48692 or carboplatin do not influence the expression of MRP2, ATP7A and ATP7B, but the combination of NTSR1 antagonist with carboplatin strongly downregulates these three key player of drug efflux and would explain the enhancement of carboplatin efficiency.

One of major characteristics of NTS is to be a survival factor. This was demonstrated in several cancerous cellular models from diverse origin (10). In particular, in breast cancer cells, NTS agonist inhibited apoptosis induced by serum deprivation, which was accompanied by the enhancement of Bcl-2 gene transcription and protein cellular content (37). Platinum salt-based drug efficacy is obtained by the induction of apoptosis, mediated often concomitantly, by the activation of several signal

transduction pathways, including calcium signaling, death receptor signaling, and the activation of mitochondrial pathways. Cells which escape drug induced apoptosis are closely associated with the development of platinum-based therapy resistance (49). The Bcl-2 (B-cell lymphoma-2) family plays a crucial role in the regulation of apoptosis. The Bcl-2 family contains three subfamilies, the BH3 only family (Bid, Bim, Bad), the anti-apoptotic family (Bcl-2, Bcl-xL), and the pro-apoptotic family (Bax, Bak). In our model, effector proteins in control of apoptosis are essentially not stimulated by carboplatin treatment alone, but in contrast these effectors are strongly stimulated or downregulated accordingly in the combined treatment (see figure 3 BCL-2, BCL-xL, BCL2L11 BAK, BAX). As a consequence, the initiator (caspase-2,8,9) and executioner (caspase-3,6) caspases of the apoptotic pathway are also over stimulated when NTSR1 antagonist is combined with carboplatin. We also noted the overstimulation of caspase-8 by the combined treatment, as compared to the carboplatin treatment alone, suggested that the extrinsic apoptotic pathway was also implicated in better drug efficiency when associated with NTSR1 antagonist. This cascade merits a further exploration.

In summary, we have shown that a NTSR1 antagonist enhanced the response to platinum salt-based chemotherapy by improving the drug-target interaction and enhancing the chemotherapy-induced apoptosis. These results suggest that patients with ovarian cancer expressing both NTS and NTSR1 could benefit of a treatment based on platinum salt drug associated to a blocking agent of the NTS/NTSR1 complex. Our initial studies suggest that could concern 67 % of the patient with ovarian cancer.

Method and materials

Cell culture

Ovarian adenocarcinoma cells SKOV-3 and A2780 were cultured in RPMI1640 supplemented with 10% fetal bovine serum (Gibco®) and 2 mM glutamine. The corresponding NTSR1-overexpressing clone A2780-R1, was cultured in RPMI1640 supplemented with 10% fetal bovine serum, 2 mM glutamine and 0.5 mg/mL G418.

Transfection procedure

A2780 cells were transfected with NTSR1 expressing pcDNA3 vectors using Lipofectamine 2000 (Invitrogen™) according to the manufacturer's protocol. Selection was performed 2 days after transfection with 1mg/mL of G418 (Invitrogen™). The stable transfectants were selected with G418 1mg/mL and were screened for NTS and NTSR1 expression by RT-PCR and immunofluorescence.

Cell viability assay

The effect of Carboplatin and SR48692 on tumor cell growth was evaluated by PrestoBlue® Cell Viability Reagent (Invitrogen). Cell suspension (100 µL) containing 8000 cells was seeded in 96-well plate and cells were treated accordingly. After 72 hours, 10 µL presto blue was added to each well and cells were incubated for 2h at 37°C. The fluorescence was measured by a multimode plate reader (EnSpire®, PerkinElmer).

Tumor xenografts

2.5 millions A2780 cells were suspended in 50% PBS and 50% Matrigel (BD Biosciences) then inoculated in the right flank of nude mice (NMRI-Nude Foxn1). All the procedures were in accordance with the "Guide of the Care and Use of laboratory Animals". Institutional Review Board approval was obtained by «Le Comité d'Ethique en l'Expérimentation Animale Charles Darwin # B751201». 14 days after injection, 4

groups of 6-7 mice were randomized as follows: $116.62 \pm 9.54 \text{ mm}^3$ for control group, $120.25 \pm 10.63 \text{ mm}^3$ for SR48692 group, $113.15 \pm 9.63 \text{ mm}^3$ for carboplatin group and $110.28 \pm 7.11 \text{ mm}^3$ for carboplatin and SR48692 group. Mice were treated for 11 days *per os*, with PBS containing DMSO, or 1 mg/kg SR48692, or 4 mg/kg carboplatin or both.

RNA extraction RT-PCR and quantitative RT-PCR

Total RNA was extracted with guanidinium thiocyanate-phenol-chloroform acid method modified by Souaze et al (50) see details and primer sequence in supplementary data. 2 μg of total RNA was subjected to reverse transcription, during 1 hour at 37°C , using 1 μg of non-specific hexameric random primers dN, 1 mM dNTP, 10 mM dithiothreitol, 24 units RNaseOUT and 200 units of M-MLV RT enzyme (Invitrogen). The PCR amplification was performed on 400 ng of the reverse transcription reaction using 0.2mM dNTP, 2.5mM MgCl_2 , 1 unit of ThermoStart Taq DNA polymerase (ThermoFisher Scientific), and 25pmol of each specific primer: Quantitative RT-PCR was performed using Absolute SYBR Green qPCR Mix (ThermoFisher Scientific) and amplification detected in a 7900HT Fast Real-Time PCR System (ThermoFisher Applied Biosystems).

Annexin V-FITC/PI dual staining analysis

SKOV3 and A2780-R1 cells were plated in 100-mm dishes in complete medium. After 24 hours, cells were treated SR48692 5 μM , carboplatin 150 μM or both for 72 hours for SKOV3 or 48 hours for A2780-R1. Cells were dissociated using Accutase[®] solution (Sigma[®]), washed with ice-cold PBS, resuspended in 100 μL incubation reagent (20 μL annexin V-FITC and 20 μL PI diluted in 1 mL incubation buffer, Annexin-V-FLUOS Staining Kit, Roche) then incubated for 15 minutes at room temperature in the dark. Samples were then analyzed by BD FACS Canto™ II (Becton Dickinson).

Hoechst 33258 staining assay

Cells were seeded on 12 mm-diameter glass slides in 24-well plate at the density of 3×10^4 cells/well in 500 μ L medium and incubated 37 °C with 5% CO₂ overnight. Cells were treated for 48 hours then washed with PBS for 3 times and fixed with 4% paraformaldehyde for 20 min at RT. Cells were stained with Hoechst 33258 solution in the dark for 10 min at RT and immediately imaged by a fluorescence microscope with the excitation wavelength of 340 nm at 200x.

Caspase activity protease assay

Caspase activity was detected using ApoTarget™ Caspase Colorimetric Protease Assay Sampler Kit (KHZ1001, Invitrogen) and was performed according to the manufacturer procedure (see detail in supplementary data).

Measurement of platinum uptake in whole cell and accumulation in DNA

Platinum accumulation in whole cell and DNA was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis. At each data point cells were trypsinized, lysed in 1% Triton X-100, 0.1% SDS(w/v) buffer for 15 min on ice, then incubated in 52.5%(v/v) nitric acid at 65°C for 2 h, finally the acid concentration was reduced to 5 % for storage. For DNA platinum accumulation, cells were digested overnight at RT in lysing buffer (50 mM Tris-HCl pH=8, 10 mM EDTA, SDS 0.6% (w/v), 50 μ g/mL RNase A, 400 mM NaCl, 166 μ g/mL proteinase K). After digestion, the samples were homogenized in 1M NaCl by shaking. Samples were centrifuged at 3000 rpm for 15 minutes at 4°C, and 2 volumes of ethanol were added to the supernatants. The DNA was retrieved by winding the DNA around a glass pipette then washed in 70% ethanol, dried in the air then resuspended in water. Platinum concentration was measured using ICP-MS. The results were normalized by the protein levels or DNA amounts.

Immunofluorescence

Immunocytochemistry was performed according to the procedure previously described in (8) and detailed in supplementary data.

SDS-PAGE and Western blotting analysis

Western blots were processed as previously described (8). Western blots were revealed with antibodies directed against Anti-ATP7A (1:500), anti-ATP7B (1:500), anti-MRP2 (1:500), anti-BCL-2 (1:500), anti-BCL-xL (1:500), anti-BAK (1:1000) and HRP conjugated anti-Actin purchased from Santa Cruz Biotechnology, Anti-active caspase-3 (1:1000) from BD pharmingen. Primary antibodies were incubated overnight at 4°C according to the manufacturer's instructions. Secondary anti-rabbit (Santa Cruz Biotechnology®), anti-goat (Santa Cruz Biotechnology®) or anti-mouse (Sigma®) antibodies conjugated to HRP were used at 1:2000 dilutions for 1h at RT and visualized by enhanced chemiluminescence (Pierce™ ECL 2 Western Blotting Substrate, Thermo Scientific™).

Analysis of patient data

The mRNA expression was analysed by (Agilent, Affymetrix HuEx, Affymetrix U133A. Ovarian Serous Cystadenocarcinoma TCGA dataset (<http://cancergenome.nih.gov/>) was obtained via cbiportal (<http://www.cbiportal.org>) (51,52). Clinical data, main molecular features, NTSR1 and NTS mRNA z-scores (RNASeqV2), available in 491 cases (53).

Population, Clinical Data, and Tissues

Forty-six consecutive cases of ovarian tumor tissues with the main clinical data were retrospectively retrieved from the files of the Department of Pathology (CHU Cochin Port-Royal, AP-HP, Paris, France). Clinical and histological characteristics of the patients are detailed in table 1 supplementary data. Ten nonmalignant ovarian samples were also retrieved (CHRU of Nancy, Nancy, France).

The histological subtype, the grade according to the criteria of the WHO

classification, neoplastic emboli, local and nodal invasion, were reviewed by two experienced pathologists (GG, PAJ).

Immunohistochemistry

For all cases, histologic slides of primary tumors were obtained by paraffin wax embedded tissues. Deparaffinized tissue sections (5 μ m) were subjected to heat-induced epitope retrieval in citrate buffer (pH 6.0). The sections were labeled for the target proteins using the avidin-biotin-peroxidase complex method as described in (54), see detail in supplementary data. All slides were scored by pathologist (GG). Cytoplasmic staining for NTSR1 and NTS were evaluated using a semi-quantitative score. The staining intensity was graded as following: 1, weak; 2, medium; and 3, strong. A score was obtained by multiplying the percentage of positive cells by the intensity level (range: 0-300). Samples with score < 50 were considered as negative or weak, and samples \geq 50 moderate or strong.

Ethic

Informed consent was obtained from all patients before surgery. The database including pathological variables was established in accordance with the French data protection authority.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc. La Jolla, USA). Statistical analysis was carried out using t test or Bonferroni Multiple Comparisons Test: **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. A p value of less than 0.05 was considered significant.

The comparison of NTS and NTSR1 mRNA z-scores or IHC semi-quantitative score between clinicopathological groups were performed using the U-Mann Whitney test (two groups) or Kruskal-Wallis test with Duns post-analysis (more than two groups). A p-value of 0.05 was accepted as significant. These statistical analyses were conducted using the R 2.3.3 software.

Supplementary data

RNA extraction

Total RNA was extracted by the acidic phenol/chloroform guanidine thiocyanate. Three cycles of phenol-chloroform 5:1 extraction (pH 4.7) were preceded by two ethanol precipitations in GTC buffer (4 M Guanidium Thiocyanate, 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 30% N-lauroylsarcosine sodium salt, 1% β -mercaptoethanol) and followed by a final extraction with chloroform-isoamyl alcohol 25:1 (v/v). Two final ethanol precipitations in acetic acid and NET buffer (150mM NaCl, 15mM Tris-HCl pH 7.5, 1mM EDTA) were successively performed. After washing in 70% ethanol diluted by deionized diethyl pyrocarbonate-treated water, pellets were resuspended in 20 μ L of sterile deionized diethyl pyrocarbonate-treated water and titrated by NanoDrop® ND-1000 Spectrophotometer at 260 nm.

Primer sequences

	Forward primer	Reverse primer
<i>NTS</i>	5'-CAGCTCCTGGAGTCTGTGCT-3'	5'-AGTATGTAGGGTCTTCTGGGTT-3'
<i>NTS(qPCR)</i>	5'-ACAGCAGGGCTTTTCAACAC-3'	5'-AGGGTCTTCTGGGTTTATTCTCA-3'
<i>NTSR1</i>	5'-CCTTCAAGGCCAAGACCCTC-3'	5'-CAGCCAGCAGACCACAAAGG-3'
<i>NSTR1(qPCR)</i>	5'-CGAGCACAGCACATTTCAGCA-3'	5'-CAGCCAGCAGACCACAAAGG-3'
<i>NTSR2</i>	5'-TGACCAACACACTTTTCTAC-3'	5'-TTATTGAGCATCTACTCGC-3'
<i>NTSR3</i>	5'-TGGACAGTCCAAGCTATATCG-3	5'-CTTGTGGTA TCCTTATCAGCCA-3'
<i>BCL-2</i>	5'-GGACGGGGTGAAGTGGGGGA-3'	5'-AGTTCCACAAAGGCATCCCA-3'
<i>BCL-xL</i>	5'-GTAAACTGGGGTCGCATTGT-3'	5'-TGCTGCATTGTTCCCATAGA-3'
<i>BCL2L11</i>	5'-TCCCTACAGACAGAGCCACA-3'	5'-TCCAATACGCCGCAACTCTT-3'
<i>BAX</i>	5'-AGCTGAGCGAGTGTCTCAAG-3'	5'-TAGAAAAGGGCGACAACCCG-3'

<i>BAK</i>	5'-AGCCTGTTTGAGAGTGGCAT-3'	5'-CAGAACCACACCCAGAACCA-3'
<i>ATP7A</i>	5'-ACCCTCTAGGAACAGCCATAACCA-3'	5'-ATACCACAGCCTGGCACAACCT-3'
<i>ATP7B</i>	5'-GGACCACAACATCATTCCAGGAC-3'	5'-ATGAGCACGTCCATGTTGGCTG-3'
<i>ABCC2</i>	5'-ATGCTTCCTGGGGATAATCAGCA-3'	5'-TGCTCAAAGGCACGGATAACT-3'
<i>36B4</i>	5'-CCTGGAGGGTGTCCGCAAT-3'	5'-CAGGGGCAGCAGCCACAAA-3'
<i>18S</i>	5'-AGGAATTGACGGAAGGGCAC-3'	5'-GTGCAGCCCGGACATCTAAG-3'

Caspase activity protease assay

SKOV3 cells were plated in 150-mm dishes at the density of 3×10^6 cells/plate. Apoptosis was induced by SR48692 5 μ M, carboplatin 150 μ M or both for 48 hours then cells were lysed with 50 μ L Cell Lysis Buffer supplied in the kit and incubated on ice for 10 minutes. The lysate was centrifuged for 1 minute at 10,000x g then the supernatant was transferred to a fresh tube. Caspase activity assays were performed in 96-well plates and 50 μ g protein in 45 μ L buffer was combined with 50 μ L of 2x Reaction Buffer containing 10 mM DTT and 5 μ L of the 4 mM substrates (VDVAD (for caspase-2), DEVD (for caspase-3), VEID (for caspase-6), IETD (for caspase-8), and LEHD (for caspase-9)). After incubated at 37 °C for 2 hours, caspase activities were measured using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific) at 405 nm and the results were normalized by the protein levels.

Immunofluorescence

Cells were seeded on 12 mm-diameter glass slides for 24 hours. After treatment, the cells were fixed in 4% paraformaldehyde for 20 min at RT, permeabilized in PBS /0.1% Triton X-100 for 30 min, and saturated in PBS /0.1% Triton X-100 containing 10% horse serum for 20 min. Cells were then incubated overnight at 4 °C with the primary antibody diluted in PBS/0.1% Triton X-100 containing 1.5% horse serum. The primary

antibodies included anti-NTS (1:200, mouse monoclonal anti-NTS long fragment), anti-NTSR1 (1:200, SC-7596, Santa Cruz Biotechnology), anti-phospho-Histone H2A.X(Ser139) (1:400, 05-636, Merck Millipore) anti-ATP7A (1:500, SC-376467, Santa Cruz Biotechnology). Then the slides were incubated for 1 hour with a fluorescent secondary antibody (1:1000) including Alexa Fluor 488 conjugated donkey anti-goat IgG(H+L) (A-11055, Invitrogen) and Alexa Fluor 555 conjugated donkey anti-mouse IgG(H+L) (A-31570, Invitrogen). Nuclei were counterstained for 5 min with DAPI 1:50000 then mounted using ProLong® Gold Antifade Reagent (Life technologies).

Immunohistochemistry

Five µm paraffin sections were immersed in a 10mM sodium citrate buffer (pH 6) for 20 minutes at 97°C for dewaxing and antigen retrieval. Staining for NTSR1 was evaluated with a primary anti-NTSR1 antibody (1/50; goat polyclonal sc-7596, Santa Cruz, USA). Additionally, staining for NTS was evaluated using an antibody aiming NTS peptide (1/200 overnight; mouse monoclonal, homemade antibody). Immunohistochemistry was performed with Dako Autostainer Plus (Dako Cytomation, Glostrup, Denmark) and Flex+ Envision revelation system (Dako) in the Department of Pathology (CHRU of Nancy, France). Negative controls were used throughout the experiment.

Table 1. Clinical and histological characteristics of the patients.

Clinical data (n=46)	Mean \pm SD or percent of patients
Age (years)	56 \pm 13.8
Body Mass Index (kg/m ²)	26 \pm 3.6
Parity	1.28 \pm 1.15
<i>BRCA</i> mutation	10.8%
Menopause hormone therapy	18%
Endometriosis history	13%
FIGO Stage (n=33)	
I	18.2%
II	3%
III	48.5%
IV	30.3%
Histological type (n=33)	
Serous Adenocarcinoma	66.7%
Endometrioid Adenocarcinoma	12.1%
Clear cell Adenocarcinoma	12.1%
Mucinous Adenocarcinoma	6.1%
Mixt Adenocarcinoma	3%
Histological Grade (n=33)	
Low Grade	13%
High grade	87%

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Figures

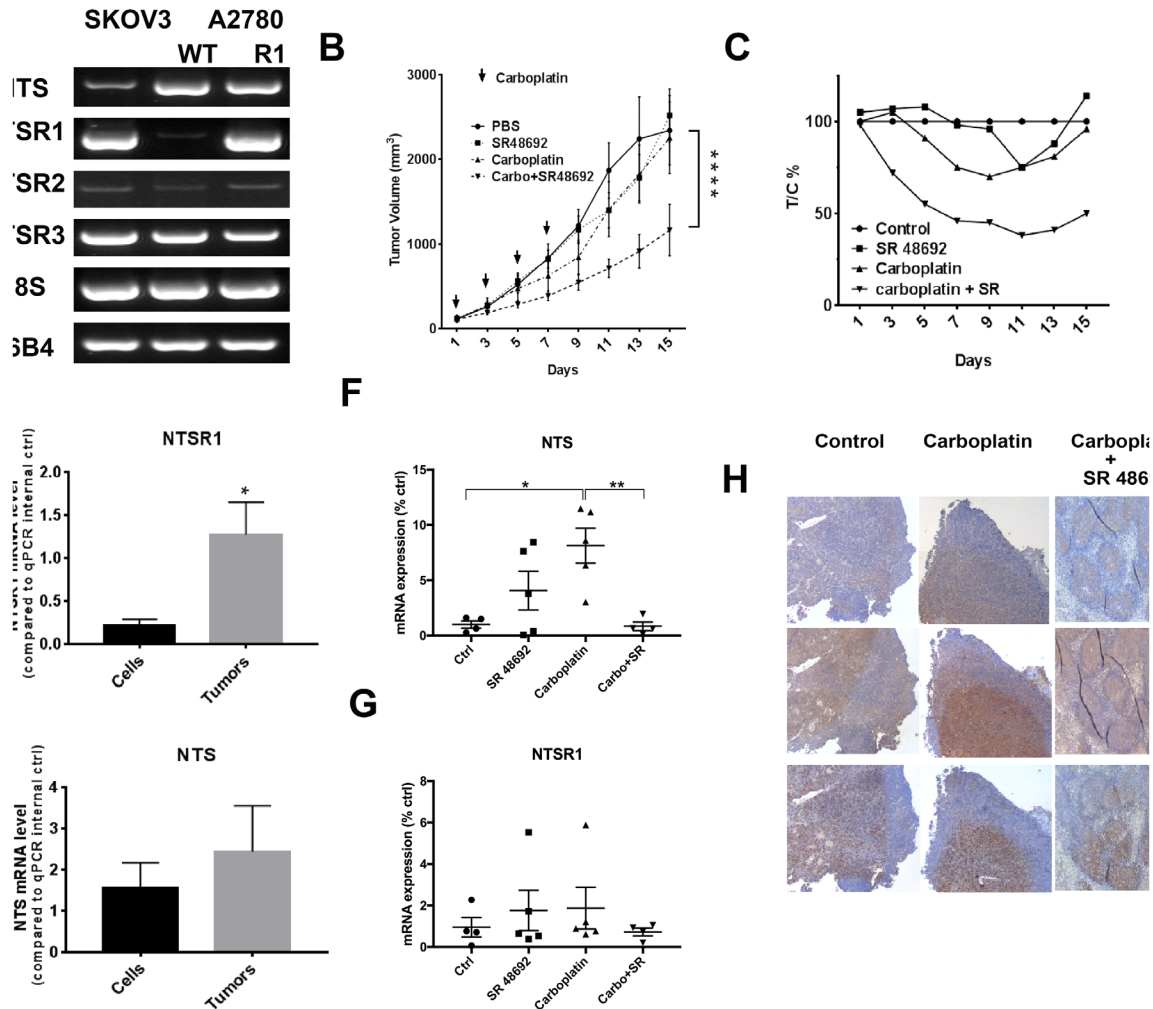


Figure 1. Contribution of NTSR1/NTS complex to the growth of ovarian experimental tumor

(A) mRNA expression of NTS and NTSR1 in ovarian cancer cell lines SKOV3 and A2780, and A2780 over-expressing NTSR1, A2780-R1(right).

(B) A2780 cells (3×10^6) were injected subcutaneously into female NMRI-Nude Foxn1 mice (n=7). When tumors reached 100 mm³, mice were treated with PBS (per os), SR 48692 (1mg/kg/day per os), carboplatin (4mg/kg, four times, i.p.) (see arrow), or with the combination of carboplatin and SR 48692. Tumor volume was calculated using the ellipsoid formula, every two days. In two-way ANOVA statistical analysis, **p<0.01; ****p<0.0001.

(C) T/C % ratio of tumor size to the average of the control tumor size.

(D) NTSR1 mRNA quantification, and (E) NTS mRNA quantification in A2780 cells and experimental tumors by quantitative RT-PCR. Results represent the mean \pm SEM of 8 independent cell and tumor extracts, *p<0.05

(F) NTS mRNA quantification, and (G) NTSR1 mRNA quantification by qRT-PCR in tumors treated with carboplatin, SR 48692 and the combination from 4 to 5 mice for each group.

(H) Example of NTS, NTSR1, and KI67 immuno-histological labelling of experimental tumors from animals treated with PBS, carboplatin, and the combined treatment at day 15.

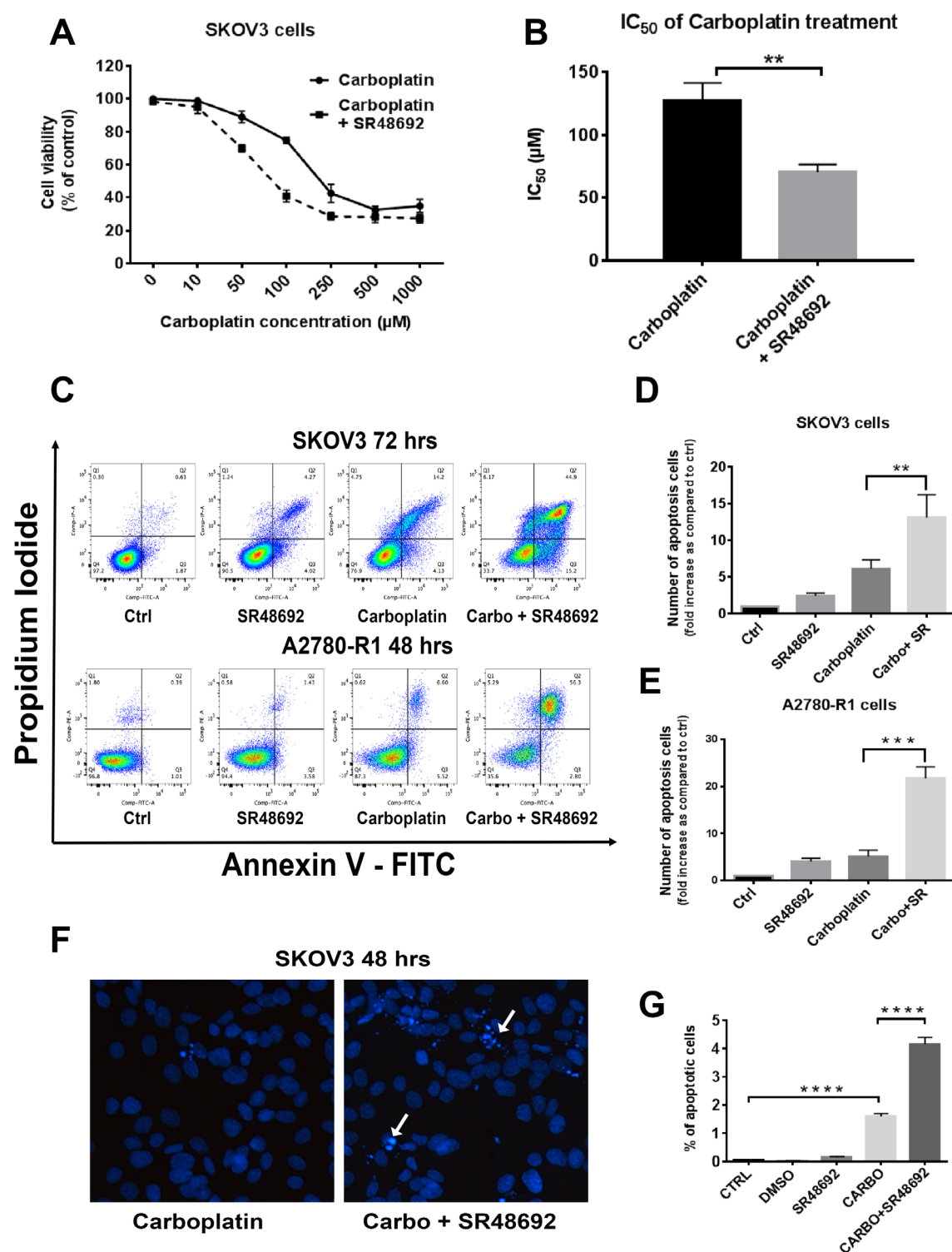


Figure 2. NTSR1 antagonist enhances cell death induced by carboplatin

(A) Effect of SR 48692 on SKOV3 cells viability inhibition induced by increasing concentration of carboplatin at 72 hours of treatment.

(B) IC₅₀ calculation of SKOV3 cells viability inhibition by carboplatin in the presence or not of 5 µM SR 48692. Results represent the mean \pm SEM of 4 independent

experiments, **p<0.01.

(C) Flow cytometry analysis of apoptotic cells determined by Annexin V/Propidium iodide dual staining. Cells were incubated with 150 μ M carboplatin corresponding cells viability at the IC₅₀ concentration, with or without 5 μ M SR 48692. Treatment was for 72 hours or 48 hours for SKOV3 cells and A2780-R1 cells, respectively.

(D) and (E) Calculation of fold increase of cellular apoptosis from flow cytometry experiments SKOV3 cells and A2780-R1 cells, respectively. The results represent the mean \pm SEM of 3 independent experiments; in one-way ANOVA statistical analysis **p<0.01, ***p<0.001

(F) Example of Hoechst 33258 staining of SKOV3 cells treated with 150 μ M carboplatin for 48 hours in the presence or not of 5 μ M SR48692.

(G) Graph represents the proportion of apoptotic cells under each condition as compared to control. 5000 cells from 8 images were counted by condition. In a one-way ANOVA statistical analysis ****p<0.0001

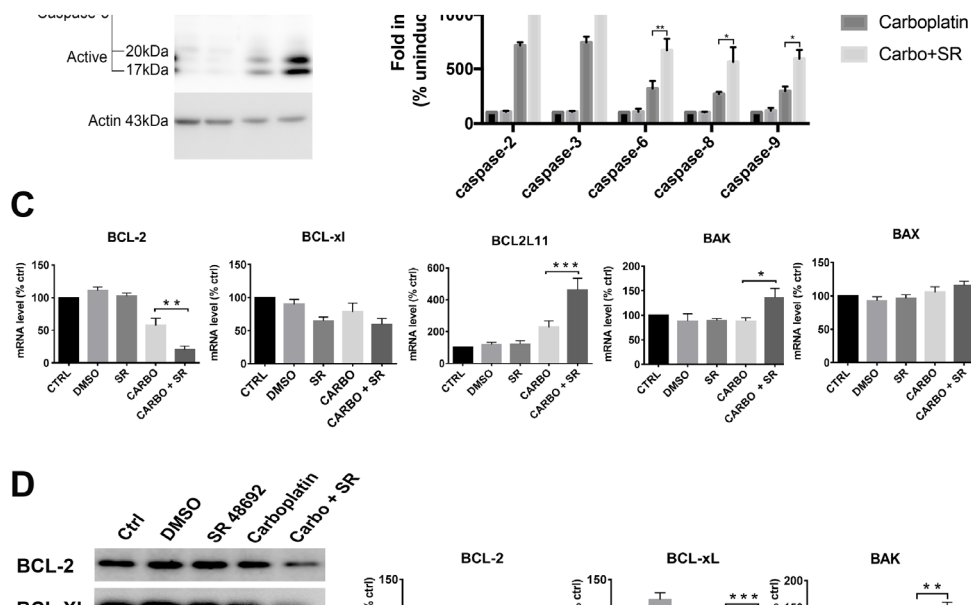


Figure 3. NTSR1 antagonist stimulates caspases activation and facilitates the apoptosis via the regulation of Bcl-2 family protein in cells exposed to carboplatin

(A) Effects of carboplatin and SR 48692 on the expression of active caspase-3

analyzed by western blot.

(B) Caspases activation by carboplatin and SR48692 determined by colorimetric protease assay kit. 50µg of SKOV3 whole cell lysates were derived from SKOV3 cells treated with carboplatin (150 µM), SR48692 (2 µM) or both for 48 h. The result is expressed as fold-increase from non-induced control cells, and represents the mean \pm SEM of 3 independent experiments; in a two-way ANOVA statistical analysis *p<0.05, **p<0.01, ****p<0.0001.

(C) The gene expression of 5 major Bcl-2 family transcripts was measured by quantitative RT-PCR. SKOV3 cells were treated with 150 µM carboplatin, 2 µM SR 48692 or both for 24 hours. 18S and 36B4 were used for the normalization. The result represents the mean \pm SEM of 3 independent experiments; in a one-way ANOVA statistical analysis *p<0.05, **p<0.01, ***p<0.001.

(D) The regulation of carboplatin and SR48692 on the expression of BCL-2, BCL-xL and BAK protein analyzed by western blot (50 µg whole cell lysate per lane). *Graph*, Semi-quantitative analysis of the band intensity analyzed with Image J software. The result is expressed as fold induction as compared to control from three independent experiments. The result represents the mean \pm SEM of 3 independent experiments; in a one-way ANOVA statistical analysis, *p<0.05, **p<0.01.

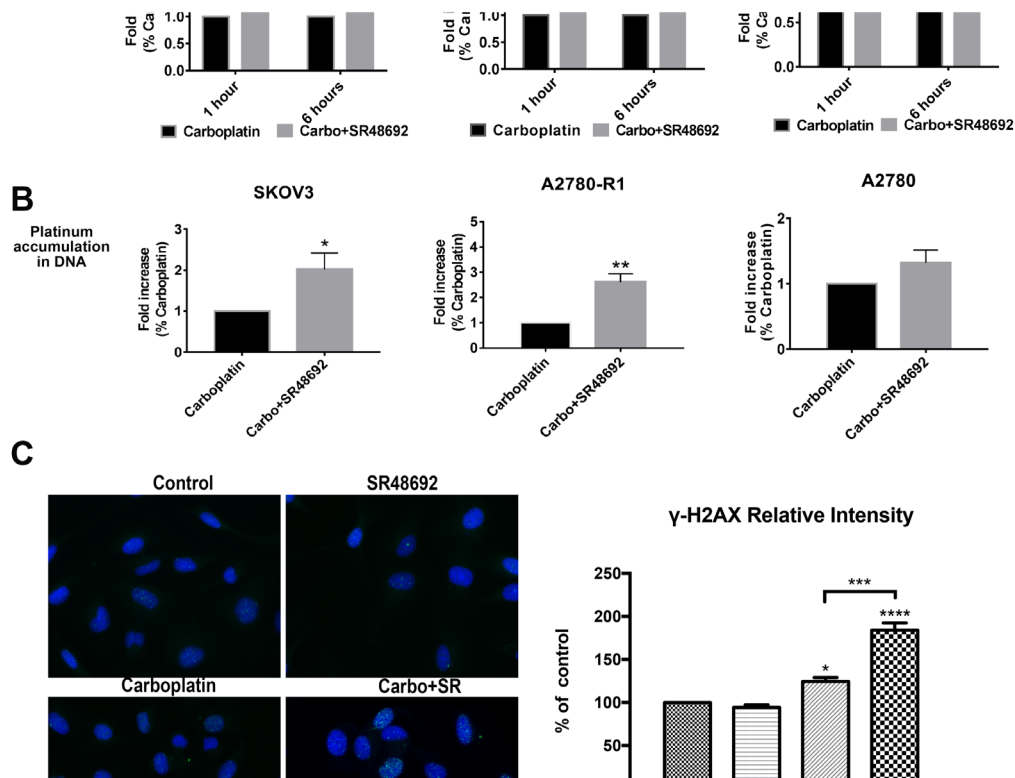


Figure 4. NTSR1 antagonist increases platinum accumulation and the DNA damage in the SKOV3 cells.

(A) Accumulation of platinum in SKOV3 whole cell extracts for 1 h or 6 h, and (B) in purified DNA of SKOV3, A2780 and A2780-R1 cells. The cells were treated with 500 μ M carboplatin without or with 5 μ M SR 48692. The platinum content was determined by inductive coupled plasma mass spectrometry. The results were normalized by the protein (whole cell accumulation) or DNA concentration (DNA accumulation) respectively. The result represents the mean \pm SEM of 3 independent experiments; in a two-way ANOVA statistical analysis * p <0.05, *** p <0.001

(C) γ -H2AX labelling in nucleus of SKOV3 cells treated by 150 μ M carboplatin in the presence or not of 5 μ M SR48692 for 6 hours. Images were taken by the fluorescence microscope (magnification x400). *Graph*, quantitative analysis of γ -H2AX staining in nucleus of SKOV3 cells. The result represents the mean \pm SEM of 3 independent experiments in which 50 cells were counted; in a two-way ANOVA statistical analysis * p <0.05, **** p <0.0001

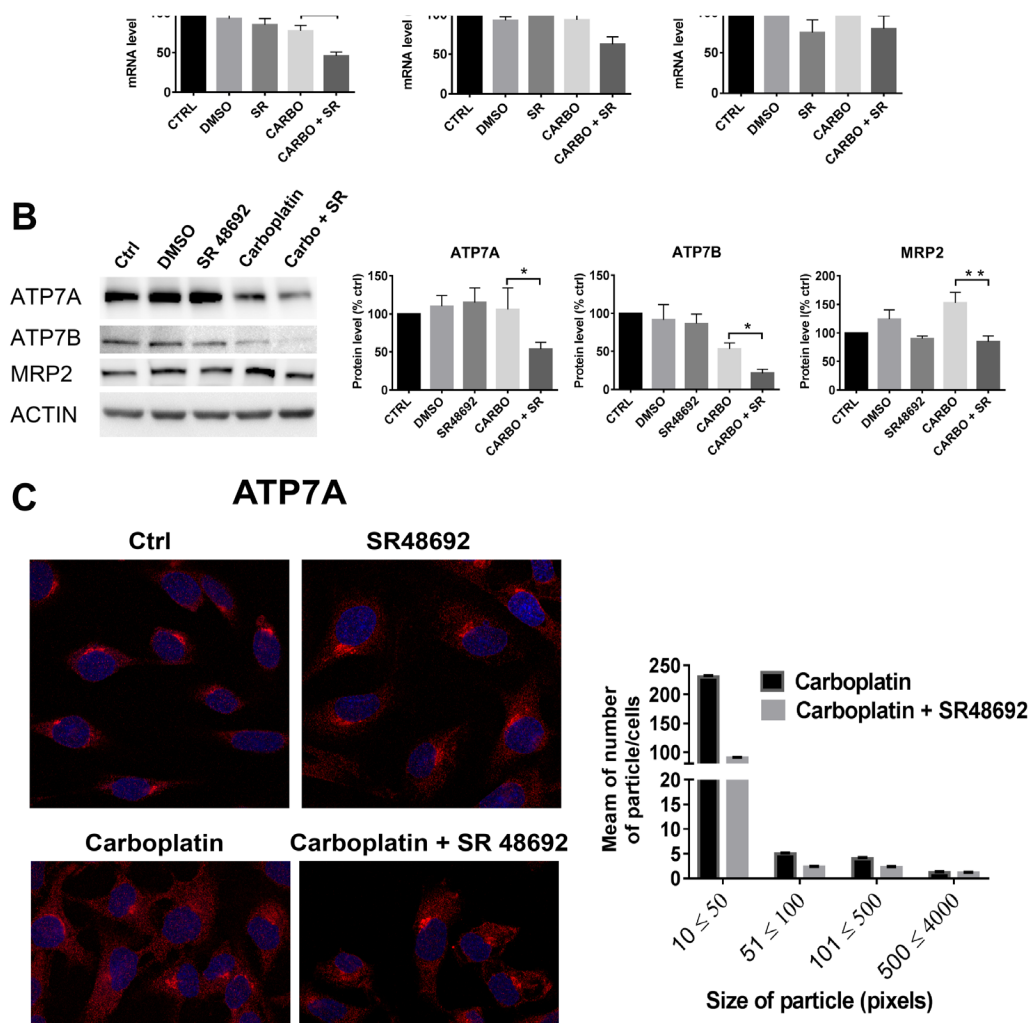


Figure 5. NTSR1 antagonist reduces the efflux of platinum in SKOV3 cells.

(A) The mRNA quantification by qRT-PCR of 3 major transporters involved in the efflux of carboplatin, ATP7A, ATP7B and MRP2. The RNA were extracted from SKOV3 cells treated with 150 μ M carboplatin, 2 μ M SR 48692 or both for 24 hours. 18S and 36B4 were used for the normalization. In a two-way ANOVA statistical analysis, * $p < 0.05$, ** $p < 0.01$.

(B) The protein expression of ATP7A, ATP7B, and MRP2 was analyzed by western blot from SKOV3 cells treated as described above. *Graph*, semi-quantitation of the band intensity, performed by Image J software, from three independent experiments. In a two-way ANOVA statistical analysis * $p < 0.05$, ** $p < 0.01$.

(C) Cellular localization of ATP7A, in SKOV3 cells after exposure to 500 μ M carboplatin with or without 5 μ M SR4 8692 for 1 hour. Cells were fixed then stained with ATP7A antibody (red) and nuclei were counterstained with DAPI in blue. *Graph*,

distribution of the red particles (ATP7A) according to their size in pixels, counting was performed in 50 cells treated with carboplatin or carboplatin and SR 48692 with Fiji software.

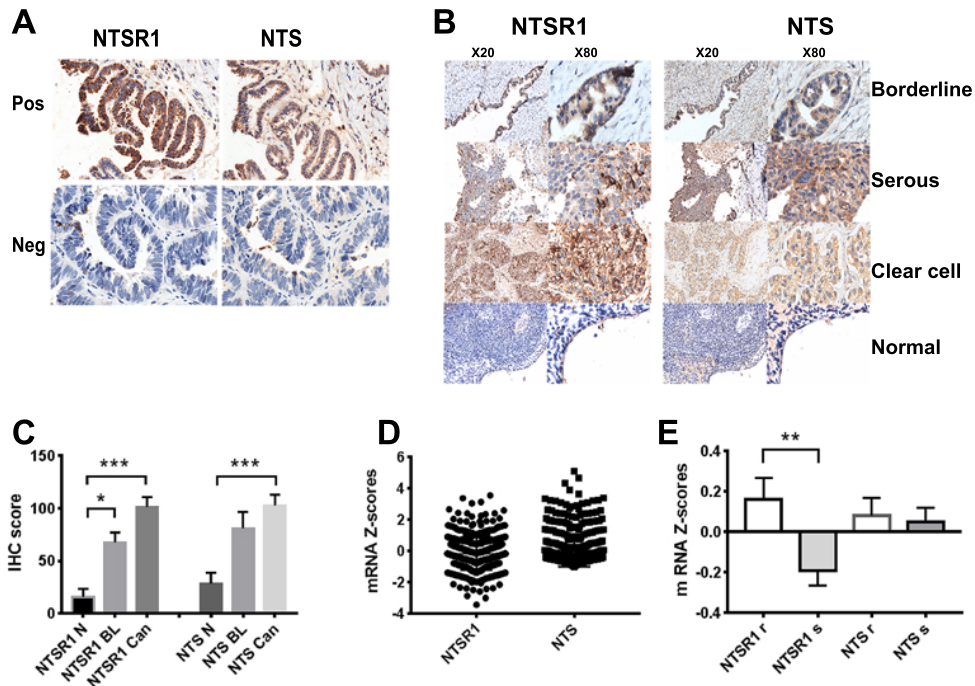


Figure 6. NTSR1 and NTS are expressed in ovarian cancer, NTSR1 expression is correlated with carboplatin resistant patient.

(A) Example of positive and negative staining for NTSR1 and NTS showed in low grade serous carcinomas.

(B) Positive staining for NTSR1 and NTS in borderline, serous, clear cell carcinomas and normal tissue. Slides were scanned with 3DHISTECH scanner and analyzed with Panoramic viewer at the X20 and X80 magnification.

(C) IHC score distribution for NTSR1 and NTS in normal tissue (N), borderline (BL), or cancerous tissues (Can).

(D) Distribution of NTSR1 and NTS Z-scores, in a cohort of 491 high-grade ovarian serous cystadenocarcinoma from the TCGA database.

(E) Within the same database, distribution of NTSR1 and NTS Z-scores within the patient resistant or sensitive to platinum salt-based therapy (287 patients).

Chapter 6. Supportive results

6.1 The mRNA level in the tumors treated with carboplatin and SR 48692

We analyzed the mRNA expression of several genes involved in the drug response and resistance, in tumors treated with carboplatin, SR 48692, or both. *BRCA1*, the main component involved DNA HR repair pathway, showed a slight decrease (not significant) in the tumors treated with carboplatin plus SR 48692 as compared to tumors treated with SR 48692, or carboplatin individually (Figure 29 top left). On the other hand, *ERCC1* in NER repair pathway was observed the same regulation as *BRCA1* but the controls for *ERCC1* were irregular (Figure 29 top right). This result suggests a diminution of DNA repair ability and thus induction of cell death when NTSR1 antagonist is combined with carboplatin.

Moreover, survivin, encoded by the *BIRC5* gene, belongs to the inhibitor of apoptosis (IAP) family. The mRNA of *BIRC5* was upregulated by carboplatin but this effect was abolished by the addition of SR 48692, indicating that SR 48692 may facilitate the apoptosis induced by carboplatin through inhibiting surviving (Figure 29 bottom left). HSPs (Heat Shock Proteins) such as HSP90 have attracted a great deal of interest as a potential anticancer target because HSPs are required for the stabilization of various proteins in oncogenesis, such as the stabilization of mutant proteins, angiogenesis, and metastasis (Shrestha et al., 2016). The stress generated by carboplatin led to an upregulation on the tumor mRNA level of HSP90 (Figure 29 bottom right). However, in the combination group, HSP90 mRNA expression was inhibited by SR 48692, suggesting that NTSR1 antagonist may counteract the protective effect of HSPs against platinum-based drugs (Galluzzi et al., 2012; Shoshan and Linder, 2008).

Although the regulation on some of these genes didn't show the statistical significance, it is noteworthy to investigate further.

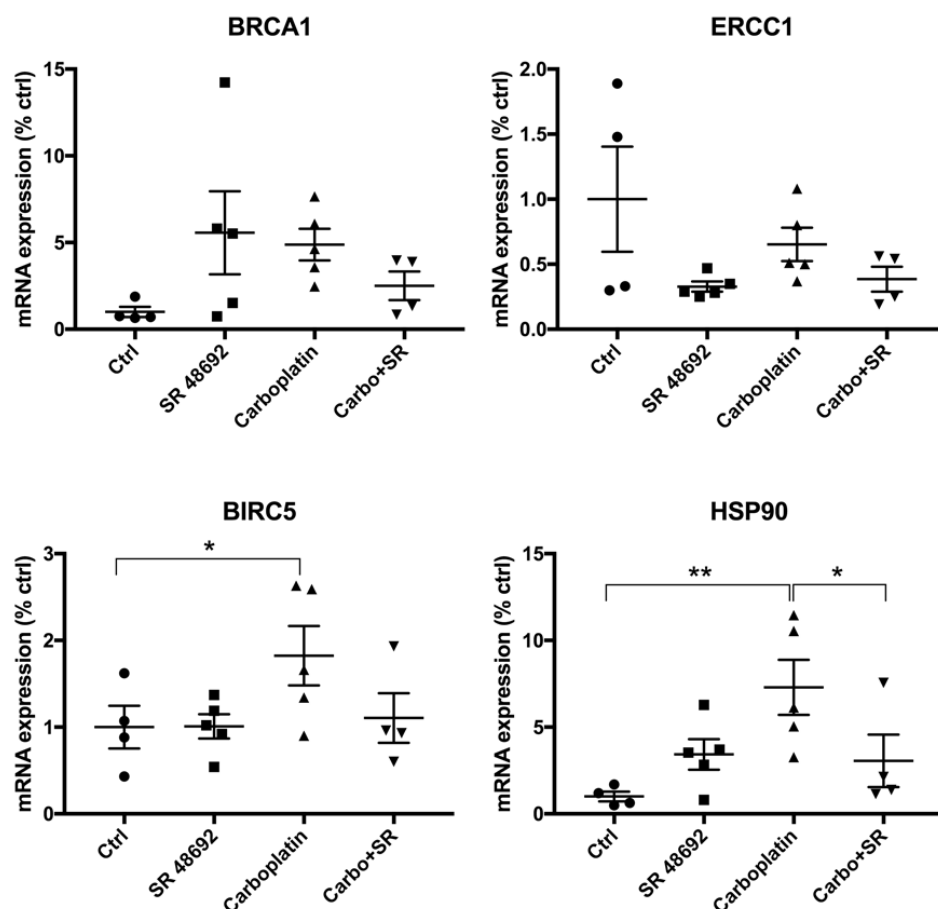


Figure 29. The mRNA expression of genes involved in DNA repair, apoptosis inhibitor and stress-response protein in the tumors treated with carboplatin, SR 48692, or both. The mRNA quantification of BRCA1, ERCC1, BIRC5 and HSP90 was analyzed by QRT-PCR in tumors treated with carboplatin (4mg/kg carboplatin every two days, 4 times totally), SR 48692 (1mg/kg/day) and the combination from 4 to 5 mice for each group, in a one-way ANOVA statistical analysis, * $p < 0.05$, ** $p < 0.01$.

6.2 Cell cycle arrest and pro-apoptosis induced by carboplatin is intensified by NTSR1 antagonist-SR 48692

DNA damage induced by platinum-based drugs influences the cell cycle progression and generates DNA damage response. We determined the cell cycle in cells treated with carboplatin and SR 48692, for 24 hours. Cells were fixed in 70% ethanol ice-cold. Cells were incubated in 1 mg/mL RNase A for 30 minutes at room temperature to

remove RNA, then stained in 50 µg/mL propidium iodide (PI) solution. The measurement was carried out by BD FACSCanto II flow cytometer and the data were analyzed in FlowJo 7.0 software.

Three phases, G1, S and G2/M were outlined by the pink curve. Green, yellow and blue surface represent G0/G1 phase, S phase; and G2/M phase respectively. A S-phase arrest (yellow surface) was observed in low- and high-dose carboplatin treated cells. More cells were accumulated at S-phase when treated with the combination. Furthermore, an obvious sub-G0/G1 peak only presented at high dose carboplatin plus SR 48692, suggesting that the apoptosis was accelerated in the presence of SR 48692 (Figure 30).

p21^(CIP1/WAF1), encoded by *CDKN1A* gene, functions as a regulator of cell cycle progression at G1 and S phase through p53-dependent or independent pathways (Gartel and Radhakrishnan, 2005). The induction of p21 by platinum-based drug causes cell cycle arrest. During this period cells can repair the damage DNA. We found that p21 mRNA level was upregulated by carboplatin around 4-fold and 8-fold by carboplatin combined with SR 48692, respectively (Figure 31 right). This result reflects that SR 48692 enhances the DNA damage induced by carboplatin and therefore cell cycle arrest and apoptosis (Figure 30).

JNK pathway plays an important role in the response to cellular stress and chemotherapeutic drugs. Pro-apoptotic signaling pathways were initially described in the NGF (nerve growth factor)-deprived sympathetic neurons. JNK pathway activation leads to the phosphorylation of the AP-1 transcription factors c-Jun and ATF2 (activating transcription factor 2). This intensify the ability of c-Jun to activate the transcription of target genes such as, *bim*, *dp5* (death protein 5), and *c-jun* itself (Kristiansen and Ham, 2014). The increased level of Bim and DP5, two BH3-only protein, promotes the mitochondrial pathway of apoptosis then MOMP (Mitochondrial Outer Membrane Permeabilization) and cytochrome C release. The JNK pathway plays an important in the apoptosis mediated by platinum-based drugs and chemoresistance (Li et al., 2005; Mansouri et al., 2003). We found that mRNA of c-jun,

consistent with bim (result shown in the manuscript), was upregulated by carboplatin around 4 fold, and 7 fold with the combination, respectively (Figure 31 left), suggesting that increased C-jun contributed to the induction of apoptosis and this effect is likely to be enhanced by NTSR1 antagonist, SR 48692.

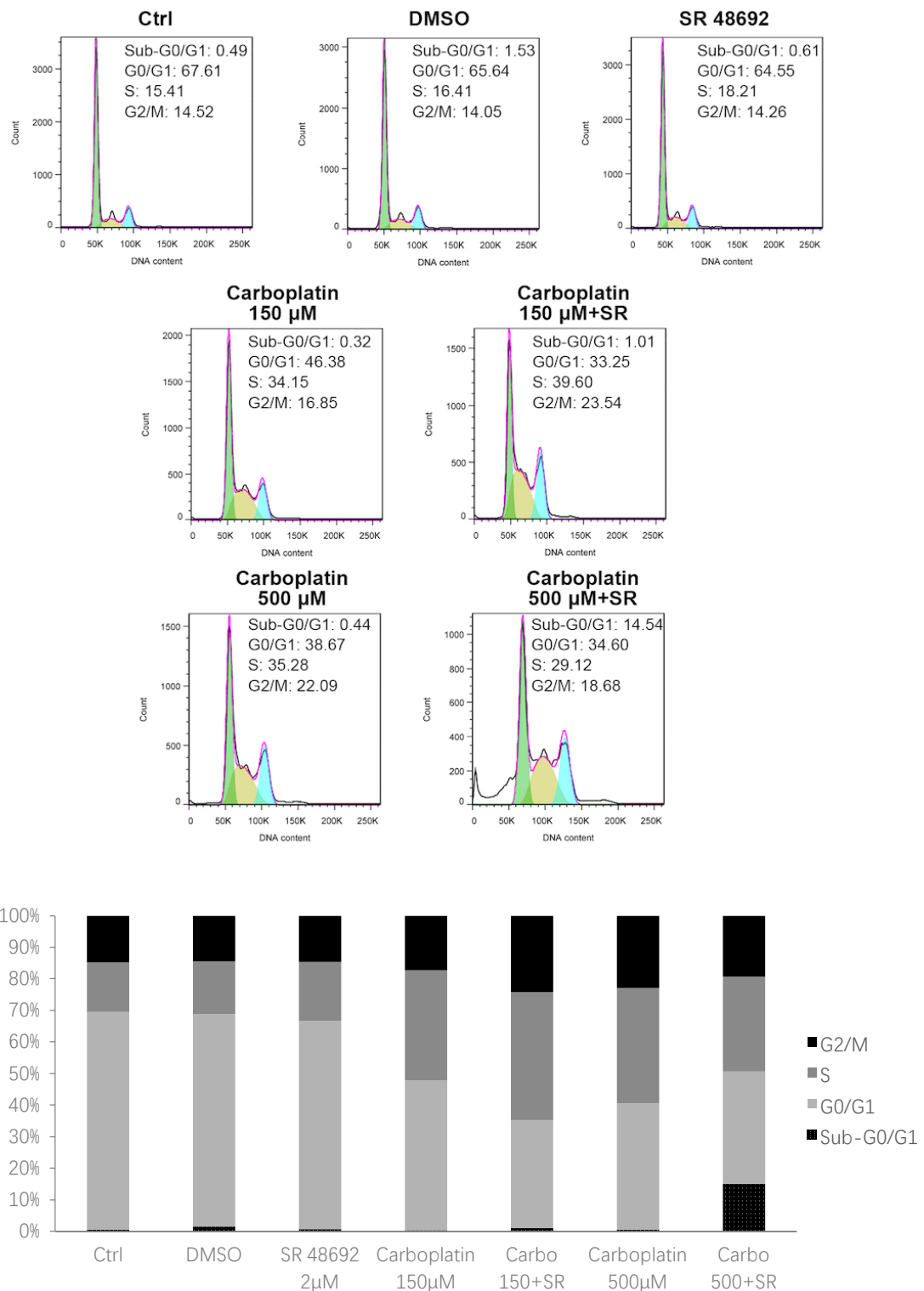


Figure 30. NTSR1 antagonist accelerates the carboplatin-induced arrest and pro-apoptosis. Cells were treated as indicated in the figure for 24 hours. Green surface - G0/G1 phase; yellow surface - S phase; Blue surface - G2/M phase. The distribution of cell cycle phase was shown in the bar graph.

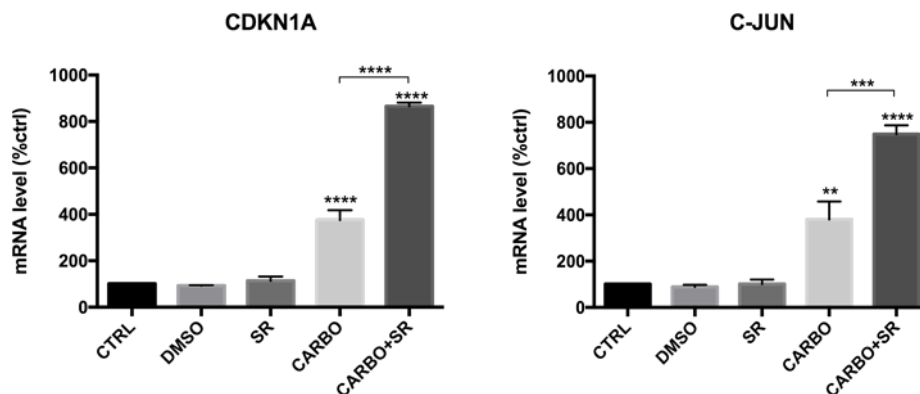


Figure 31. The mRNA expression of c-jun and CDKN1A in cells treated with carboplatin, SR 48692 and both. The mRNA quantification was analyzed by QRT-PCR in cells treated with carboplatin (150 μ M carboplatin), SR 48692 (2 μ M) and the combination for 24 hours. The result represents the mean \pm SEM of 3 independent experiments; in a one-way ANOVA statistical analysis, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.3 NTS mAb inhibits ovarian cancer tumor growth alone or in combination with cisplatin

Two NTS monoclonal antibodies were developed and successfully tested for their ability to neutralize NTS oncogenic effects. NTS mAb directed against the mature peptide, and LF NTS mAb directed against the uncleaved peptide.

We generated the experimental tumors by A2780 and mice were treated with NTS mAb (15 mg/mL every two days, i.p), cisplatin (1mg/kg, 3 times totally, i.p) and the combination. Interestingly, unlike SR 48692, the tumor growth was inhibited dramatically by NTS mAb antibody individually. The experimental tumors showed better response to antibody than cisplatin single treatment. When cisplatin was used in combination with NTS mAb antibody, the tumor growth was immediately efficient (Figure 32). This result exhibited the similar efficacy with carboplatin plus SR 48692.

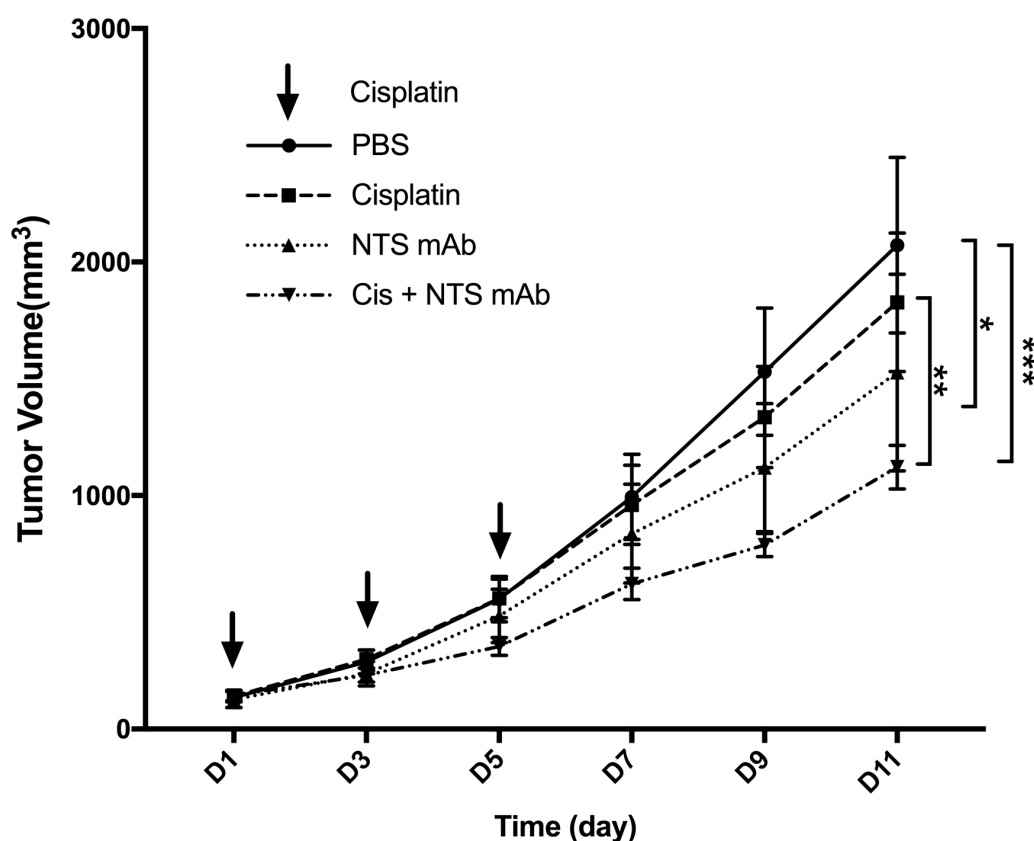


Figure 32. NTS mAb monoclonal antibody inhibits ovarian cancer tumor growth alone or in combination with cisplatin. A2780 cells (5×10^6) were injected subcutaneously into female NMRI-Nude Foxn1 mice ($n=7$). When tumors reached 100 mm³, mice were treated with PBS (*per os*), NTS mAb antibody (15mg/kg, every two day, i.p.), carboplatin (1mg/kg, four times, i.p.) (see arrow), or with the combination of cisplatin and NTS mAb antibody. Tumor volume was calculated using the ellipsoid formula, every two days. In two-way ANOVA statistical analysis, * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$.

6.4 LF NTS mAb facilitates carboplatin to generate DNA damage

Since NTS mAb showed excellent effect of anti-proliferation in experimental tumors, we analyzed the DNA damage by γ H2AX staining to investigate whether NTS mAb antibody could facilitate the drug access to DNA. γ H2AX is a marker for DNA double strand break. In the montage containing 4 pictures, barely any positive cells could be detected in the control group, while a few of scattered positive cells appeared in the antibody, or carboplatin alone treated cells. However, we observed an increased γ -H2AX labeling (number of cell labelled and intensity) in cells treated with carboplatin

combined with LF NTS mAb as compared to carboplatin alone (Figure 33 top). All the cells in each group were taken into account for the analysis. We measured the intensity of γ -H2AX labeling in the nucleus in ImageJ and compared all the results to control group. The intensity of γ -H2AX labelling was increased from 119.47 ± 9.31 % for carboplatin treated cells to 172.62 ± 20.65 % for the combine treatment as compared to the control cells (Figure 33 bottom).

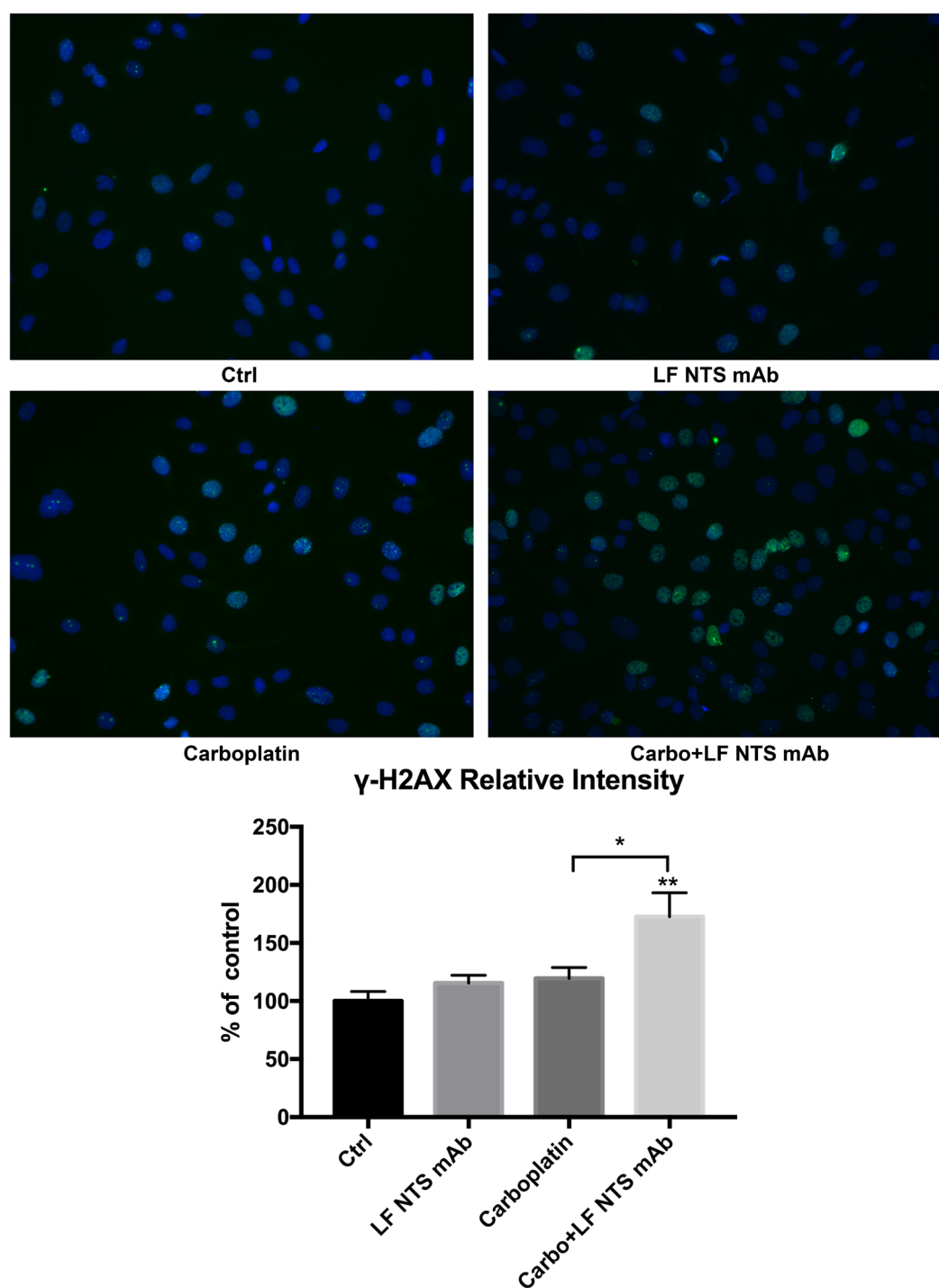


Figure 33. LF NTS mAb antibody enhances carboplatin-mediated DNA damage in SKOV3 cells. γ H2AX staining in SKOV3 cells treated with carboplatin (150 μ M carboplatin), LF NTS mAb (50 μ g/mL), or combination for 6 hours. At least five images were taken by the fluorescence microscope (magnification x200). The analysis of γ H2AX intensity was shown in the bar graph below. 300-600 cells were analyzed by condition. In a one-way ANOVA statistical analysis, *p<0.05, **p<0.01.

6.5 NTS mAb induces apoptosis in ovarian cancer SKOV3 cells

Apoptotic cells were detected by Annexin V/PI dual labeling assay in the flow cytometry performed on SKOV3 cells. A higher number of cells were detected in Annexin V positive quadrants (quadrants of top right and bottom right), when cells were treated with NTS mAb, cisplatin and the combination for 24 hours, as compared to control cells (Figure 34 left). NTS mAb induced equivalent apoptosis as cisplatin alone. A slighter increase was observed in cells treated with the combination as compared to cisplatin or NTS mAb alone. The number of apoptotic cells was increased by eight-fold for the SKOV3 cells treated with NTS mAb antibody and nine-fold for cells treated with the combination (Figure 34 right).

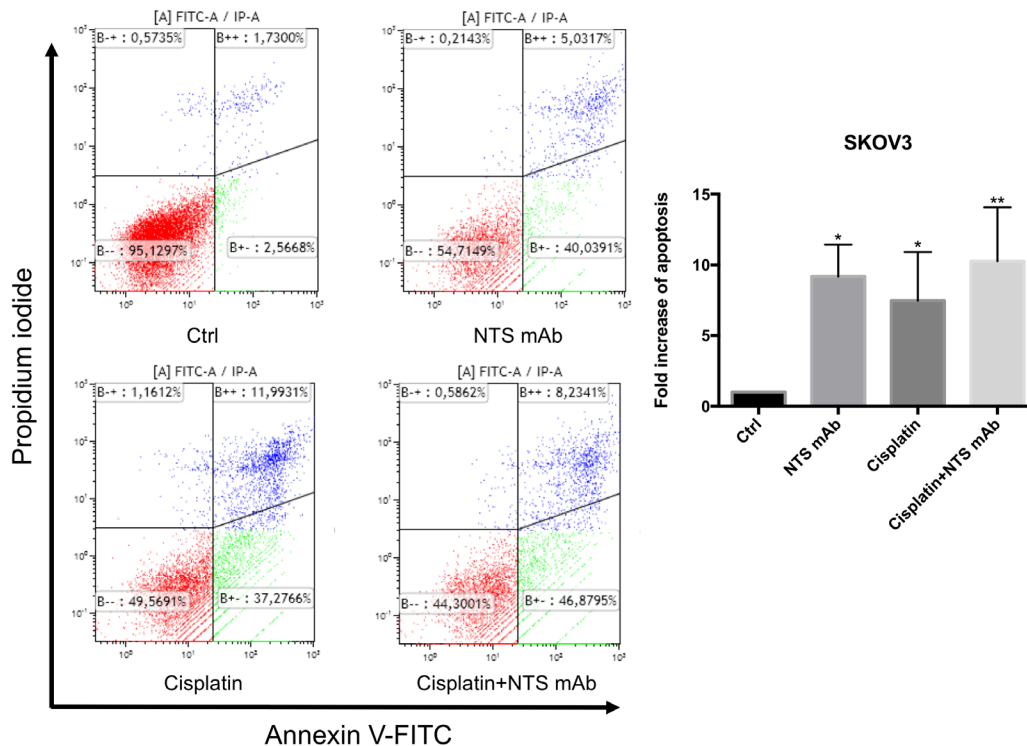


Figure 34. Flow cytometry analysis of apoptotic cells determined by Annexin V/Propidium iodide dual staining. SKOV3 cells were incubated with cisplatin (20 μ M), NTS mAb (50 μ g/mL) or both for 24 hours. *Graph*, calculation of fold increase of cellular apoptosis from flow cytometry experiments. The results represent the mean \pm SEM of 3 independent experiments; in a one-way ANOVA statistical analysis, * p <0.05, ** p <0.01 as compared to control.

General discussion

Chapter 7. General discussion

Improving the present therapeutic treatment and decreasing the mortality is the primary goal in oncology. Biological understanding of the etiology and pathogenesis of cancers aims at improving early diagnosis and patient management. This discipline, linking researchers and clinicians, ensures a continuum between cognitive biological research and clinical research. This includes the elucidation of new mechanisms of tumor progression, the research of potential diagnostic and prognostic biomarkers, the identification and validation of relevant targets for therapeutic purposes, the development of innovative therapies or new clinical protocols. Taking the ovarian cancer as an example, early detection and new therapeutic approaches are the main impediments in the treatment of ovarian cancer. Ovarian cancer is mostly diagnosed in advanced stage because this disease often has no apparent symptoms at the early stages. Therefore, understanding the genetic profile and pathogenesis are conducive to the early diagnosis and the development of targeting treatment.

7.1 The expression of NTS-NTSR1 complex in ovarian cancer

Our candidate, the complex of neurotensin and its high affinity receptor 1, has been shown to contribute to the tumor progression in several types of cancers. The pejorative correlations between the abnormal expression of the NTSR1 receptor in tumor cells, and the tumor size or the parameters of tumor aggressiveness (lymph node invasion, tumor grade and mortality of the patients), oriented us towards two potential effects of the NTS-NTSR1 complex, such as, the action on tumor growth and on the metastatic process. These two aspects were studied by our team in lung cancer, breast cancer and hepatocellular carcinoma (Alifano et al., 2010; Dupouy et al., 2014; Sehgal et al., 1994; Toy-Miou-Leong et al., 2004b; Ye et al., 2016; Younes et al., 2014)

Therefore, our first goal was to establish the expression status of NTS proform and NTSR1 in ovarian cancer. Studies performed on 46 ovarian cancer specimens, showed that 67 % of the samples were positive for both NTSR1 and NTS labeling. In addition, the expression of NTS and NTSR1 was significantly increased in higher grade

malignant specimens than in lower grade and nonmalignant specimens. In this context, the neurotensinergic system presents the characteristics of a good candidate for the development of molecules for therapeutic purposes. Our results exposed in this manuscript, indicate that the compound inhibiting NTS/NTSR1 signaling could be used in combination with other treatment. Platinum-based drugs have been used in the first-line chemotherapy for several types of cancer, such as lung cancers, head and neck cancer and ovarian cancer either alone or combined with other anticancer agents. Carboplatin is second generation of platinum salt-based agent and shows less toxic and side effects than cisplatin. Carboplatin shows equivalent efficacy as its predecessor for the treatment of non-small-cell lung cancer and ovarian cancer (Go and Adjei, 1999). However, drug resistance is a major obstacle for the therapy of ovarian cancer because most relapsed patients eventually acquire resistance to the platinum-based treatment. We analyzed platinum status of 287 patients from TCGA database, including 90 (26.1%) resistant cases and 197 (57.1%) sensitive cases. NTSR1 mRNA higher expression was significantly associated with platinum resistant status ($p=0.0076$). All these cumulative evidences suggest that the addition of NTS/NTSR1 as a potential sensitizing agent could optimize the first-line chemotherapy or platinum resistant recurrent treatment.

7.2 The enhancement of carboplatin treatment by the NTSR1 antagonist

In order to study the contribution of NTS/NTSR1 complex to the chemotherapy, we studied the synergy effect of SR 48692, the specific NTSR1 antagonist, in the ovarian cancer cells and experimental tumors. The mice were treated with sub-toxic dose of platinum salt-based therapy. The tumors generated with the A2780 cells were only weakly responsive to the platinum salt-based therapy treatment, but the addition of NTS antibody or NTSR1 antagonist to the treatment largely improved the chemotherapy response. The unexpected findings can be attributed to the increase of NTS mRNA and protein levels by platinum salt based drug in tumors. NTS/NTSR1 complex is known to confer a more aggressive phenotype to tumor cells. Therefore, NTSR1 antagonist and NTS antibody abolish both NTS overexpression induced by

platinum salt based drug and enhance the response to the drug.

In parallel, *in vitro* studies we used another cell line the ovarian cancer adenocarcinoma SKOV3 cells and A2780 cells stably expressing NTSR1, A2780-R1. We confirmed that the inhibition of cell proliferation caused by carboplatin is enhanced when NTS/NTSR1 pathway is blocked by SR48692. We also confirmed that NTS/NTSR1 complex participate in the drug response since IC_{50} for the combination of carboplatin and SR48692 was significantly higher in A2780-R1 than in A2780 wild type cells. These results corroborate our hypothesis that when the activation of NTS-NTSR1 signaling pathway is blocked, either antagonized by SR 48692 or neutralized by NTS mAb, cisplatin and carboplatin achieve better effect. More importantly, as the consequence of specific overexpression of NTS and/or NTSR1 in cancer cells, the employment of NTS mAb or NTSR1 antagonist will specifically target tumor and metastasis.

7.3 The mechanisms associated with the enhancement of platinum salt-based chemotherapy response by inhibiting NTS/NTSR1 complex

The platinum-based drugs have been characterized to induce cytotoxicity, cell cycle perturbations, and cell death in ovarian cancer cells. Our rationale was that inhibiting NTS/NTSR1 activation may improve the action of the platinum-based drug, and/or additionally induce cell death.

7.3.1 The involvement in drug uptake

Carboplatin alone or combined with taxane is the standard of treatment for ovarian cancer patients. Carboplatin is often effective when first given, however, its application is limited inevitably by the development of drug resistance. Reduced uptake and increased detoxification of drug is one of the proposed mechanisms for platinum resistance. The cytotoxic effect induced by platinum complexes is strongly related to the quantity of platinum accumulated in cells and in the nucleus. The dose response curves of carboplatin alone or the combination have provided measures for the combined drug action, indicating that antagonized NTSR1 signaling produces higher

response to carboplatin. Herein lies our first hypothesis that increased response firstly stems from increased cellular platinum accumulation.

In cells expressing both NTS and NTSR1, as in SKOV3 and A2780-R1 cells, the amount of platinum in the cell and in the nucleus, is significantly higher in the combined treatment than in the individual carboplatin treatment. On the contrary, in A2780 wild type cells lacking of NTSR1 no extra platinum accumulation could be seen when cells were exposed to the combination. This result suggests a regulation of drug uptake by NTS/NTSR1 complex. Carboplatin enters into the cell either via passive infusion, or facilitated and active uptake by a number of transport proteins. CTR1 (copper transporter 1) is the most studied transporters responsible for the platinum influx. Platinum can be extruded either via copper efflux system (ATP7A/B), or by GS-X pumps (MRP 1-5) after chelation with glutathione (Hall et al., 2008). Multidrug resistance-associated protein 2 (MRP2) belongs to ATP-binding cassette sub-family C, and is encoded by the *ABCC2* gene (Jedlitschky et al., 2006). Glutathione (GSH) can quench the DNA-platinum monoadducts before their conversion to cytotoxic DNA crosslinks and form glutathione-platinum complex. MRP2 together with GS-X pump (ATP-dependent glutathione S-conjugate export system) can effuse the glutathione-conjugated platinum from cells (Ishikawa and Ali-Osman, 1993; Ishikawa et al., 1994; Taniguchi et al., 1996). Upregulation of CTR1 or downregulation of ATP7A/ATP7B/GS-X pump have been extensively reported to contribute to the platinum resistance (Kalayda et al., 2008; Samimi et al., 2004; Taniguchi et al., 1996). Among several candidates involved in the drug influx and efflux, we found that SR 48692 or carboplatin do not influence the expression of MRP2, ATP7A and ATP7B, but blocking NTSR1 chronic activation by SR 48692, downregulates the three proteins involved in the carboplatin efflux (MRP2, ATP7A and ATP7B). However, how the neurotensinergic system interacts with platinum efflux remains an open question.

Cisplatin has been shown to trigger redistribution of ATP7A and ATP7B from the perinuclear region to the more peripherally located sites in the cytosol. Interestingly, localization of both proteins in the area close to the nucleus is fully restored within 1 h

after removal of the drug from the culture medium (Kalayda et al., 2008). Moreover, platinum-resistant cell and platinum-sensitive cell display different distribution of ATP7A and ATP7B. We found that carboplatin changes the localization of ATP7A in the cytoplasm, and cells treated with the combination showed different distribution of ATP7A and with bigger size of dispersed particles. To confirm this result, further investigation is necessary. We can use markers of TGN, lysosome and exosome which are essential for the copper and platinum transporting to better identify and quantify the cellular compartment involved. We also can determine the difference of localization of ATP7A/B when both platinum-resistant cells and platinum-sensitive cells are exposed to carboplatin alone or in combination with SR 48692.

7.3.2 The influence of the combine treatment in the Pt-DNA adducts

It is generally accepted that DNA is the primary target for platinum salt-based drugs. The increased cellular platinum accumulation directly leads to the enhanced DNA-adduct formation followed by cell cycle arrest and DNA repair. H2AX contributes to the formation of nucleosomes and, consequently, to the structure of DNA. H2AX is phosphorylated at serine 139, in reaction to double-strand breaks in DNA (DSBs) and is called gamma-H2AX.

Interstrand crosslinks can obstruct DNA replication fork progression in dividing cells resulting in the formation of DNA double strand breaks (Clingen et al., 2008). Also, intrastrand crosslinks can also induce H2AX phosphorylation due to replication arrest, resulting in fork collapse and generation of DNA double strand breaks (Kobayashi et al., 2009). Likewise, DSBs generated in the course of DNA fragmentation in apoptotic cells induce also the H2AX phosphorylation (Huang et al., 2004). Typically, phosphorylation occurs immediately after formation of DNA double-strand breaks (DSBs). Phosphorylation of histone H2AX has been used as an indicator of exposure to a variety of DNA damaging drugs. Phosphorylation of H2AX occurs preferentially in S phase cells and is elevated up after cisplatin exposure for 6 to 18 hours (Ikeda et al., 2010; Olive and Banath, 2009). γ -H2AX foci begin to appear after 6-hours treatment, and the number of cells with foci as well as the size of foci increased in cells treated

by carboplatin combined with either SR 48692 or NTS mAb. The degree of H2AX phosphorylation was relatively high in many cells treated with the combination making difficult to count individual foci. An alternative approach is to measure the γ -H2AX labelling intensity in the nuclei and calculate the relative expression by comparing to non-treated control. As expected, semi-quantitation of γ -H2AX labelling intensity in nuclei show that the blockage of the neurotensinergic system increase the carboplatin-induced DSBs. The relative expression of γ -H2AX appears to evaluate carboplatin performance by the degree of DNA damage.

Perturbation of cell cycle progression induced by platinum-based drugs occurs after the formation of Pt-DNA adducts. The regulation of cell cycle progression is complicated and the pattern of cell cycle arrest varies between cell type. Carboplatin caused an accumulation of SKOV3 cells in the S phase. When cells are exposed to higher dose of carboplatin or to the combined treatment an increase in the G2/M cell fraction was observed. More interestingly, cells treated with high dose of carboplatin and SR 48692 displayed a significant sub-G1 cell population but not in the cells treated with high dose carboplatin alone, reflecting that the combination induced superior effects on cell cycle arrest and apoptosis. p21^{WAF1/CIP1}, a CDK inhibitor, is known to negatively regulate cell cycle progression and is usually induced by p53. However, the transforming growth factor- β (TGF- β) can also induce p21^{WAF1/CIP1}. Actually, the induction of p21^{WAF1/CIP1} in many types of malignancies has been shown to be p53-independent (Ikeda et al., 2010). A substantial increased of p21 mRNA expression was noted in cells treated with carboplatin but this increase was even higher in the combined treatment group as compared to the single treatments (carboplatin or SR 48692). The overexpression of p21 eventually stops the cell growth and may play a role in the apoptosis when cells are under the stress induced by DNA damage. p21 overexpression acts as an anti-apoptotic factor in the absence of UVB-induced DNA damage but acts in a pro-apoptotic manner in the presence of UVB-induced DNA damage, displaying an “antagonistic duality” similar to other growth-promoting oncoproteins. A number reports have shown that overexpression of p21 enhances the

apoptosis in response to a chemotherapeutic agent, as cisplatin in glioma and ovarian carcinoma cell lines by both p53-dependent and -independent mechanisms (Gartel and Tyner, 2002). Overexpression of p21 in the p53-deficient human ovarian carcinoma cellular models, SKOV3 and OVCAR3 led to increased apoptosis in response to cisplatin treatment (Lincet et al., 2000). Besides, the p21 expression was shown to correlate with the induction of Bax protein (Kang et al., 1999; Li et al., 1999) and members of tumor necrosis factor family (Goke et al., 2001; Hingorani et al., 2000). These findings along with our results suggest that p21 may not only participate in the cell cycle regulation but also facilitate apoptosis in our cellular model when exposed to platinum-based agent and NTSR1 antagonist.

The efficacy of chemotherapeutic drugs depends not only on their ability to induce DNA damage but also on the cell's ability to detect and respond to DNA damage. Following DNA damage, cells may either repair the damage and start progressing through the cell cycle or induce cell death if they cannot repair the damage (Basu and Krishnamurthy, 2010). The fate of cell directly depends on whether cells succeed in recognizing and repairing the Pt-DNA adducts. Cisplatin-resistant cells acquire the ability to repair adducts at an increased pace, or become able to tolerate unrepaired DNA lesions (Galluzzi et al., 2012). The increased repair proficiency contributes to the development of drug resistance, such as the increased NER and HR expression level. We didn't conduct intensive study in terms of DNA repair, but we have been encouraged by some preliminary results. The studies on ERCC1 and BRCA1 expression were included in the gene expression profile of tumors treated with carboplatin and SR 48692. The mRNA expression level of BRCA1, the main component involved in DNA HR repair pathway, showed a slight diminution in the tumors treated with carboplatin plus SR 48692 as compared to tumors treated with SR 48692, or carboplatin alone. On the other hand, ERCC1 involved in NER repair pathway follows the same regulation than BRCA1. Although the changes on these two genes are not significant, it is reasonable to infer that blocking NTSR1 activation may interfere in DNA repair pathway and thus enhance the carboplatin-induced cell death.

7.3.3 The role in the apoptosis induced by platinum-based drugs

Platinum-based agents primarily induce cell death by apoptosis when cells fail to repair. Besides, a defect in apoptotic signaling also contributes to the development of platinum resistance. It has been reported that NTS has anti-apoptotic property (Somai et al., 2002; Zhang et al., 2014). The apoptosis was detected by Annexin V/PI dual labeling assay and Hoechst staining assay and the enhancement of apoptosis was strongly linked to the blockade of NTS-NTSR1 signaling. The number of apoptotic cells (Annexin V positive cells) was significantly increased in SKOV3 and A2780-R1 cells when the combined treatment of carboplatin plus SR48692 treatment was compared with carboplatin alone treatment. This effect was confirmed by Hoechst staining assay. Although SR 48692 alone has been reported to be able to induce apoptosis in tumor cells, I didn't observe any pro-apoptotic effect when SR 48692 was applied individually. SR 48692 merely intensifies the carboplatin-induced apoptosis in cells expression both NTS and NTSR1.

It has been observed that cisplatin-induced apoptosis in ovarian cancer cells is associated with an increased level of Bax and Bak proteins (Gonzalez et al., 2001). The Bcl-2 (B-cell lymphoma-2) family plays a crucial role in the regulation of apoptosis. Bcl-2 family contains three subfamilies, the BH3-only family (Bid, Bim, Bad), the anti-apoptotic family (Bcl-2, Bcl-xL) and pro-apoptotic family (Bax, Bak). In carboplatin treated cells, SR48692 overstimulates the expression of Bim, inhibits Bcl-2 expression and stimulates Bak and Bax expression. BH3-only proteins promote apoptosis by directly or indirectly activating Bak and Bax, leading to MOMP (Mitochondrial Outer Membrane Permeabilization). In the direct activation model, certain BH3-only proteins, particularly the truncated form of BID (tBID), BIM and perhaps PUMA, directly engage and activate the pro-apoptotic effectors BAX and BAK. BH3-only proteins that lack this direct 'activator' function, such as BAD, are designated 'sensitizers' to indicate that they exclusively engage pro-survival BCL-2 family members, thereby releasing bound BH3-only activator proteins (Czabotar et al., 2014).

As noted in chapter 2, JNK pathway plays an important role in the response to

cellular stress and chemotherapeutic drugs. Pro-apoptotic signaling pathways were initially described in the NGF-deprived sympathetic neurons. JNK augmentation leads to the phosphorylation of the AP-1 transcription factors c-Jun and ATF2 (activating transcription factor 2). This increases the ability of c-Jun to activate the transcription of target genes such as *bim*, *dp5* (death protein 5) and *c-jun* itself (Kristiansen and Ham, 2014). The increased level of two BH3-only protein, Bim and DP5, promote the mitochondrial pathway of apoptosis then the MOMP (Mitochondrial Outer Membrane Permeabilization) and cytochrome C release. On the other hand, elevated AP-1 activity also can stimulate transcriptional activation of *FasL* which promotes the apoptosis in a Fas-induced apoptotic pathway (Jia et al., 2008; Roos and Kaina, 2006). The simultaneous overexpression of *c-jun* and *bim* indicates a potential cooperation between these factors to induce apoptosis, and this effect is enhanced in the combination treatment. In this context, it is worth studying JNK pathway and its potential targets to understand its role in the response to chemotherapeutic agents.

MOMP and the release of cytochrome C from mitochondria consequentially induces the formation of apoptosome and the activation of caspase (Akiyama et al., 2009). The activation of caspase cascade leads to the irreversible cell death in response to carboplatin. SR48692 overstimulates both the initiator (caspase-2,8,9) and executioner (caspase-3,6) caspases activated by carboplatin. Caspase-2 and -3 are prominently upregulated by carboplatin and SR 48692. Caspase-3, the primary downstream effector, was observed more pronouncedly stimulated in cells treated with the combination than carboplatin alone. Caspase-2 is unique member in caspase family and it displays dual functions of both initiator- and effector- caspases. Caspase-2 plays an important role of apoptosis in the response to DNA damage or other cellular stress signaling. Caspase-2 acts upstream of MOMP and is possibly activated by different complexes, such as the PIDDosome (protein scaffold formed in response to DNA damage) or DISC (death-inducing signaling complex). Once activated, caspase-2 cleaves and activates BID and this causes MOMP and cyt-c release. On the other hand, p53 activates BH3-only protein and BAK or BAX activation lead to MOMP, cyt-

c release, formation of the APAF1 apoptosome, which in turn activates caspase-9 and initiates the caspase activation cascade, such as caspase-2 and caspase-3 (Kumar, 2009). In our models, caspase-2 activation plays a critical role in the apoptosis in response to DNA damage. Caspase-2 acts in the p53-independent pathway in SKOV3 cells and it may activate MOMP and cyt-c then activate the effector caspase.

We can conclude from our studies that the blockage of NTS/NTSR1 signaling improves response to platinum salt-based chemotherapy and enhance its efficacy in ovarian cancer. We already confirmed from several aspects involved in the drug action and thorough research can be placed on the participation in the platinum resistance.

Chapter 8. Conclusion and perspectives

In our studies, we have shown that SR48692 enhanced the response to chemotherapy in ovarian cancer cells and experimental tumors. After the investigation of several actions involved in the process of platinum-based drugs, we confirm that the addition of NTSR1 antagonist augments the intracellular platinum accumulation, increases the DNA damage and enhances apoptosis. Additionally, the results from NTS antibodies also show similar effects as NTSR1 antagonist. NTS antibody inhibits the growth of ovarian experimental tumors, increases DNA damage and apoptosis. In the clinical context, the high expression of NTS and NTSR1 is correlated to ovarian cancer at higher stages and high level of NTSR1 is linked to the platinum resistance. It is worth of studying that whether the NTS/NTSR1 complex influences platinum resistance.

Based on our studies in ovarian cancer together with our previous published results, we propose that the neurotensinergic system could use as a marker for cancer progression and a therapeutic target in ovarian cancer. As a whole, all the results suggest a combination of NTS or NTSR1 inhibitor with platinum salt-based agents for ovarian cancer to achieve better efficacy as the primary treatment. Besides, NTS or NTSR1 inhibitor may also sensitize tumor cells which are resistant to platinum because the several aspects involved in the action of carboplatin investigated in our studies also are related to the development of platinum resistance. NTS or NTSR1 inhibitor is also could be used in the platinum-resistant recurrent treatment but this need to be investigated thoroughly in the future.

Annexe

Article 1

Activation of EGFR, HER2 and HER3 by neurotensin/neurotensin receptor 1 renders breast tumors aggressive yet highly responsive to lapatinib and metformin in mice

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Activation of EGFR, HER2 and HER3 by neurotensin/neurotensin receptor 1 renders breast tumors aggressive yet highly responsive to lapatinib and metformin in mice

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ABSTRACT

A present challenge in breast oncology research is to identify therapeutical targets which could impact tumor progression. Neurotensin (NTS) and its high affinity receptor (NTSR1) are up regulated in 20% of breast cancers, and NTSR1 overexpression was shown to predict a poor prognosis for 5 year overall survival in invasive breast carcinomas. Interactions between NTS and NTSR1 induce pro-oncogenic biological effects associated with neoplastic processes and tumor progression. Here, we depict the cellular mechanisms activated by NTS, and contributing to breast cancer cell aggressiveness.

We show that neurotensin (NTS) and its high affinity receptor (NTSR1) contribute to the enhancement of experimental tumor growth and metastasis emergence in an experimental mice model. This effect ensued following EGFR, HER2, and HER3 over-expression and autocrine activation and was associated with an increase of metalloproteinase MMP9, HB-EGF and Neuregulin 2 in the culture media. EGFR over expression ensued in a more intense response to EGF on cellular migration and invasion. Accordingly, lapatinib, an EGFR/HER2 tyrosine kinase inhibitor, as well as metformin, reduced the tumor growth of cells overexpressing NTS and NTSR1. All cellular effects, such as adherence, migration, invasion, altered by NTS/NTSR1 were abolished by a specific NTSR1 antagonist. A strong statistical correlation between NTS-NTSR1-and HER3 ($p < 0.0001$) as well as NTS-NTSR1-and HER3- HER2 ($p < 0.001$) expression was found in human breast tumors.

Expression of NTS/NTSR1 on breast tumoral cells creates a cellular context associated with cancer aggressiveness by enhancing epidermal growth factor receptor activity. We propose the use of labeled NTS/NTSR1 complexes to enlarge the population eligible for therapy targeting HERs tyrosine kinase inhibitor or HER2 overexpression.

INTRODUCTION

The mortality rate of breast cancer has been stabilized due to early detection and constant progress in therapy. Nevertheless, breast cancer remains the leading cause of cancer-related deaths among women in most of the western countries [1,2]. The progression of tumors to a metastatic disease is the primary cause of death in most patients and the main target of cancer research.

The human epidermal growth factor receptor (HERs) signaling pathways have been shown to largely contribute to this process. The success of therapies employing HERs immunotherapy or tyrosine kinase inhibitors, while limiting the progression of the disease and extending the disease free survival time, demonstrates the contribution of HERs at the clinical level [3]. In breast cancer, where human epidermal growth factor receptor 2 (HER2) gene amplification is detected in 25% of patients [4], a monoclonal antibody targeting HER2, trastuzumab, (Herceptin) significantly improves the survival of these patients [5]. Nevertheless, contribution of the HERs in breast cancer progression is complex, and studies have focused more specially on HER3/HER2 or HER2/EGFR dimers. HER3 and HER2 overexpression and activation driven growth and metastasis are associated with a worse prognosis [6,8]. The availability of the EGFR inhibitors, proposed to patients in association with HER2 immunotherapy, has been a real benefit [9]. For example, patients with metastatic breast cancer, which had experienced progression under trastuzumab, found a benefit from the association of trastuzumab and Pertuzumab [5], a recombinant humanized monoclonal antibody binding to the HER2 dimerization domain and preventing dimerization of HER2 with other HER receptors (HER3, HER1, and HER4) [10]. More recently, investigations have focused on autocrine HERs activation in cells lacking HER2 amplification. This cellular context generates regulatory mechanisms involved in cancer progression and is suspected to induce drug resistance. These mechanisms remain to be clarified, and the identification of factors inducing this cellular context would help to better characterize and treat breast tumor.

Amongst the factors contributing to tumor aggressiveness, neurotensin (NTS) and its cognate high-affinity receptor (NTSR1) have been shown to contribute to breast cancer progression [11,12]. High proportion of pro NTS in the blood is also correlated with a higher risk of breast cancer [13]. NTS and NTSR1 are concomitantly overexpressed in patients with breast cancer [14]. The deregulated expression profile of the NTSR1 was correlated with negative prognostic parameters such as tumor size, the number of invaded lymph nodes, Scarff, Bloom and Richardson's histoprognostic grade, and patient mortality [14]. In corroborating clinical experiments, a direct effect of the neurotensinergic system on breast tumor growth in mice, and the anti-apoptotic

property of NTS on breast cancer cellular models was also shown [15,16].

Under physiology, NTS, a 13 amino acids peptide, is mainly localized in endocrine N-cells of the gastrointestinal tract, where it regulates various functions such as inhibition of gut motility and gastric acid secretions, stimulation of pancreatic and biliary secretions and facilitation of fatty acids intake [17,19]. The effects of NTS are mediated by three subtypes of receptor. NTSR1 and NTSR2 belong to the class A GPCRs [20] and exhibit high (sub-nanomolar) and low (nanomolar) affinity for NTS, respectively. NTSR3 or gp/95/sortilin is a single transmembrane domain receptor. The NTSR1 often overexpressed in tumors, activates many physiological effectors participating in cell proliferation, migration, invasion and remodeling of the actin cytoskeleton, like MAPK, focal adhesion kinase (FAK), and RhoGTPases. NTSR1 activation also transactivates the EGFR receptor in colonic, prostatic and pancreatic cancer cell lines [21,23].

We propose that a cross-talk occurs between neurotensinergic and the HER pathways in breast tumors which effects breast cancer progression. In this report, we analyzed this hypothesis, and showed that a NTS autocrine signaling loop dramatically accelerates the tumor growth and the metastatic progression of a poorly aggressive breast adenocarcinoma cell line. We analyzed the oncogenic cellular effects enhanced by NTS, and showed a possible synergic interaction between EGF and NTS, and observed a complete remodeling of HERs basal and activated profiles in cells under the constant stimulation by NTS.

RESULTS

The NTS-NTSR1 complex enhances tumor growth and metastasis emergence from breast experimental tumor

MCF-7 cells, which constitutively express NTSR1, were transfected with the neurotensin full length coding sequence to evaluate the influence of an autocrine neurotensinergic signaling loop on the tumorigenicity of the ER-positive breast cancer. Amongst the selected clones, two showed a differential NTS expression based on transcription levels the NTS high expressing clone, NTS-h, and the NTS low expressing clone, NTS-l (Figure 1A inset). The NTS expression levels were confirmed by quantitative RT-PCR in both clones, showing a 500-fold and a 4-fold NTS transcript's induction for NTS-h and NTS-l, respectively as compared to the MCF-7 parental cells (Figure 1SA). High expression levels of NTS alter the cell morphology by reducing the size of the cytoplasm. The nucleocytoplasmic ratio is similar for NTS-l and parental cell lines (1/3.2 and 1/3.3 respectively) whereas the ratio decreases to (1/2.5) for NTS-h cells (Figure 1S B). The size of the nucleus remained similar for the three cell

lines (Fig 1S B). Immunofluorescent staining experiments show the sub-cellular localization of NTS (Figure 1S C - panels 1, 2, 3), and NTSR1 (Figure 1S C - panels 4, 5, 6). In wild type cells, no or an extremely weak NTS labeling is noted. In the NTS-l cells, NTS immunoreactivity is dispersed in small dots throughout the cytosol. The same pattern is observed in NTS-h cells with larger dots of stronger intensity due to the higher NTS expression level. In parallel, in MCF-7 cells, NTSR1 labeling is localized at the cell membrane, whereas an intense intracellular granular labeling of an endocytosed receptor is seen in NTS-h cell. NTS-l cells show a dual pattern of NTSR1 localized both at the membrane and inside the cytoplasm.

To evaluate the influence of the NTS autocrine signaling loop on tumor growth, the MCF-7 parental line and the two NTS-overexpressing clones, NTS-l and NTS-h, were xenografted in the mammary fat pad of female athymic mice. Tumor growth rose with NTS expression levels (Figure 1A). The group bearing the MCF-7 cells began to develop tumors after 77 days to reach a small size of $108 \pm 56 \text{ mm}^3$ at day 105. During the same period of time mice bearing NTS-overexpressing cells developed much bigger tumors, with a volume of $483 \pm 102 \text{ mm}^3$ for NTS-l at day 105 and $1239 \pm 251 \text{ mm}^3$ for NTS-h at 91 days. The corresponding tumor weights were $0.2 \pm 0.1 \text{ g}$, $1.0 \pm 0.2 \text{ g}$ and $2.0 \pm 0.2 \text{ g}$, respectively. None of the animals xenografted with MCF-7 cells developed metastases during the experiment (Figure 1B). On the contrary, metastases were observed in 41% of the NTS-l group and 76% of the NTS-h group. The metastases were preferentially found in the homolateral lymph nodes (24% and 59% respectively) and the lungs (35% and 47% respectively). Furthermore, 18% of the animals in the NTS-h group also showed a metastatic spread in the contralateral lymph nodes reflecting a more advanced metastatic stage. Immunohistochemical slides representing examples of a lymph node, a lung, and a kidney metastatic lesion are shown in figure 1C.

The NTS/NTSR1 complex enhances EGFR, HER2, and HER3 expression and activation in breast cancer and tumoral cells

In order to identify the mechanisms underlying NTS induced growth and metastasis, we searched for the possible interrelation between NTS/NTSR1 complex and epidermal growth factor receptors (HERs). We first observed the amplification of HER2 and HER3 protein levels in tumors obtained after the xenograft of NTS-h and NTS-l cells, as compared to MCF-7 cells. In these cases, the membranes of the labeled tumoral cells was often thicker and more intense (Figure 2 A).

Analysis of NTS-l, NTS-h, and MCF-7 cellular protein content, showed an increase of 150–275% of EGFR, HER2, and HER3 (Figures 2B and C). This effect

was confirmed by the stronger labeling of all three HER receptors by immunocytochemistry in NTS-l cells as compared to MCF-7 cells (Figure 2D). In NTS-l cells, EGFR is accumulated in clusters close to the nucleus, while in MCF-7, EGFR is at the membrane, HER2 membrane labeling, and HER3 cytosol labeling are more intense in NTS-l than in MCF-7 cells (Figure 2D see white arrow). The specific NTSR1 antagonist, SR 48692, reduced HERs increases in NTS expressing cells, but not in wild type cells (Figures 2C and 2S A), validating the contribution of NTS/NTSR1 complex in this HERs overexpression. As HERs transcript levels were essentially the same in MCF-7 and NTS-l cells (Figure 2S B), HERs protein accumulation in the cells suggest that recycling and degradation are altered due to NTS exposure.

In parallel, an increase in the activation states for all three receptors was observed in NTS-l cells. The phosphorylation levels were enhanced up to 400 and 500% for EGFR and HER3, respectively (Figures 2E and F), and was partially abolished by the antagonist SR 48692 (Figure 2S C). Similar observations were made with NTS-h cells and a 200% increase was seen for the three receptors (not shown).

Subsequently, we searched for a possible EGFR and HER3 autocrine activation by the release of EGF “like” ligands from the cell membrane following a proteolysis process mediated by NTS. In the culture media from NTS-l cells, the amount of HB-EGF was twofold higher than MCF-7 cells. The presence of SR 48692 in the culture media abolished this increase (Figure 3 A). In the same vein, a stronger release of neuregulin 2 could be detected in the culture media of NTS-l cells compared to MCF-7 cells, and again counteracted by SR 48692 (Figure 3 B). The release of these specific ligands for EGFR and HER3 suggests an activation of metalloproteases due to NTS exposure. MMP9 transcripts increased (Figure 3 C) in NTS-l cells as compared to MCF-7, as was the case for MDA-MB 231 cells [16]. In addition, MMP9 anchored at the membrane was activated, as a 180% increase of MMP9 released in culture media was detected in NTS-l as compared to MCF-7 cells and abolished by SR 48692 (Figure 3 D).

We confirmed the contribution of EGFR and HER2 activation in NTS induced tumor growth by treating xenografted mice with NTS-h and lapatinib. Lapatinib inhibits the tyrosine kinase activity of both HER2 and EGFR. This property is currently used in breast cancer treatment. We confirmed that NTS-h cells do not carry the following EGFR active mutations: exon 19 deletion, exon 20 insertion, exon 18 Q719A, Q719C, Q719S and exon 21 L858R, L861Q point mutations. These cells do not possess the HER2 exon 20 insertion, or the KRAS point mutations: G12D, G12S, G12C, G12R, G12A, G12V, G13D KRAS. Figures 4 A and B show that the tumor growth rate is reduced when mice are treated with lapatinib. The final tumor volume after 23 days of

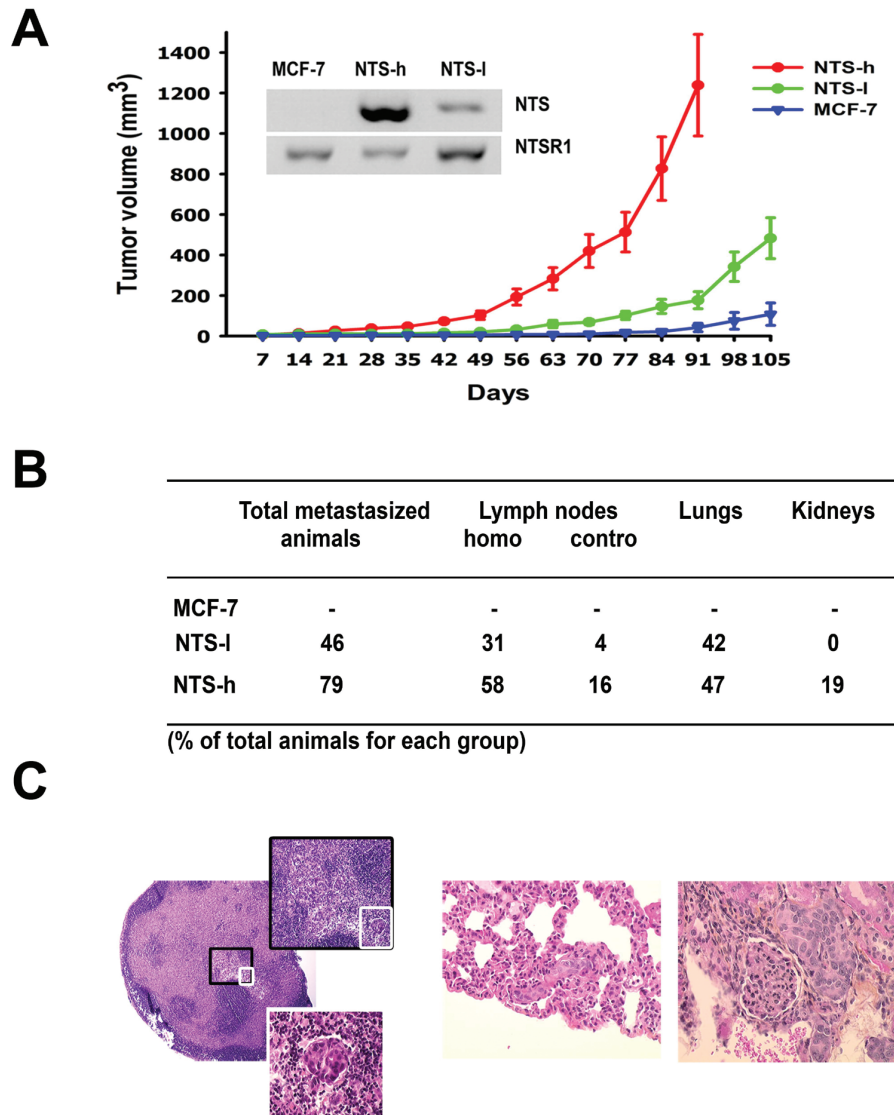


Figure 1: NTS/NTSR1 complex enhanced experimental tumor growth generated in human breast cancer cell lines. (A) Experimental tumors were generated from the breast cancer cell line, MCF-7 and the NTS-overexpressing subclones. Comparative growth curves of MCF-7, NTS-h (high NTS expression) and NTS-l (low NTS expression) cells xenografted in 10, 20, and 25 mice, respectively. Tumor volumes were measured every week. Inset, NTS and NTSR1 transcript analysis from 200ng of MCF-7, NTS-h, and NTS-l total RNA. (B) Proportion of animals with metastases, and metastases distribution within organs and lymph nodes. (C) Typical H&E staining performed on paraffin sections of (left) invaded lymph node at 50X magnification, 200X magnification (black square) or 400X magnification (white square); (middle) lung metastasis at 400X magnification; (right) kidney metastasis at 400X magnification.

treatment was $352.3 \pm 87 \text{ mm}^3$ for the control, and $134.5 \pm 44.15 \text{ mm}^3$ ($p = 0.02$ vs control) for lapatinib. We also tested metformin on our model. Metformin is suspected to provide anticancer effects in breast cancer and several clinical trials are currently under way [24]. In parallel, metformin was shown to disrupt the crosstalk between insulin receptor and NTS receptor in pancreatic cancer cells [25]. In addition to inhibiting the mTOR pathway, metformin prevents ERK activation induced by NTS and insulin [26]. In a breast cancer cellular model with NTS overexpression, metformin reduced the tumor growth with

the same efficiency as lapatinib. No additional effects were detected when both drugs were employed. The final volumes were $135.9 \pm 30.4 \text{ mm}^3$ and $177.7 \pm 51.3 \text{ mm}^3$ when animals were treated with metformin or both drugs, respectively (Fig 4 A and B). The tumor doubling time was in agreement with tumor volume with 11.7 ± 1.35 days, for controls. For lapatinib, metformin, and metformin + lapatinib double time cannot be calculated for because within the group 2 to 3 tumors shrank, the others grow very slowly. The absence of additional response suggested a common signaling cascade was targeted by both drugs.

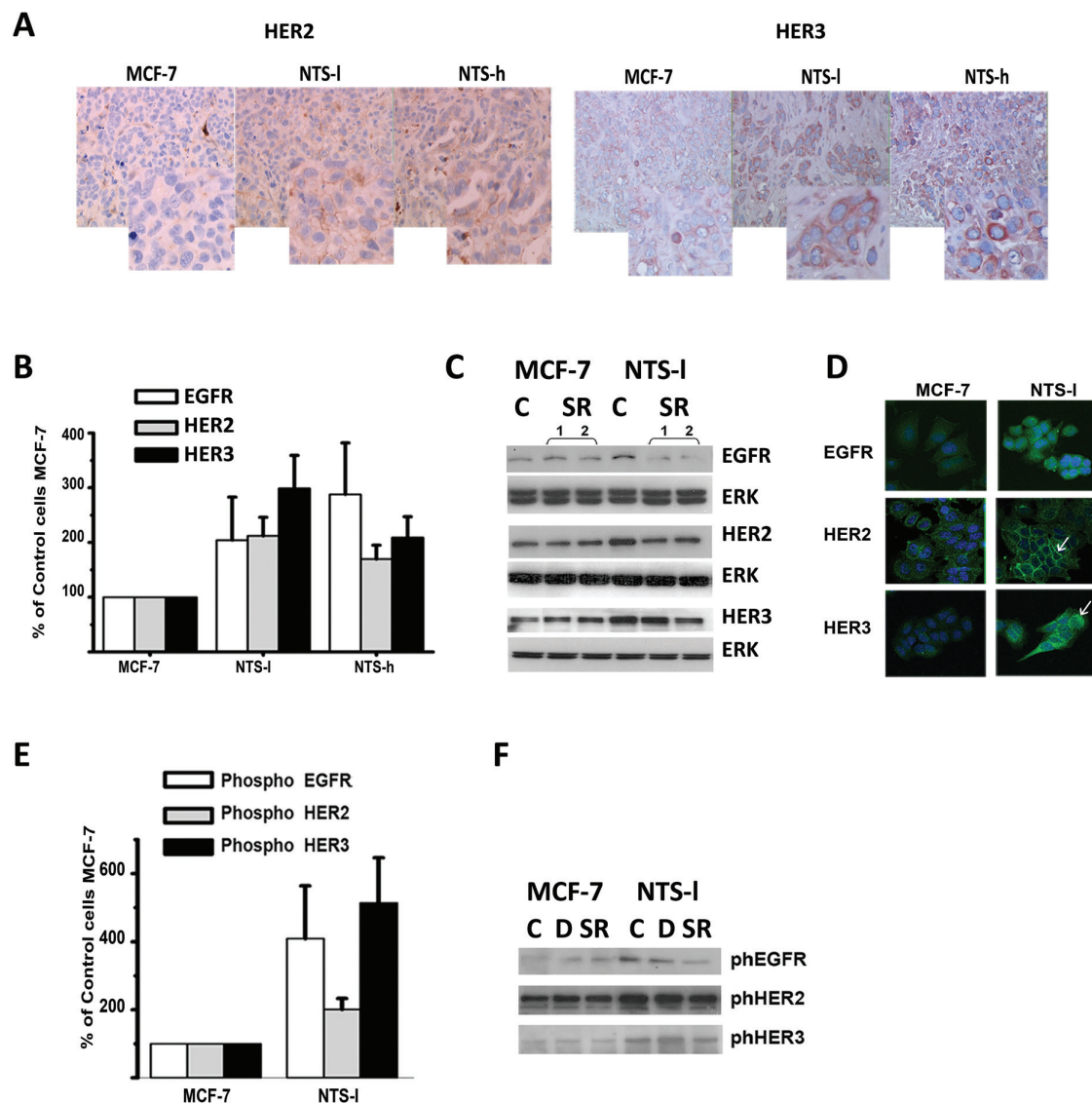


Figure 2: NTS autocrine and paracrine regulation enhanced EGFR, HER2, and HER3 basal expression and activation in human breast cancer cell lines. (A) HER2 and HER3 immunohistochemistry performed on paraffin embedded tumors from mice xenograph with MCF-7, NTS-I or NTS-h. 200X magnification and computer enlargement of specific areas. (B) Breast cancer cells MCF-7, NTS-I and NTS-h, with the histograms representing intensity-based quantification of Western blot bands of basal total protein, EGFR, HER2, and HER3, using Morpho Expert software (Explora Nova, France). Values are expressed as the percentage of the control MCF-7 cells and are the mean \pm SEM of 5 to 7 independent experiments. (C) Representative western blot analyses of EGFR, HER2, HER3 and ERK 1/2 total protein from MCF-7 and NTS-I cells treated with 5×10^{-6} M SR 48692. (D) EGFR, HER2, and HER3 immunolabeling in MCF-7 and NTS-I cells treated after 48h of seeding. (E) Breast cancer cells MCF-7 and NTS-I, with the histograms representing intensity-based quantification of Western blot bands of phosphorylated protein, EGFR, HER2, and HER3. Values are expressed as the percentage of the control MCF-7 cells and are the mean \pm SEM of 5 to 7 independent experiments. (F) Representative western blot analyses of Phospho EGFR, phosphoHER2, and Phospho HER3 protein from MCF-7 and NTS-I cells treated with DMSO or 5×10^{-6} M SR 48692 for 48h.

The NTS/NTSR1 complex enhances pro-metastatic cellular effects

We evaluated the oncogenic cellular effects impacted by NTS. The basal growth capacity of MCF-7 in an anchorage-independent context was doubled in the

presence of NTS. In MCF-7 (Figure 5A), the colonies were organized in a spherical conformation around an inside lumen delimited by a single cell monolayer in wild type cells whereas, NTS-h or NTS-I the colonies were larger and the cells forming compact spheroids, filled up with cells (Figure 3S A).

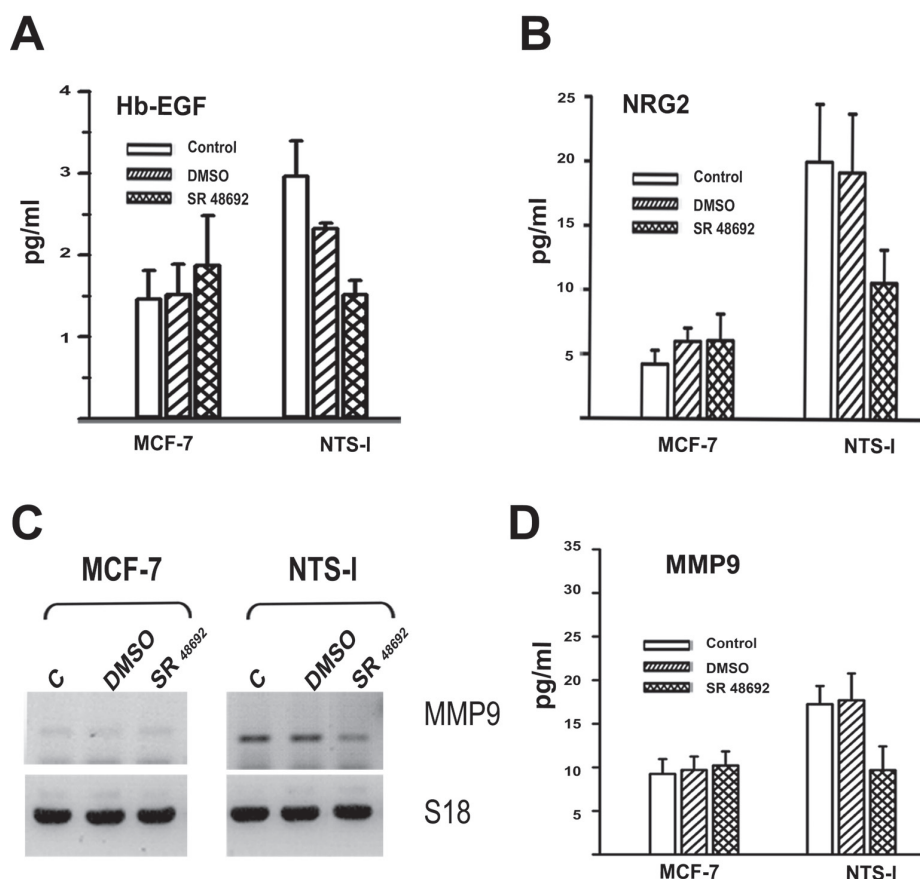


Figure 3: EGF like ligands and metalloprotease released by NTS. (A) Amount of Hb-EGF (pg/ml), assayed in 0% FCS culture media of MCF-7, or NTS-I, cells. Cells were not treated, or treated for 24h with DMSO, 5×10^{-6} M, SR 48692. Using Paired t test $p = 0.0088$ between DMSO and SR 48692 NTS-I treated cells, $n=4$; with unpaired test $p=0.03$ between MCF-7 and NTS-I, $n=5$. (B) Amount of NRG2 (pg/ml) assay in 0% FCS culture media of MCF-7 or NTS-I cells not treated or treated for 48 h with DMSO, 5×10^{-6} M SR 48692. Using Paired t test $p = 0.005$ between DMSO and SR 48692 NTS-I treated cells, $n=5$; with unpaired test $p=0.016$ between MCF-7 and NTS-I, $n=4$. (C) MMP9 transcript analysis of total RNA from MCF-7, and NTS-I treated with DMSO or 5×10^{-6} M SR 48692 for 48h. (D) Amount of MMP9 (pg/ml) assay in 0% FCS culture media of MCF-7 or NTS-I cells not treated or treated for 48 h with DMSO, 5×10^{-6} M SR 48692. Using Paired t test $p = 0.005$ between DMSO and SR 48692 NTS-I treated cells, $n=5$; with unpaired test $p=0.003$ between MCF-7 and NTS-I, $n=5$.

Cell detachment is a pre-requisite in metastatic spreading. For this purpose, we studied the adhesion ability of our cellular models on various matrices. Cells were initially detached from their support and the kinetics of reattachment was evaluated. After 1 hour of seeding, the NTS-h cells displayed only 68% and 60% of the adhesion capacity on collagen (Figure 5 B) and matrigel (Figure 3S B) respectively, as compared to MCF-7. NTS-I cells displayed 55% and 52%, respectively. Interestingly, this decline in cellular adhesion ability stabilizes over time. At 48 hours of post seeding, the percentage of adherent cells is similar to those after 1 hour (Figure 5 B and 3S B). This suggests that NTS alters the basal cell adhesion capacities of the tumor cells.

We performed migration assays on type I collagen or matrigel supports, because migration requires cell

interactions with the extracellular matrix. We developed an experimental procedure, in order to estimate the migration speed of the cells. As described in detail in the methods section, this procedure allows measuring the average distance covered by the cells within 48h, while preserving the matrix coating on the dishes. As shown in figure 5 C and 3S C, NTS expression significantly affects the basal migration speed of the NTS-I cells on both collagen and matrigel coated dishes. The migration speed on collagen was $2.50 \pm 0.07 \mu\text{m/h}$ and $3.10 \pm 0.09 \mu\text{m/h}$ ($p < 0.0001$; $n=10$) and on matrigel was $3.99 \pm 0.21 \mu\text{m/h}$ and $5.31 \pm 0.24 \mu\text{m/h}$ ($p = 0.003$; $n=12$) for MCF-7 and NTS-I, respectively. As EGFR expression was enhanced under NTS autocrine regulation, we inquired on the effect of EGF on cell speed migration. When cells were treated with EGF, a clear synergic effect between NTS and EGF

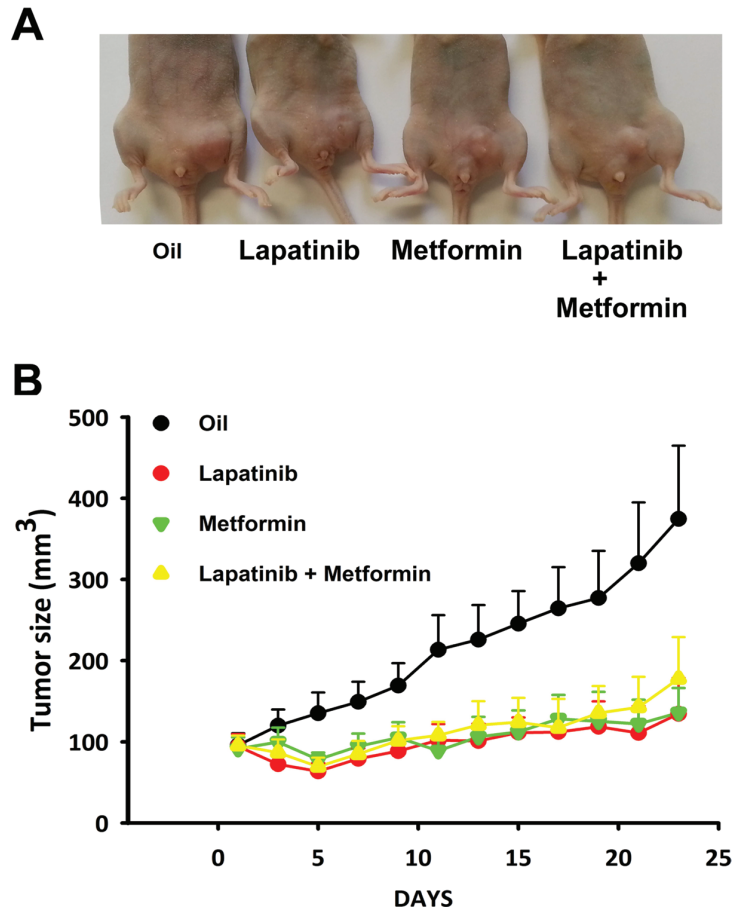


Figure 4: NTS/NTSR1 expressing tumors response to EGFR/HER2 inhibitor treatments. (A) NTS-h cells were inoculated in the left mammary gland of the mice. Here is shown an example of a mouse from each group after 23 days of treatment. (B) Tumor growths generated by NTS-h cells treated for 23 days with sesame oil 6% DMSO, or 75 mg/kg lapatinib, or 200 mg/kg metformin, or both. At day one, 7 mice per group were randomized on tumors size reaching approximately 95 mm³.

was observed. The NTS-I cells migrate 38% and 42% faster when treated with EGF on collagen and matrigel respectively, compared to EGF-treated MCF-7 cells (Figure 5 C and 3S C). In order to confirm the contribution of the NTS-NTSR1 complex in this synergic effect, MCF-7 and NTS-I were treated with EGF and the NTSR1 specific antagonist SR 48692. As shown in figure 5 C and 3S C, this treatment had no effect on the EGF-induced migration of MCF-7 on collagen and matrigel, whereas, SR 48692 inhibited the acceleration of the migration speed in the EGF-treated NTS-I cells. Specific PKC and PLC inhibitors were applied and clearly abolished the synergic effect of NTS and EGF observed in NTS-I cells. In the parental cells, EGF's contribution to the migratory effect was insensitive to these inhibitors although the basal migration speed did increase in matrigel matrix (Figures 5 D and 3S D). As control, a specific inhibitor of EGFR, the AG1478, completely abolished the EGF-induced

migration in both MCF-7 and NTS-overexpressing cells (Figure 5 D and 3S D).

The invasiveness properties of NTS-overexpressing cells, was studied using a 3 dimensional collagen invasion assay. Results are expressed as the invasion index corresponding to the number of invading cells related to the number of total seeded cells. The introduction of NTS expression into MCF-7 cells induced a small increase in invasiveness properties (Figure 5 E). However, EGF-induced invasion doubled in NTS-overexpressing cells (20% invasion index) as compared to MCF-7 (10% invasion index). The induction of invasiveness was inhibited by PKC inhibitors only in NTS-overexpressing clones, suggesting dependence of this effect on GPCR activation.

The synergic effects of NTS and EGF on cellular migration and invasion suggest that a new pattern of HER heterodimers, with a higher EGF response potency, is generated in the cells. When cells were stimulated with

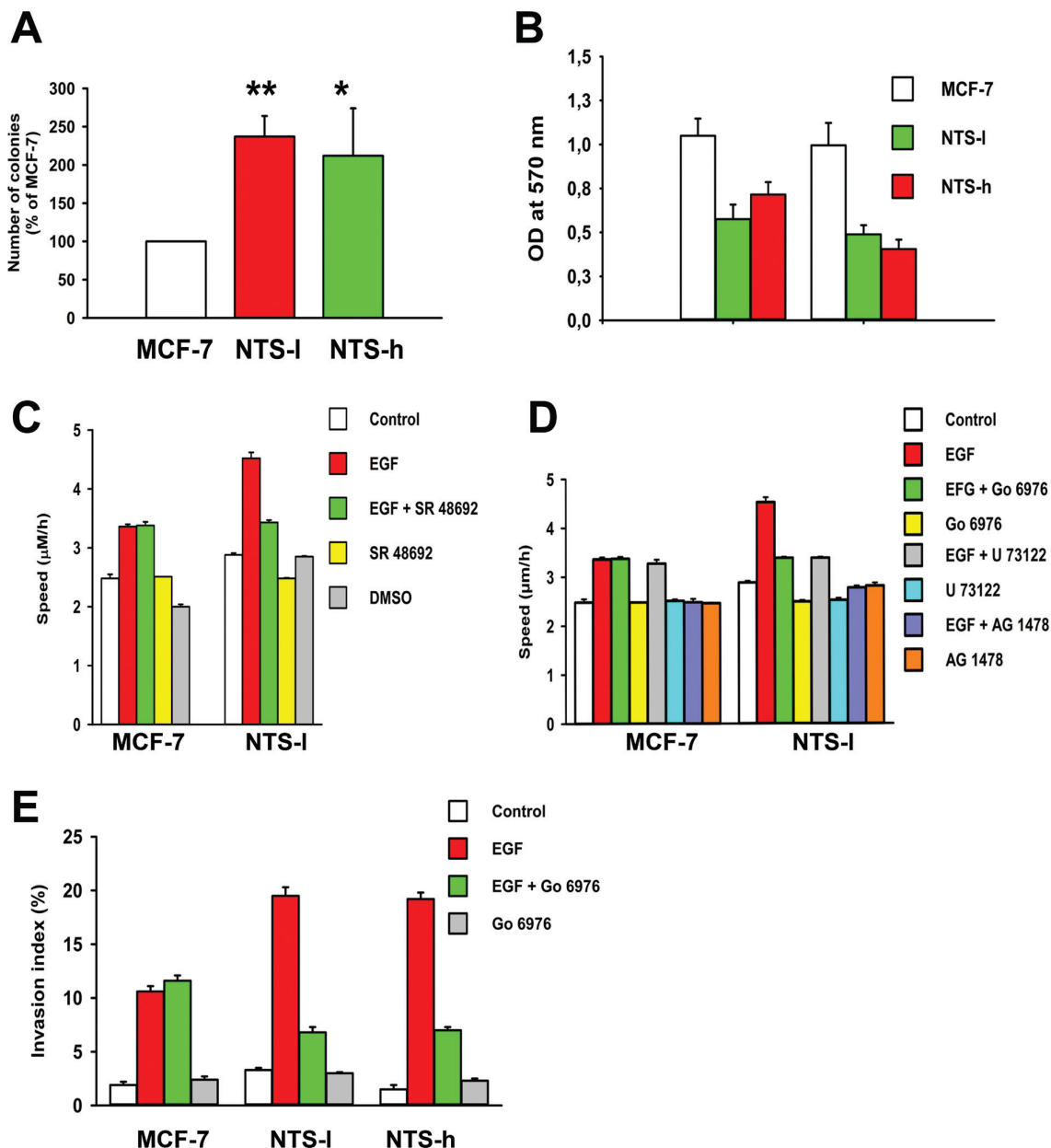


Figure 5: NTS autocrine and paracrine regulations enhanced oncogenic cellular effects on EGF-induced migration and invasion. (A) Number of colonies formed on semi-solid medium after 12 days expressed as the percentage of MCF-7 cells. (B) Adhesion assays were performed on type I collagen supports. After 1h or 48h of seeding, cells were gently washed and the remaining attached cells were quantified by spectrophotometric analysis of crystal violet staining. Results represent the mean optic density \pm SEM of 4 experiments. (C) Speed of migration on type I collagen of MCF-7 and NTS-I cells, control or treated with EGF (10 ng/mL), in the presence or not of SR 48692 (5×10^{-6} M). Results represent the mean \pm SEM of 9 to 10 independent experiments. (D) Speed of migration on type I collagen of MCF-7 and NTS-I cells control or treated with EGF (10 ng/mL), in the presence or not of the PKC-inhibitor Gö6976 (5×10^{-8} M) or the PLC-inhibitor U73122 (5×10^{-6} M) Results represent the mean \pm SEM of 4 independent experiments. Results represent the mean \pm SEM of 3 to 4 experiments. Student-Newman-Keuls Multiple Comparisons Test was performed on the data : *** $P < 0.001$, ** $P < 0.01$, and * $P < 0.05$. (E) Synergism between NTS and EGF on invasion in a type I collagen invasion assay. Cells were seeded on the top of a type I collagen gel and treated with EGF (100 ng/mL) in the presence or absence of Gö6976 (5×10^{-8} M). Results represent the mean \pm SEM of 3 to 4 experiments.

EGF, the amount of phosphorylated proteins EGFR, HER2, and HER3 in the NTS-overexpressing cells is higher than the phosphorylated proteins in wild type cells,

with an increase of 290, 190, and 275%, respectively as compared to MCF-7 EGF treated cells (Figure 6 A and B).

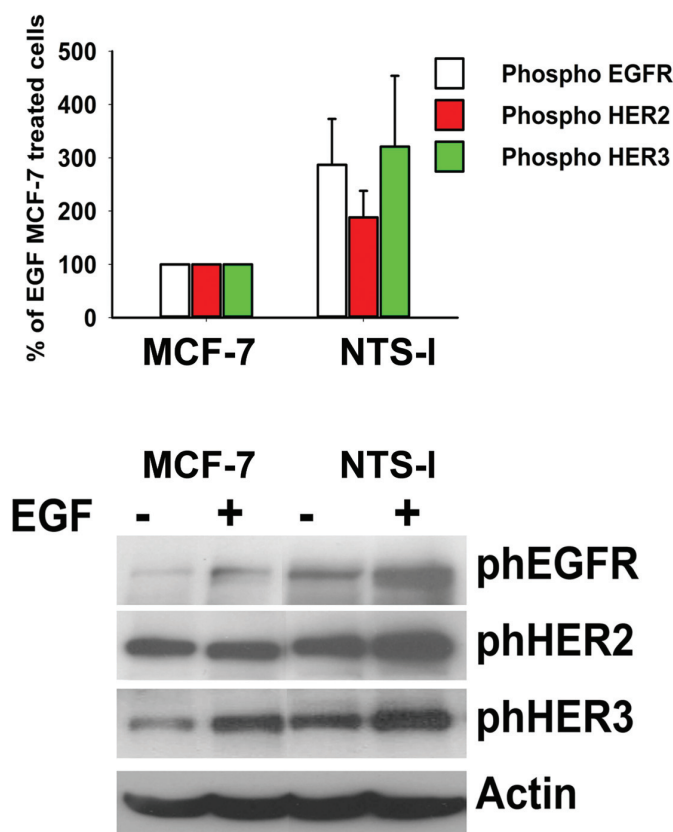


Figure 6: Synergy between NTS and EGF to activate EGFR, HER2, and HER3. (A) Breast cancer cells NTS-I or MCF-7, with the histograms representing intensity- based quantification of Western blot bands of phosphorylated protein, EGFR, HER2, and HER3 treated for 10 min with 10ng/ml EGF. Values are expressed as the percentage of the EGF treated MCF-7 cells and are the mean \pm SEM of 5 independent experiments. (B) Representative Western blot analyses of phosphoEGFR, phosphoHER2, phosphoHER3 and actin from MCF-7 and NTS-I cells treated or not with 10ng/ml EGF for 10 min.

Overexpression of NTS and NTSR1 correlates with HER2 and HER3 overexpression in breast human tumors

Co expression of NTS, NTSR1, HER2 and HER3 was analyzed in breast cancer tissue microarrays (TMA). Seven TMAs containing 269 samples each, was scored from 0 to 3 according to the labeling intensity and the proportion of stained cells. Correlations between the expression of NTS/NTSR1 complex and the expression of HER2 and/or HER3 were evaluated. We considered that a score of 2 and 3 indicate the overexpressed condition, and consequently we performed contingent's analysis on these combined scores. Results are summarized in table 1. NTS and NTSR1 were found in 23% of the samples, in agreement with our previous studies [14], whereas HER2 and HER3 were found in 28% and 53% of the samples, respectively. Amongst this NTS-NTSR1 positive population a higher proportion overexpressed HER3 (16%) than HER2 (7%). HER3 expression was positively associated with those for NTS and NTSR1 (OR= 19.073,

95% Confidence Interval [13.633-26.684], $p < 0.0001$). In contrast, no significant relation between HER2 and NTS/NTSR1 was found. Only 5% of the samples overexpressed the four markers, nevertheless HER2 and HER3 expressions remained strongly significantly associated to NTS/NTSR1 expression (OR = 12.117 95% Confidence Interval: to [7.121-20.618], $p < 0.0001$). It should be noted that an absence of correlation between high expression of HER2 and HER3 was detected. Two examples of the same tumor sample labeled for the four markers are shown in figure 7.

DISCUSSION

The organization of tumor cell signaling networks evolves with accumulation of genetic and epigenetic alterations in connection with the local stroma, vessels and immune system. Identifying factors which contribute and predict tumor aggressiveness are means to understand how the progression of the disease evolves across individuals. Unfortunately, the biological and clinical

Table 1: Contingent analysis for NTS, NTSR1, HER2 and HER3 expression on samples from breast cancer tissue microarray

	NTS	NTSR1	HER2	HER3
n total	1408	1347	1268	1410
Positive n (%)	1029 (73)	375 (28)	361 (28)	753 (53)
NTS, n (%)		301 (23) $p<0.0001$	274 (22)	599 (43) $p = 0.006$
NTSR1, n (%)			102 (8)	244 (19) $p<0.0001$
HER2, n (%)				218 (18)
NTS & NTSR1, n (%)			85 (7)	204 (16) $p<0.0001$
NTS & NTSR1 & HER2, n (%)				62 (5) $p<0.0001$

n total= number of sample readable; Positive = number of sample scored 2 or 3; P = Fisher's Exact Test

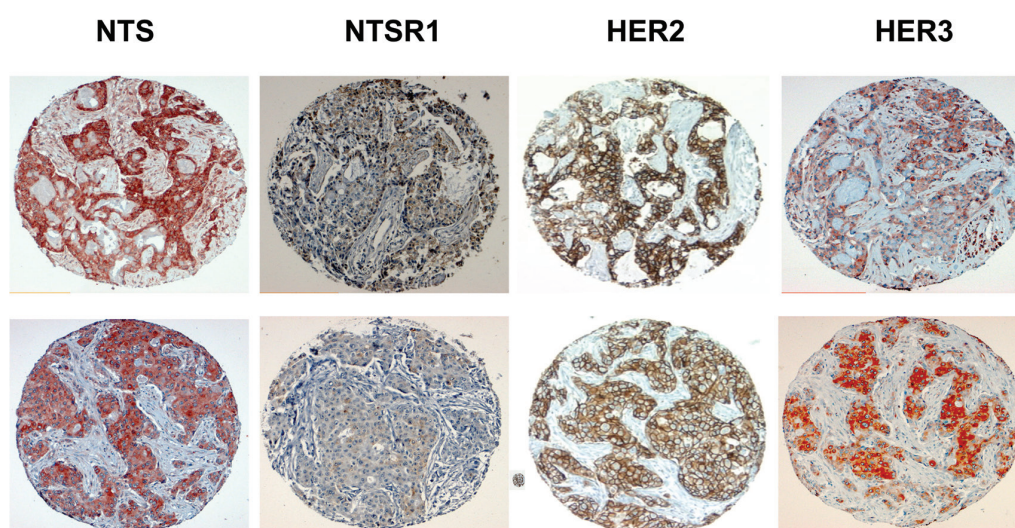


Figure 7: NTS, NTSR1, HER2, and HER3 immunohistochemistry on breast and lung cancer tumors. Two examples of positive labeling scored 2 or 3 for NTS, NTSR1, HER2, HER3 from breast tumor TMA, labeling was performed on consecutive slides. 100X magnification.

validation of these factors is difficult because tumors are often heterogeneous and their molecular characteristics evolve over time. In addition to the clinical parameters, genetic alterations on key genes provide additional information on disease outcome. These genetic alterations can be modulated by regulatory mechanism which may cause changes to the amplitude of cell aggressiveness within the tumor. While genetic alterations acquired by tumors are irreversible, their regulatory process can be de-programmed to restore a tumor phenotype to a less aggressive and more differentiated form.

In this article we show that the NTS/NTSR1 complex enhances tumor aggressiveness (tumor growth and metastasis emergence) by enhancing HERs expression, and their activation by the release of specific

EGFR and HER3 ligands. This concept can be extended to other tumors, as we observed similar results in lung cancer cells and subsequent experimental tumors [27]. In lung cancer cells all three receptors (but mainly HER2 and HER3) are over expressed under NTS autocrine regulation. EGFR and HER3 activation occurred with HB-EGF and NGR1 as well as MMP1 released from the cells [51].

In breast cancer cells, NTSR1 activation alters many cellular effects having oncogenic characteristics including proliferation, survival, adherence, migration and invasion, with a synergic effect between NTS and EGF on cellular migration and invasiveness. This latter point may explain the exacerbation of the metastasis process seen due to NTSR1 activation. Synergy between NTS and EGF was

previously described on DNA synthesis in primary adult rats' hepatocytes [28], and the regulation between these two factors appears to be independent of oncogenic characteristics of the cells. EGF was detected in normal and tumoral breast tissues, with a higher expression found in normal tissues adjacent to tumor [29]. We previously showed that NTS is expressed in normal breast epithelial cells. Its expression is regulated by estrogen [14]. The synergy between NTS and EGF may therefore occur during the breast carcinogenesis.

Our data showed that under NTS stimulation, EGFR, HER2 and HER3 are up-regulated and intrinsically activated. This over expression was not associated with gene transcriptional or post-transcriptional activity, suggesting that NTS induced a new equilibrium in HERs cellular traffic and a new pattern of HERs formed after stimulation. HERs internalization pathways depend on the expression of ligands and/or their receptors [30,31]. Under physiological conditions (low concentration of ligands and moderate EGFR expression <200 000/cell), EGFR internalization is dependent on clathrin coated pits, with a half-life of 6-10 hours. However, under conditions of receptor overexpression or high concentration of ligands, the clathrin pathway becomes saturated, and the complex (ligand-receptor) is internalized with a slow kinetics in a clathrin independent pathway. Under these conditions, the half-life can reach 24 hours [32,33].

In cells overexpressing HER2, receptors are mainly localized at the cell membrane, indicating that overexpression of HER2 does not lead to accelerating its endocytosis [34]. The HER2 heterodimerization with EGFR influences the endocytosis pathway for both receptors. Treatment by EGF of cells expressing low HER2, resulted in HER2 down-regulation [35]. In contrast, EGFR activation in cells overexpressing HER2, does not affect the membrane expression of HER2 or its degradation [34,36]. In addition, overexpression of HER2 had a dominant-negative effect on the down regulation of stimulated EGFR, and stabilized both receptors by altering autophosphorylation patterns [34,37]. HER2 overexpression may also prevent EGFR internalization by clathrin-dependent endocytosis, and it's re-directed from the degradation to the recycling pathway [36,38,39].

The activation of HER3 leads to its internalization and its down regulation. However, HER3 internalization is slower than EGFR internalization [40]. In addition, HER3 is inefficiently sorted to the degradation pathway, apparently due to the lack of the C-terminal domain, which contains patterns used for targeting to lysosomes [41,42]. It has also been suggested that neuregulins do not guide HER3 to degradation due to the early dissociation of complexes (ligand-receptor) in endosomes [41]. In these situations the stabilization of HERs cells are independent of gene transcription, activation and amplification, as in the case for NTS.

Associated with HERs regulation, we also observed an increase of NRG2 and HB-EGF secretion by NTS mediated by MMP activation. The release of these growth factors caused the concomitant activation of HER3 and EGFR. EGF "like" ligands are largely implicated in breast cancer progression, yet, most reports have only studied the expression of ligands specific to EGFR, or those specific to HER3. For example, in one case a 10 fold increase of HB-EGF in cancer tissues was reported, and in another a high proportion of the four neuregulins and major isoforms were expressed in the cytoplasm of DCIS and IDC of the breast [29,43,44]. In both cases, deleterious effects of growth factors are often in HER2 overexpressing cells, indicating that EGFR/HER2 and/or HER3/HER2 dimers are related to biological aggressiveness [29]. Globally, the overexpression of both HER2 and HER3 participates in the stabilization of the HERs dimer, and subsequently the sustained activation of the HERs, and the survival pathway. By enhancing the overexpression and activation of EGFR, HER2 and HER3, the NTS/NTSR1 complex becomes an upstream factor that modulates this regulatory mechanism.

In agreement with *in vitro* studies, tumor growth induced by NTS/NTSR1 can be restrained by a specific tyrosine kinase inhibitor EGFR and HER2, lapatinib. The cascade of biological events from the interaction between NTS and NTSR1 to the activation of HERs receptor appears to be a major contributor to rapid cancer cell growth.

This cascade is inhibited by metformin, known to interfere with activated mTOR and ERK pathway [26,45]. The lack of additional effects from the combination of the two drugs suggests that consecutive cellular events lead to increase tumor aggressiveness by NTS. We previously showed that sustained NTSR1 activation generated a permanent PKC dependent activation of ERK signaling [46]. It is therefore coherent that metformin and other ERK or PKC inhibitors counteract the same NTS oncogenic cellular effect. PKC appears to be a central signaling hub to generate cell aggressiveness by NTS/NTSR1 through the sustained overexpression and activation of HERs.

Experimental tumors report the tumorigenic performance of single cell lines or clones. Nevertheless, heterogeneity is an important characteristic of human tumors. For instance, we found that in 35% of patient with IDC, NTSR1 was expressed in at least 80% of tumoral cells, and only 20% of patients express high level of NTSR1 and NTS [47]. In human tumors, NTS and NTSR1 are expressed in cells clusters with diverse sizes. Tumoral cells with potential aggressiveness characteristics could be detected with NTS/NTSR1 labeling, and specific treatment could be proposed accordingly. Lapatinib is proposed in a second line of treatments, in combination with other

drug in advanced or metastatic breast cancers whose tumors overexpress HER2. Our results suggest that a more restrained (or targeted) population, can be determined by taking into account NTS and NTSR1 co expression. The resulting subpopulation will provide a significantly better performance for this drug.

CONCLUSION

The activation of the neurotensinergic system in breast tumors induces the overexpression of the EGFR, HER2 and HER3 receptors and their concomitant autocrine activation. The presence of this regulatory mechanism would have a significant impact on cancer progression in tumor cells by accelerating the process of metastasis. It also modulates the response to tyrosine inhibitor HER2 and EGFR therapy. NTS/NTSR1 overexpression in breast cancer cells creates a tumoral context for EGFR tyrosine kinase inhibitor responsiveness, and therefore a new population of patients would be eligible to these specific tyrosine inhibitors.

MATERIALS AND METHODS

Cell culture procedure

The human breast adenocarcinoma cell line MCF-7 and the corresponding NTS-overexpressing clones, NTS-h (high level of NTS) and NTS-l (low level of NTS), were grown at 37°C, in a humidified atmosphere of 5% CO₂, in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine and G418 0.5 mg/mL (Invitrogen™).

Tumor xenografts

3 millions cells (MCF-7, NTS-h, NTS-l) resuspended in Matrigel (BD Biosciences) were then inoculated in the right mammary gland of the mice. Tumor growth was induced by a daily intra-peritoneal injection of 2µg estradiol per mouse. Tumors, axillary and inguinal lymph nodes, lungs, liver and bones (vertebra, sternum) were taken, weighed and fixed in formol or frozen. All the procedures were in accordance with the "Guide of the Care and Use of laboratory Animals". Institutional Review Board approval was obtained by «Le Comité d’Ethique pour l’Expérimentation Animale Charles Darwin # Ce5/2010/049 ». For lapatinib experiments NTS-h cells were inoculated in the left mammary gland of the mice. 51 days after injection, 4 groups of 7 mice were randomized as follows : $95.9 \pm 14.57 \text{ mm}^3$ for control group, $94.5 \pm 15.0 \text{ mm}^3$ for lapatinib group, $91.5 \pm 14.09 \text{ mm}^3$ for metformin group and $95.6 \pm 12.20 \text{ mm}^3$ for lapatinib and metformin group. Mice were treated for 21 days per os, with sesame oil containing 6% DMSO, or 75 mg/kg lapatinib, or 200 mg/kg metformin or both.

Western blots

Samples in Laemmli buffer were loaded on 10% SDS-PAGE and transferred to PVDF membranes. Membranes were exposed to primary antibody overnight. Total anti-EGFR (1:500), anti-phospho-EGFR (1:500), anti-phospho-HER2 (1:500), anti-HER3 (1:2000), anti-phospho-HER3 (1:1000), anti-ERK 1/2 (1:2000) were from Cell Signaling Technology®. Total anti-HER2 (1:2000) were purchased from Neomarkers and anti-βactin (1:50000) from Sigma®. Secondary anti-rabbit (Santa Cruz Biotechnology) or anti-mouse (Sigma®) antibodies conjugated to HRP were used at 1:2000 dilutions for 1h at room temperature and visualized by enhanced chemiluminescence (GE Healthcare®). See details in supplementary information.

Adhesion assays

The assay was performed in 96-wells plates coated or not with 50 µg/mL of type I collagen (Sigma®) overnight at 4°C or with 1:10 diluted-growth factor reduced Matrigel (BD Biosciences®) for 1 hour at 37°C. Cells were harvested with PBS containing 0.5 mM EDTA for 20 min at 37°C, pelleted and suspended in adhesion buffer (DMEM, 15 mM HEPES, 1.2g/L sodium bicarbonate, 0.2% BSA). 5×10^4 cells per well were seeded and incubated at 37°C for 1 or 48 hours. Wells were washed with culture medium and adherent cells fixed with 5% paraformaldehyde for 45 min, then colored with 0.1% cristal violet during 30 min at room temperature. Cells were subjected to 30 min lysis in 1% SDS under agitation. Absorbance at 570 nm was determined by spectrophotometric measures and correlates with the number of adherent remaining cells.

Anchorage-independent growth assay in soft agar

Colony growth assays were performed by seeding on the top of a 0.6% low gelling temperature agarose layer, 5×10^3 cells in 0.5 mL of cultured medium containing 0.3% agarose solution ± EGF (10 ng/mL). Culture medium and EGF were replenished every 2-3 days. Cells were incubated at 37°C for 12 days and colonies $\geq 50 \mu\text{m}$ were counted in the whole well.

Cell migration assays

12 wells-culture plates were coated with type I collagen or Matrigel as mentioned in adhesion assay. 1×10^5 cells were plated for 4 hours in an 8 mm-cloning ring placed in the center of the well to form a confluent circle of monolayer cells. The cylinder was removed and cells grow for 16 hours. Cells were then treated in serum-free medium ± EGF (10 ng/mL) in the presence or absence of various cell signaling inhibitors. Four pictures were

acquired per well at the initial time of treatment and 48 hours later for comparison with an inverted microscope at 200X magnification. Migration speed was determined using the Morpho Expert software (Explora Nova) and was expressed as the average cells covered distance divided by the experimental duration ($\mu\text{m/h}$). Presented results are expressed in percentage of the non-treated condition.

Collagen invasion assays

Invasion potential was evaluated by a single-cell collagen invasion model, extensively described [48]. Briefly, 6-wells plates were coated with 1.25 mL of a 1 mg/mL collagen type I solution, allowed to gel for at least 1 hour at 37°C in a humidified atmosphere containing 10% of CO_2 . 2×10^5 viable single-cells, obtained by mild enzymatic dissociation with trypsin/EDTA solution and filtration, are seeded on the top of the gel in presence or not of EGF (100 ng/mL) and/or G66976 (4×10^{-8} M) for 24h. Invasion index (cells with invasive extensions versus total number of cells $\times 100$) was determined by counting the number of invading and non-invading cells present in 10 to 15 random fields of an inverted phase-contrast microscope.

ELISA (Enzyme-linked immunosorbent assay)

Cells were seeded at 4×10^6 cells in 100 mm dish in culture media. The next day media was changed to 4.5 mL of serum free media for 48h. To assay Hb-EGF and NRG-2, the media were concentrated with dialysis tube (7 Spectra / Por® Dialysis Membrane). In all samples Protease Inhibitor Cocktail P8340 [1:100] were added. MMP9, HB-EGF and NRG2 released in the culture media were assayed by ELISA kits (DuoSet®ELISA Development System, and USCN Life Science Inc.).

RNA extraction and RT-PCR

Total RNA was extracted by the acidic phenol/chloroform guanidine thiocyanate method as documented by [49,50]. Detailed are described in supplementary information.

Immunohistochemistry

Procedure is detailed in supplementary information. The slides were incubated at 4°C overnight with primary antibody included anti-NTS (1:200, SC-20806, Santa Cruz biotechnology®), and anti-ErbB3 (1:50, NCL-c-erbB-3, Novocastra™), anti-NTSR1 (1:100; SC-7596, Santa Cruz Biotechnology®) and anti-ErbB2 (1:400, A0485, Dako) was incubated at room temperature for 1hour and 30 minutes respectively. The levels of staining were scored based on staining intensity within the tumor sample, with weak as 1, moderate as 2, strong as 3.

Patients

We studied 499 specimen from patients operated for breast cancer in 2008 in Algeria. The average patient age was 51.1 ± 11.7 years. The sizes of the tumors were 36.3 ± 21.7 mm. The SBR histoprosthetic grading was 6% grade I, 63% grade II and 31% grade III (See supplementary information). Investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to Algerian guidelines and has been approved by the authors' institutional review board from CPMC. Data were analyzed anonymously.

Statistics

Statistical analysis was carried out using test Student's t-test or Student-Newman-Keuls Multiple Comparisons Test : *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. For contingency analysis Fisher's Exact Test was applied. The Odds ratio was performed using the approximation of Woolf.

ADDITIONAL METHODS

Transfection procedure

Following the manufacturer's instructions, a pCDNA3.1 plasmid encoding the large fragment of rat neurotensin ou neurotensin full length coding sequence was transfected in MCF-7 cells using the Lipofectamine Plus Reagent (Invitrogen™). Briefly, 0.8×10^6 cells were plated in 60 mm-Petri dishes and were allowed to grow 24 hours before transfection. 2 μg of DNA were complexed together with the Plus Reagent and the Lipofectamine Reagent to form transfection complexes which were incubated during 4 hours on the cells. Complete medium was then added to reach a 10% final fetal calf serum concentration. 24 hours later, cells were harvested and placed in fresh culture medium. Selection was performed 2 days after transfection with 1mg/mL of G418 (Invitrogen™). Stable clones were obtained by cloning dilution and screened by classic RT-PCR and real-time PCR for the NTS expression level.

Western blot

2×10^6 cells were grown for 72h then serum-starved for 48h in a phenol red-free medium in presence or absence of different concentrations of 5×10^{-6} M SR 48692 and 25×10^{-9} M MMP9 inhibitor (Calbiochem®). For EGF treatments, cells were then treated 15 min with EGF (10 ng/mL) lysed (20 mM Tris pH 8.0, 150 mM NaCl, 5 mM MgCl_2 , 0,5% NP40, 0,5% glycerol, 1 mM PMSF, protease and phosphatase inhibitor cocktail) at 4°C for 30 min. Samples in Laemmli buffer were loaded on 10% SDS-PAGE and transferred to PVDF membranes.

Saturation was performed 30 min at room temperature in 5% non-fat dry milk in TBS 0.1% Tween 20. Primary antibodies were incubated overnight at 4°C according to the manufacturer's instructions. Total anti-EGFR (1:500), anti-phospho-EGFR (1:500), anti-phospho-HER2 (1:500), anti-HER3 (1:2000), anti-phospho-HER3 (1:1000), anti-ERK 1/2 (1:2000) were from Cell Signaling Technology. Total anti-HER2 (1:2000) were purchase from Neomarkers and anti-βactin (1:50000) from Sigma. Secondary anti-rabbit (Santa Cruz Biotechnology®) or anti-mouse (Sigma®) antibodies conjugated to HRP were used at 1:2000 dilutions for 1h at room temperature and visualised by enhanced chemiluminescence (GE Healthcare®).

RNA extraction

Total RNA was extracted by the acidic phenol/chloroform guanidine thiocyanate. Two cycles of phenol-chloroform extraction (pH 4.7) were preceded by two ethanol precipitations in GTC buffer (4 M Guanidium Thiocyanate, 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 30% N-lauroylsarcosine sodium salt, 1% β-mercaptoethanol) and followed by a final extraction with chloroform-isoamyl alcohol 25:1 (v/v). Two final ethanol precipitations in acetic acid and NET buffer (150 mM NaCl, 15 mM Tris-HCl pH 7.5, 1 mM EDTA) were successively performed. After washing in 70% ethanol, pellets were resuspended in 20 µL of sterile deionized diethyl pyrocarbonate-treated water and titrated by spectrophotometric measure of the absorbance at 260 nm.

RT-PCR and quantitative PCR

1 µg of total RNA was subjected to reverse transcription, during 1 hour at 37°C, using 1 µg of nonspecific hexameric random primers dN, 1mM dNTP, 10 mM dithiothreitol, 24 units RNaseOUT and 200 units of M-MLV-RT enzyme (Invitrogen). The PCR amplification was performed on 1:10 (v/v) of the 1:10-diluted reverse transcription reaction using 0.2 mM dNTP, 2.5 mM MgCl₂ and 1 unit of ThermoStart Taq DNA polymerase (Thermo Scientific), and 25 pmol of each specific primer :

NTS (5'-CAGCTCCTGGAGTCTGTGCT-3' and 5'-GAGTATGTAGGGCCTTCTGGG-3')

NTSR1 (-5'-CGTGGAGCTGTACAACCTTCA-3 and 5'-CAGCCAGCAGACCACAAAGG-3)

HER3 (5'-ATGGGGAACCTTGAGATTGTGCT-3' and 5'-ACAGCTTCTGCCATTGTCCT-3')

EGFR (5'-TTTCGATACCCAGGACCAAGCCACAGCAGC-3' and 5'-AATATTCTTGCTGGATGCGTTTCTGTA-3')

HER2 (5'-GTGCTAGACAATGGAGACC-3' and 5'-CACAAAATCGTGTCTGCTGGTAGC-3')

18S (5'-AGGAATTGACGGAAGGGCAC-3' and 5'-GTGCAGCCCCGGACATCTAAG-3')

36B4 (5'-GTGCAGCCCCGGACATCTAAG-3' and 5'-GATTGGCTACCCAACGTGTG-3')

Semi-quantitative amplification was performed in a DNA thermal cycler 9700 (Perkin Elmer Applied Biosystem), and Maxima SYBRGreen qPCR Master Mix (Fermentas) in a Mx3000P qPCR system (Stratagene) was used for quantitative PCR.

Patients

We studied 499 specimens of patients operated for Breast cancer in 2008 in Algeria. The average of patient age was 51.1 ± 11.7 years. The size of the tumor was 36.3 ± 21.7 mm. The SBR histopronostic grading was 6% grade I, 63% grade II and 31% grade III. The Subtypes were distributed as followed : 75.9% Invasive ductal carcinomas, 10% invasive lobular carcinomas, 2.2% Mucinous carcinomas, 1.20% Invasive papillary carcinomas, 1.6% invasive micropapillary carcinomas, 0.40% Invasive metaplastic carcinomas, 0.60% metaplastic carcinomas, 3.4%, invasive ductal and lobular carcinomas, 1.20% mixte Mucinous and ductal carcinomas, 3% mixte ductal and metaplastic carcinomas and 0.4% others. For TMA Three sample per specimen were analyzed.

Immunohistochemistry

Deparaffinized TMA sections (4 µm) were subjected to heat-induced epitope retrieval in citrate buffer (pH 6.0). The sections were labeled for the target proteins using the avidin-biotin-peroxidase complex method. The slides were incubated at 4°C overnight with primary antibody included anti-NTS (1:200, SC-20806, Santa Cruz biotechnology®), and anti-ErbB3 (1:50, NCL-c-erbB-3, Novocastra™), anti-NTSR1 (1:100; SC-7596, Santa Cruz Biotechnology®) and Anti-ErbB2 (1:400, A0485, Dako) was incubated at room temperature for 1hour and 30 minutes respectively. These slides were then incubated with appropriate biotinylated secondary antibodies, NTS (Trekki Biotinylates rabbit link, Biocare medical®), NTSR1 (Biotinylated anti-goat IgG, Vector laboratories, Inc), ErbB3 (Trekki Biotinylates mouse link, Biocare medical®). The antigen-antibody complex was revealed with avidin-biotinperoxidase complex, according to the manufacturer's instructions, NTSR1 (Vectastain ABC Kit, Vector laboratories, Inc.), NTS and ErbB3 (Trekavidin-HRP label, Biocare medical®). ErbB2 was biotinylated and revealed with the NovoLink™ Polymer Detection System (Leica®). NTSR1 and ErbB2 staining were done with diaminobenzidine tetrahydrochlorid, NTS and ErbB3 were done with aminoethyl carbazole. All slides were counterstained with hematoxylin then scored by an anatomopathologist (NM, FPL, or AC). The levels of staining were scored

based on staining intensity within the tumor sample, with weak as 1, moderate as 2, strong as 3.

Immunofluorescence assay

Cells were seeded on 12 mm-diameter glass slides for 24 hours, fixed in 5% paraformaldehyde for 1 hour at room temperature, permeabilized in PBS /0.5% Triton X-100 for 30 min and saturated for 20 min in PBS+ (1:100 (m/v) BSA, 1:250 (v/v) cold fish skin gelatin in PBS 1X, pH 8.0). Cells were then incubated overnight at 4°C with the primary antibody diluted to 1:100 in PBS 0.1% Triton X-100. NTS immunoreactivity was detected using a rabbit polyclonal anti-NTS immunoglobulin (NA1230, Tebu-Bio) and NTSR1 with a goat polyclonal antibody directed against the human COOH terminus of the receptor (C20, Santa Cruz Biotechnology). Slides were incubated for 1 hour with a fluorescent secondary antibody (1:100): a cyanin3 anti-rabbit immunoglobulin or a FITC-coupled anti-rabbit or goat immunoglobulin (Jackson ImmunoResearch). Nuclei were counterstained for 5 min with DAPI 1:50000.

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The authors declare that they have no competing interests

SD, VKD, ZW, JL Carried out the experiments

NM, analyzed the mouse tissues

ODW, performed the invasion assay

FPL, AC provided the human tissues and participated in the design of the clinical part of the study

AK collected the tissue, set up the TMA, and provided the clinical information

AG, PF conceived of the study

All the authors meet the authorship requirements, and have read and approved the manuscript.

ABBREVIATIONS

NTS, neurotensin; **NTSR1**, neurotensin receptor 1, **EGF**, Epidermal growth factor; **HERs**, epidermal receptors; **EGFR**, Epidermal growth factor receptor; **MMP**, metalloproteinase; **NRG**, Neuregulin; **Hb-EGF**, Heparin-binding EGF-like growth factor; **DCIS**, ductal carcinoma in situ; **OR**, Odds ratio.

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Article 2

Neurotensin regulation induces overexpression and activation of EGFR in HCC and restores response to erlotinib and sorafenib

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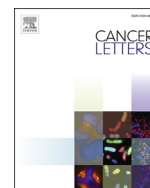
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Original Article

Neurotensin regulation induces overexpression and activation of EGFR in HCC and restores response to erlotinib and sorafenib



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ABSTRACT

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer due to the combination of late diagnosis and a lack of curative treatments. The identification of factors which promote tumor aggressiveness, and those that predict treatment responses, are a means to optimize the management of HCC patients. The complex of Neurotensin (NTS) and its high affinity receptor (NTSR1) has been shown to induce tumor growth and metastasis process in various cancers. In this paper, we propose that NTS and NTSR1 can assist in the management of HCC. Concomitant expression of NTS/NTSR1 was correlated with poor prognosis and found in 50% of HCC patients. We show that NTSR1 expression was positively correlated with the alteration of the Wnt/ β -catenin pathway. Its activation creates EGFR driver activation which consequently enhances tumor progression, and sensitizes HCC tumor cells to TKI, such as sorafenib.

The NTS/NTSR1 complex is a potential drug target for HCC, because it is an upstream regulator in the chain of cellular events involved in HCC progression. It could also be used as a theranostic biomarker for sorafenib to improve the HCC patient management.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide with over 500 000 people diagnosed each year [9]. At diagnosis, most patients have already reached metastatic stage, and are not eligible for tumor ablation. The most common treatment for late stage patients are transarterial embolization and transcatheter arterial chemoembolization (TACE). The multikinase (VEGFR-2, VEGFR-3 and PDGFR- β) inhibitor, sorafenib, was shown to prolong survival in patients with advanced HCC [22,31].

Abbreviation: NTS, neurotensin; NTSR1, neurotensin receptor 1; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinases; PKB, protein kinase B; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; RT-PCR, reverse transcriptase-polymerase reaction; OS, overall survival.

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Unfortunately, there is no standard genetic test to determine the patient subgroups that would benefit from sorafenib treatment. The identification of the factors associated with sorafenib response could, therefore, help in the management of HCC patients.

Neurotensin (NTS) is a 13 amino acid peptide, which is biologically active in the central nervous system and in the periphery [3,19]. In the periphery, NTS acts as an endocrine hormone involved in the postprandial regulation of the motor and hormonal functions of the gastrointestinal tract [20]. NTS is released into the portal vein by the endocrine cells (N) of the intestinal mucosa, after meals, especially those rich in fatty acids. NTS is subsequently metabolized by the liver [10]. NTS actions are mediated by three subtypes of NTS receptors, two G protein coupled receptors, NTSR1 and NTSR2 exhibiting high and low affinity for NTS, respectively, and NTSR3 or gp/95/sortilin, a single transmembrane domain receptor [28].

The contribution of NTSR1 and its ligand, NTS, in cancer progression has been demonstrated in many solid carcinomas [1,7,21,29]. In addition, it was shown that the sustained stimulation

of NTSR1 generated autocrine regulation of epidermal growth factor receptors, producing cell hyper-sensitivity to tyrosine kinase inhibitors, as is the case for erlotinib in lung cancer or lapatinib in breast cancer [6,33].

NTS is expressed in the fetal human liver but repressed in the normal adult liver. In fibrolamellar carcinomas, overexpression of NTS was correlated with advanced stages and chemotherapy response [5,8]. It was recently shown that NTS/NTSR1 stimulated the *wnt*/β-catenin pathway facilitating the epithelial/mesenchymal transition in HCC [32]. Earlier reports have also shown that the

NTSR1 gene is a target for the Tcf/β-catenin complex, leading to abnormal expression of NTSR1 in the early stage of breast and colon cancer [24,30].

In this paper, we show that NTS and NTSR1 are abnormally expressed in more than 50% of HCC. Elevated expression of NTS or NTSR1 mRNA is correlated with poor patient outcome. We validate that NTSR1 expression in HCC is also correlated with the alteration of the Wnt/β-catenin pathway. The resulting NTS autocrine loop generates EGFR autocrine stimulation, confers tumor aggressiveness, and responsiveness to erlotinib and sorafenib.

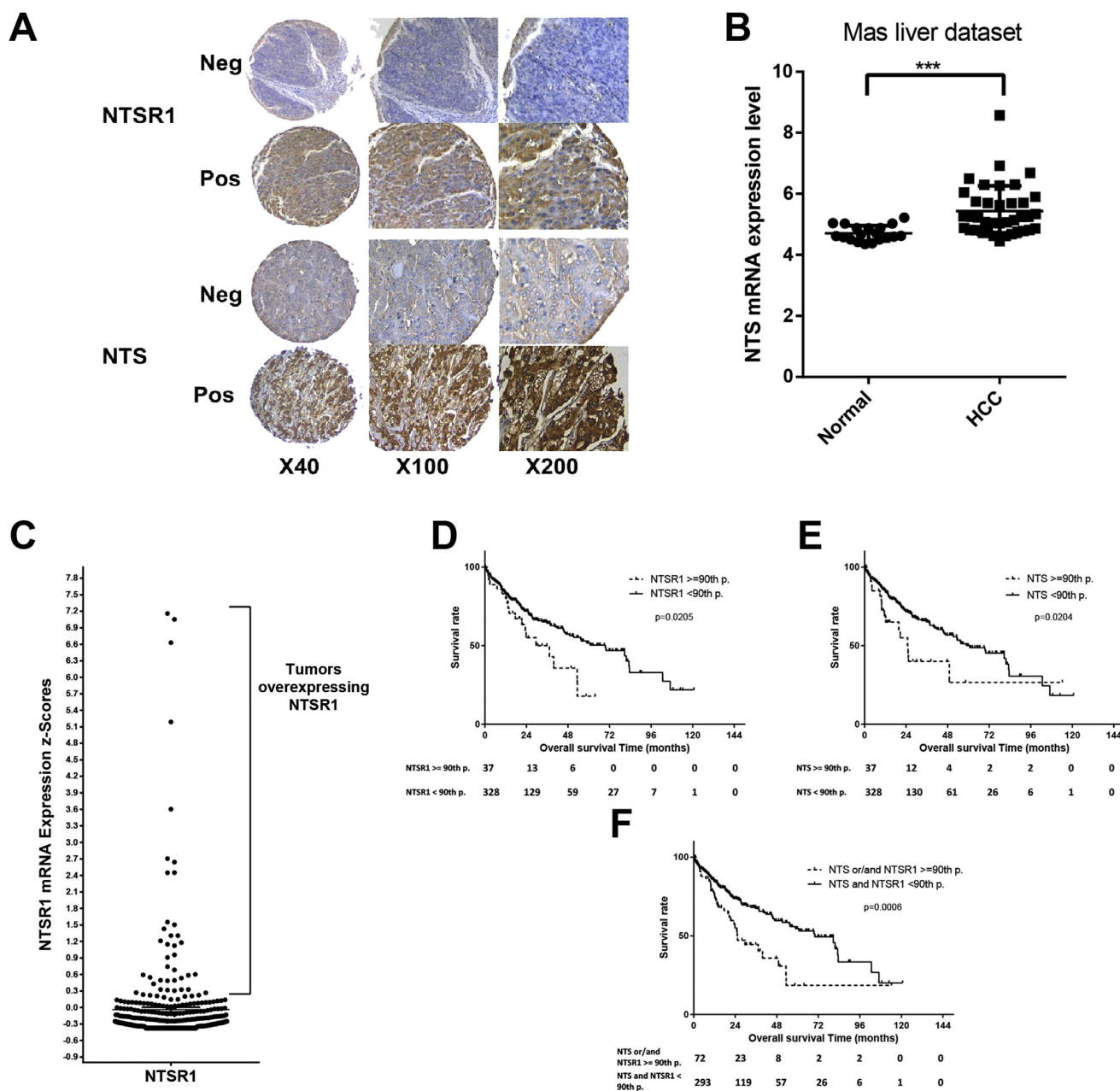


Fig. 1. HCC immuno-staining of HCC for NTSR1 and long fragment NTS. (A) Tissues Microarrays assayed for NTSR1 and LF-NTS labelling from two diagnosis centers. Examples of negative and positive labelling for NTSR1 (Top) and LF-NTS (Bottom) are shown. (B) NTS mRNA expression in normal tissues and HCCs from the Mas liver dataset (GSE14323). Signals were processed according to Robust multi-array analysis. (C) NTSR1 mRNA distribution of 373 tumor samples from Liver Hepatocellular Carcinoma (TCGA, Provisional). (D, E, and F) Overall survival of patients with HCC (n = 365) according to NTS or NTSR1 mRNA expression. A Kaplan–Meier plot according to NTS mRNA expression (D), NTSR1 mRNA expression (E), or high mRNA expression for NTS or NTSR1 (broken line) compared to patients with low expression for both NTS and NTSR1 (solid line) (F). Patients at risk are noted below the graphs.

Materials and methods

Immunohistochemistry

Deparaffinized tissue sections (4 μ m) were subjected to heat-induced epitope retrieval in citrate buffer (pH 6.0). The sections were labeled for the target proteins using the avidin-biotin-peroxidase complex method as described in [13]. Please see details in the [Supplementary Data](#) section. All slides were scored by pathologists (VP and DW). Specimens were scored positive when >20% of cancer cells showed moderate or strong labelling intensity.

Analysis of patient data

The Mas liver dataset (GEO: GSE14323) [17] was obtained from the GEO2R tool of Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/geo2r/>). The mRNA expression was analyzed by Affymetrix GeneChip HG-U133A 2.0 arrays (Affymetrix, Santa Clara, CA). The liver hepatocellular carcinoma TCGA dataset (<http://cancergenome.nih.gov/>) was obtained via cbiportal (<http://www.cbiportal.org/>) [4,11].

Cell culture and stable cell line establishment

HEP 3B, HuH7 and PLC/PRF5 cells, were cultured in Minimum Essential Medium GlutaMAX (Gibco®) supplemented with 10% fetal bovine serum (FBS) (Gibco®), MEM Non-Essential Amino Acids (Gibco®) and 1 mM Sodium Pyruvate (Gibco®). Cell lines were transfected with NTSR1 expression pcDNA3 vectors using Lipofectamine 2000 (Invitrogen™) according to the manufacturer's protocol.

RNA extraction and RT-PCR

Total RNA was extracted using guanidinium thiocyanate-phenol-chloroform acid method modified by Souza et al. [23] whose details and primer sequence are found in the [Supplementary Data](#) section.

In vitro invasion assay

Cells were seeded at one million in each cell culture insert (8 μ m, Beckton Dickinson®) coated with type I collagen (100 μ l/well, 400 μ g/ml, Sigma®), in 250 μ l of

serum free medium in the presence or absence of 1 μ g antibodies anti-NTS (C-19, SC-7592) or anti-LF-NTS (FL-170, SC-20806). After 48 h, the invading cells were fixed and stained with the Kwik-Diff staining kit (Thermo®) and the number of stained cells was counted.

In vitro migration assay

HEP 3B and HEP-R1a cells were seeded separately at 35 000 in each (ibidi®) chambers and placed in 24 well dishes coated with type I collagen (400 μ l/well at 50 μ g/ml, Sigma®). After 12 h of incubation at 37 °C, the insert was removed. Cells were treated with or without 7 μ g NTS or LF-NTS antibodies in 500 μ l serum free medium. Five photos were acquired per chamber at the initial time and after 48 h of treatment. Distance of the cellular migration front was measured using ImageJ (NIH, USA) and was expressed in μ m/h.

In vitro proliferation assay

Proliferation was determined by Prestoblue® cell viability reagent (Invitrogen™) containing resazurin after a 72-h incubation. The fluorescence signal was recorded in an EnSpire® multiwell plate reader equipped with a 560 nm excitation and 590 nm emission filter.

In vitro clonogenic assay

Cells were seeded at a concentration of 200 cells per well in 6-well plates. Cells were incubated for 2 weeks under the indicated treatment in serum free medium, and stained with crystal violet (Sigma®). Photos were acquired and the number of clones with more than 20 cells was counted using ImageJ (NIH, USA).

Western blots

Western blots were processed as previously described [33]. Western blots were revealed with antibodies directed against EGFR, ERK1/2, Thr202/Tyr204 pERK, PKB, Ser473 p-PKB (Cell Signalling Technology®), Tyr1173 p-EGFR, actin (Santa Cruz Biotechnology®).

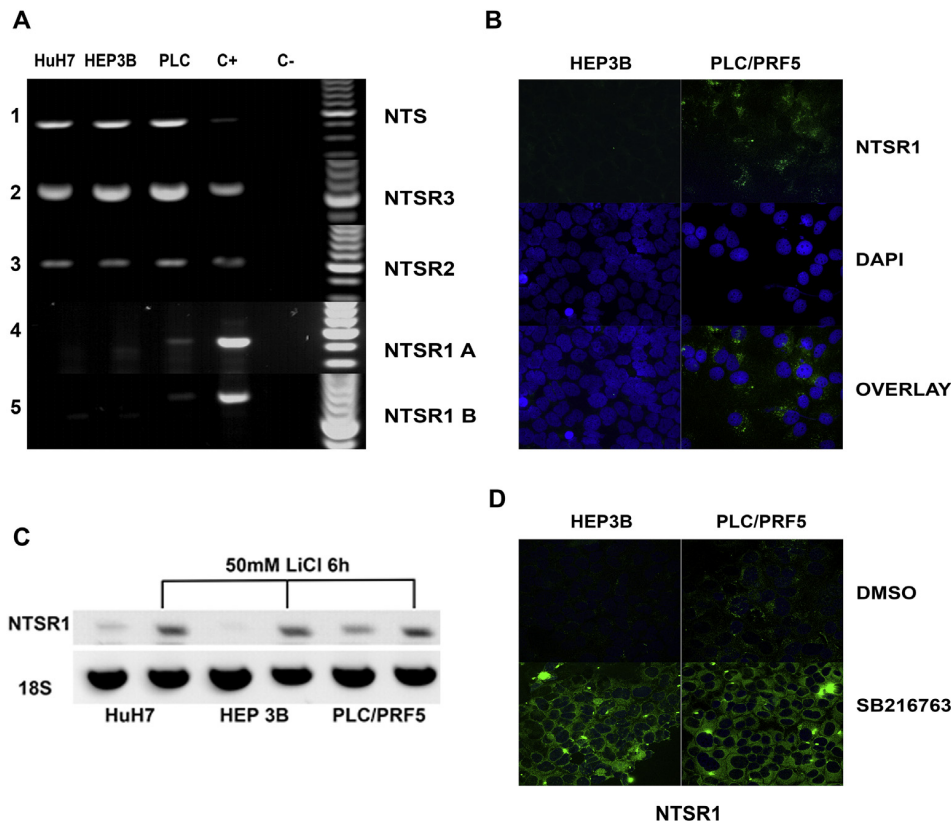
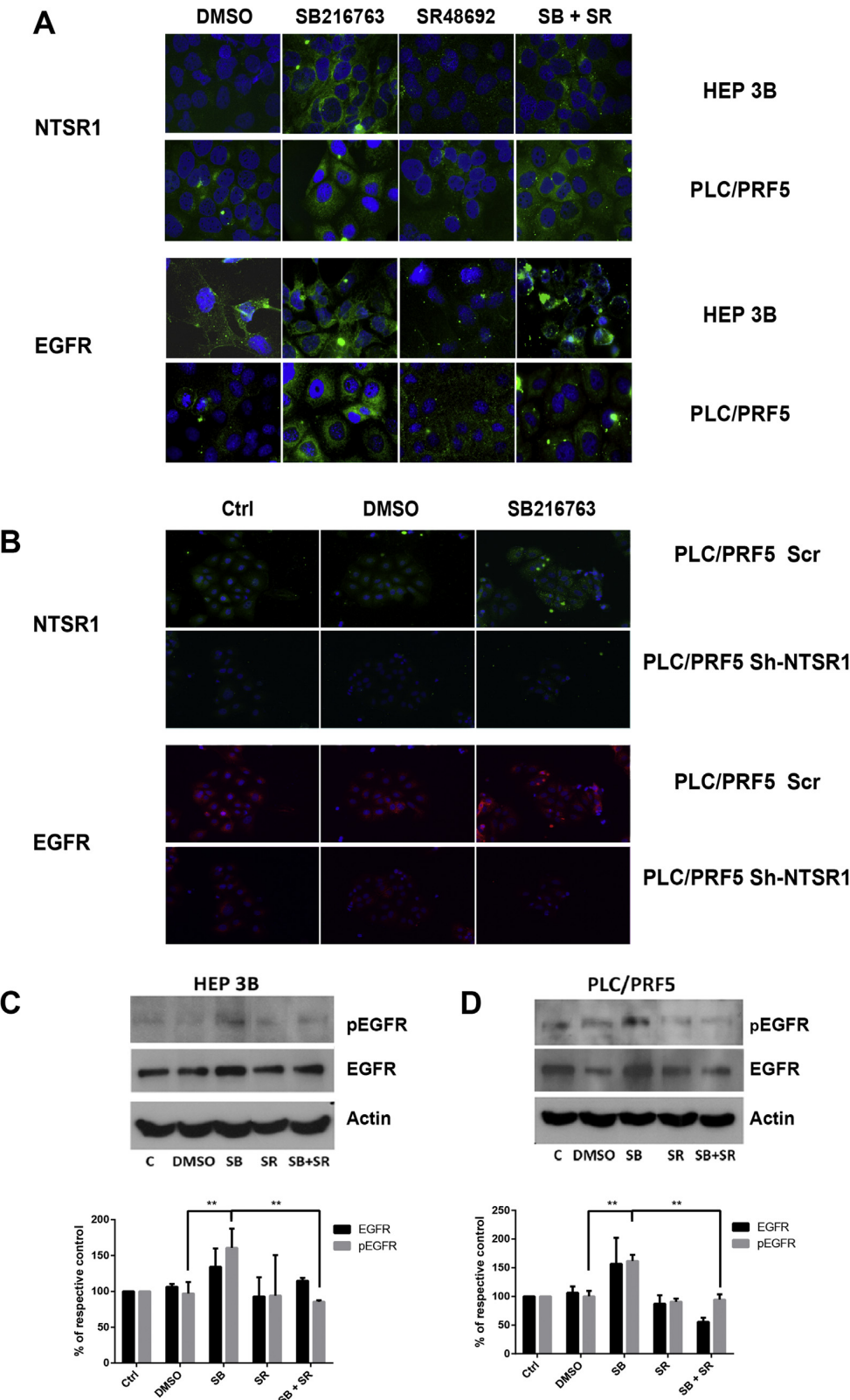


Fig. 2. Expression of NTS and NTSR1 in HCC cell lines. (A) NTS, NTSR2, NTSR3, and NTSR1 transcript analysis using 400 ng total RNA of HuH7, HEP 3B, and PLC/PRF5 cells. NTSR1 A and NTSR1 B show the amplicons obtained with two different primer sets. (B) Typical immunocytochemistry labeling for NTSR1 and DAPI in HEP 3B and PLC/PRF5 cells. Cells were seeded on glass slides, grown for 48 h, and immunocytochemistry was performed as described in the Materials and Methods section. (C) NTSR1 transcript analysis from total RNA of HuH7, HEP 3B and PLC/PRF5 cells treated or not with 50 mM LiCl for 6 h. (D) NTSR1 typical immunocytochemistry labeling of HEP 3B and PLC/PRF5 cells treated with 1 μ M SB216763 for 6 h.



Immunocytochemistry

Immunocytochemistry was performed according to the procedure previously described in [33] and detailed in the [Supplementary Data](#) section.

Tumor model in athymic mice

5×10^6 of Hep 3B, HEP-R1a, PLC/PRF5 and PLC-R1b cells were suspended in Matrigel (BD Biosciences®) and injected subcutaneously into the dorsal right flank of athymic 4-week-old male NMRI-Foxn1^{nu}/Foxn1^{nu} mice (Janvier™). Tumor volumes (mm³) were calculated using the ellipsoid formula: $4/3 \times \pi \times (L/2 \times W/2 \times H/2)$. All procedures were in accordance with the “Guide of the Care and Use of laboratory Animals”. Institutional Review Board approval was obtained by «Le Comité d’Ethique en l’Expérimentation Animale Charles Darwin # B751201». Treatments are detailed in the [Supplementary Data](#) section.

Ethics

Informed consent was obtained from all patients before surgery. The database, including pathological variables, was established in accordance with the French data protection authority.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc. La Jolla, USA). Statistical analysis was carried out using t test or Bonferroni Multiple Comparisons Test: *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$. A p value of less than 0.05 was considered significant.

Results

NTS and NTSR1 are both over-expressed and correlated with worse overall survival in HCC patients

Protein expression of NTS and NTSR1 was analyzed by immunohistochemistry on HCC tumors. NTSR1 labelling was detected in 40/72 (56%) of tumors. The staining was clustered, and was more intense in the invasion front of the tumor ([Fig. 1A](#) (top)). We also evaluated the abnormal expression of NTS by studying NTS precursor labelling (LF-NTS). Amongst the specimens, 47/73 (64%) were scored positive ([Fig. 1A](#) (bottom)). Autocrine NTS regulation, as evaluated on successive slides labelled for NTS or NTSR1 was seen in 31/63 (49%) tumors. NTS over-expression in HCC versus normal livers was confirmed with the Mas liver dataset from GEO database [17]. As shown in [Fig. 1B](#), a subset of HCC patients had a significant increase in NTS mRNA expression level.

NTSR1 and NTS mRNA expression data were extracted from a cohort of 365 HCC patients from the TCGA database. Analysis of the available clinical data showed that the expression of NTSR1 was significantly lower in patients with hepatitis B or C compared with those having no history of the primary risk factors of HCC. The high expression of NTSR1 was significantly correlated with

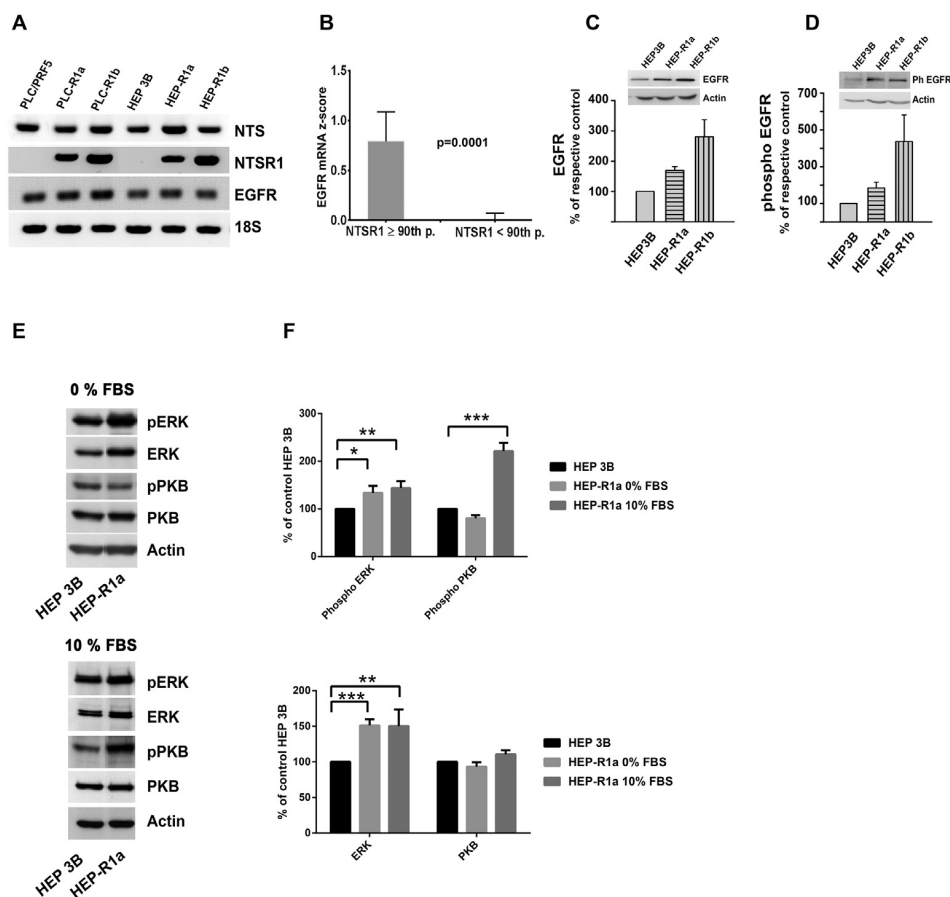


Fig. 4. Induction of EGFR expression and activation by the NTS/NTSR1 complex in HCC cell lines overexpressing NTSR1. (A) NTS, NTSR1, and EGFR transcript analysis from wild type PLC/PRF5 and HEP 3B wild types and two chosen clones stably transfected with the NTSR1 coding sequence for each cell line. (B) Correlation between NTSR1 and EGFR mRNA expression (z-score) in 375 tumor samples from Liver Hepatocellular Carcinoma (TCGA, Provisional). (C and D) Semi quantitative calculation of EGFR and phosphorylated EGFR protein content determined and normalized to actin in HEP 3B wild type cells and clones overexpressing NTSR1. Results from four experiments are expressed as relative expression compared to wild type cells. *Inset*: Representative western blot analyses of EGFR, and Tyr1173 phosphorylated EGFR using HEP 3B, HEP-R1a, and HEP-R1b cells. (E) Representative western blot analyses of phospho-ERK1/2, total ERK1/2, phospho-PKB and total PKB using HEP 3B and HEP-R1a cells cultured in serum-free (*top*) or 10% FBS medium (*bottom*). (E) Semi quantitative calculation of phospho-ERK1/2, phospho-PKB, total ERK1/2, and total PKB normalized to actin from HEP 3B and HEP-R1a cells. Results represent the average of four experiments \pm SEM as relative expression compared to respective wild type cells.

vascular invasion. The high expression of NTSR1 was more frequent in higher stages tumors, but insignificant. No correlation with NTSR1 and gender was observed (Supplementary Fig. 1). NTSR1 mRNA distribution showed that around 13% of the specimens overexpressed NTSR1 (Fig. 1C). We therefore performed the Kaplan–Meier analysis with a cutting point at the 90th percentile for NTSR1 expression z-score. The overexpression of NTSR1 was associated with a significantly worse prognosis ($p = 0.0205$). The median survival was 37.3 for patients with overexpressed NTSR1 and 69.5 months for the others. The 5-year survival rate was 17.90% [95% C.I. 3.51–41.39] vs 51.70% [95% C.I. 43.14–59.46], respectively (Fig. 1D).

Similar findings were found for NTS mRNA expression. The OS was significantly worse in patients with high NTS mRNA ($p = 0.0204$). The 5-year survival rate for patients with high NTS expression was 26.67% [95% C.I. 6.72–52.37] vs 50.05% [95% C.I. 42.16–57.93] for patients with low expression, and the median survival for these patients was 25 and 61 months, respectively (Fig. 1E).

We also compared patients with high NTS and/or NTSR1 mRNA expression to the others. A significant shorter OS was found in patients with high NTS and/or NTSR1 mRNA expression ($p = 0.0006$). The 5-year survival rate was 18.44% [95% C.I. 5.7–36.87] for patients with high NTS and/or NTSR1 mRNA expression vs 54.55% [95% C.I. 45.69–62.57] for the others (Fig. 1F). The median survival for these latter patients was 70 and 25 months, respectively.

NTS and NTSRs expression in HCC cells lines

We searched for NTS and NTSRs mRNA expression in three HCC cell lines: PLC/PRF5, HEP 3B, and HuH7. NTS, NTSR2, and NTSR3

were expressed in the three cell lines. NTSR1 mRNA was only detected in PLC/PRF5 cells (Fig. 2A lane 4 and 5), NTSR1 expression in this cell line was confirmed by immunofluorescence (Fig. 2B).

NTSR1 expression in HCC cells is regulated by the Wnt/ β -catenin pathway

Amongst the three cell lines studied, only PLC/PRF5 cells carried a deletion in Axin (E4/-), resulting in the dysregulation of the β -catenin pathway. In HCC cell lines, we forced the cellular and nuclear β -catenin relocation by exposing the cells to LiCl, or SB216763, an inhibitor of the glycogen synthase kinase (GSK) 3 β . As shown in the Fig. 2C, NTSR1 transcription was upregulated in the three HCC cell lines after LiCl exposure. NTSR1 immuno-labelling was remarkably amplified in HEP 3B and PLC/PRF5 cells (Fig. 2D) when cells were treated with SB216763 as compared to non-treated cells. The data suggest that NTSR1 overexpression is the result of the activation of the Wnt/ β -catenin pathway in HCC.

NTS/NTSR1 complex induced expression and activation of EGFR

NTS/NTSR1 complex is known to potentiate EGF signaling [2,6,33,34]. We investigated the possible relationship between NTSR1 and EGFR regulation. As shown in Fig. 3A, the treatment with SB216763 for 48 h induced an increase of NTSR1 and EGFR expression in both cell lines. Treatment with SR48692, a specific NTSR1 antagonist, decreased the expression of EGFR, whereas NTSR1 expression was not altered. In cells treated with the combination of SB216763 and SR48692 EGFR expression was decreased by the presence of the NTSR1 antagonist (Fig. 3A). The expression of EGFR dropped when NTSR1 expression was abolished in PLC/PRF5

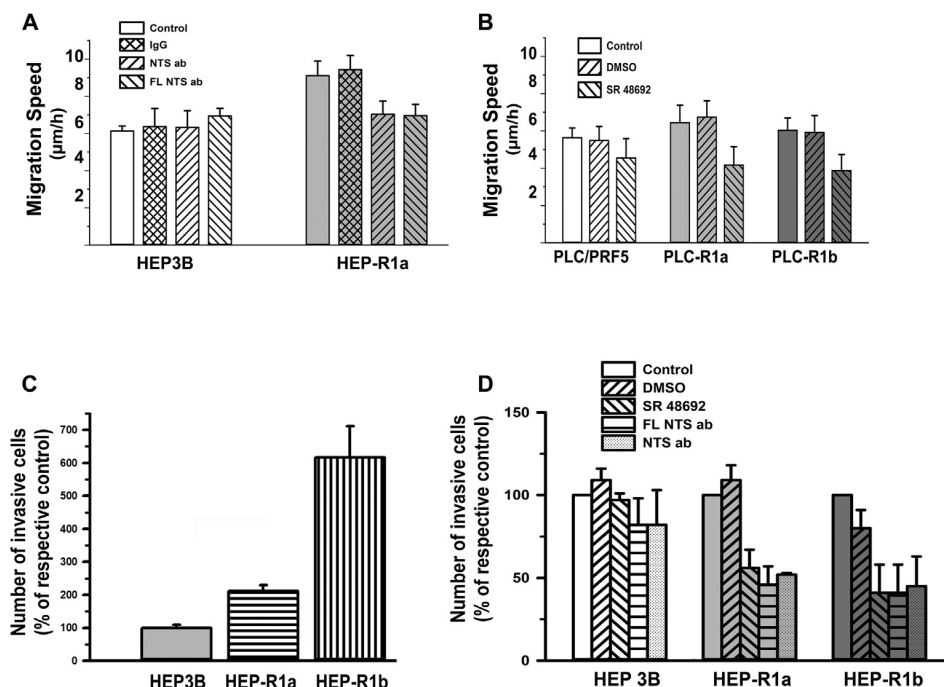


Fig. 5. NTS autocrine regulation enhanced migration speed and invasion on type 1 collagen matrix. (A) Speed of migration of HEP 3B and NTSR1 overexpressing clones treated or not with 1/100 NTS or 1/100 Long Fragment NTS antibody, on type 1 collagen. Results represent the mean \pm SEM of five independent experiments. (B) Speed of migration on type 1 collagen of PLC/PRF5 and NTSR1 overexpressing clones in the presence or absence of 5 μ M SR 48692. Results represent the mean \pm SEM of three independent experiments. (C) The numbers of invasive cells crossing the type I collagen in Boyden chamber for HEP 3B and NTSR1 overexpressing clones. Results are expressed as a ratio compared to wild type cells and represent the mean \pm SEM of five to seven independent experiments. (D) The numbers of invasive cells crossing the type I collagen in Boyden chamber when HEP 3B and NTSR1 overexpressing clones were treated or not with NTS or Long Fragment NTS antibody or 5 μ M SR 48692. Results are expressed as a ratio compared to each control group and represent the mean \pm SEM of five to seven independent experiments.

by a transient transfection with Sh-NTSR1, as compared to controls transfected with Sh-scramble (Fig. 3B).

The activated form of EGFR was evaluated by a western blot analysis. In both cell lines, the quantity of the Tyr1173 phospho-EGFR form was increased under SB216763 treatment. This increase was abolished in the presence of SR48692 (Fig. 3C and D).

Cells stably overexpressing NTSR1 were generated in order to avoid the non-specific effects of chemicals. For each cell line, clones with moderate NTSR1 expression, PLC-R1a, and HEP-R1a, and clones with high NTSR1 expression, PLC-R1b, and HEP-R1b, were selected. We noted that EGFR basal transcript levels were higher in PLC/PRF5 cells than in HEP3B cells. The overexpression of NTSR1 did not change this pattern within the cell lines. Nevertheless,

higher EGFR transcript levels ($p = 0.0001$) were correlated with patients having NTSR1 overexpression (Fig. 4A and B).

Compared to the parental cells, the basal EGFR protein expression was up-regulated in all NTSR1 expressing clones. The level of phospho-EGFR was raised by 2 fold in the clones as compared to parental cells (Fig. 4C, D and Supplementary Fig. 2).

RAF-MEK-ERK and PKB-mTOR are the two main downstream oncogenic cascades that are activated by EGFR. We examined the protein expression and activation of the major effectors of these cascades, ERK and PKB, under serum free medium or 10% FBS medium. Independently of the cell culture conditions, total ERK protein content, and the phosphorylated form were enhanced in NTSR1 overexpressing cells as compared to control cells, whereas

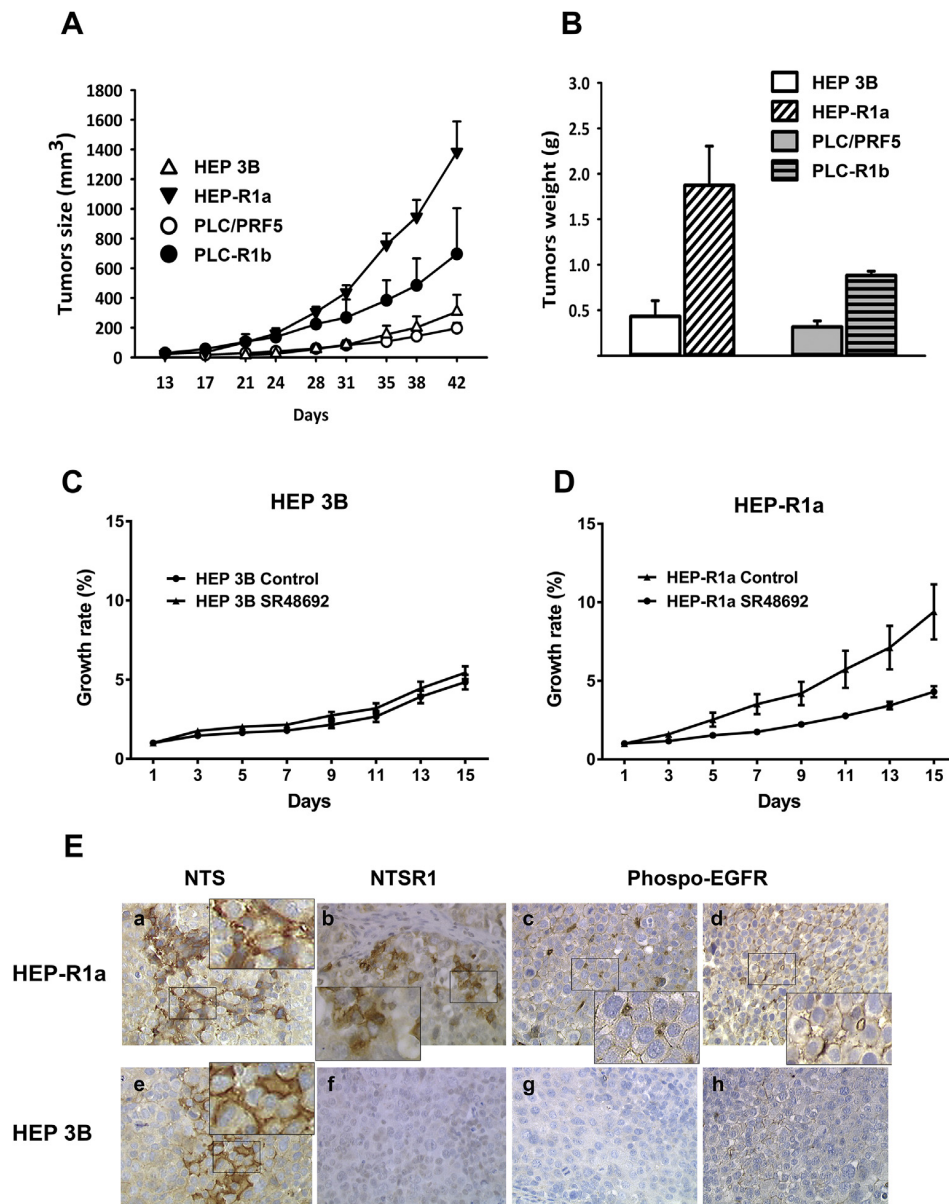


Fig. 6. NTS/NTSR1 complex enhanced experimental tumor growth using HCC cell lines. (A) Experimental tumors were generated from the HCC cancer cell lines, HEP 3B and PLC/PRF5 and the NTSR1-overexpressing sub-clones. Comparative growth curves of HEP 3B, HEP-R1a, PLC/PRF5, PLC-R1b in 8, 7, 10 and 7 mice, respectively. Tumor volumes were measured twice a week. (B) Tumor weight at 42 days. (C and D) Experimental tumors were generated from HEP 3B or HEP-R1a in different mice. When the tumor size reached 110 mm³, mice were randomly distributed into two groups for each tumor model. A control group was force fed with corresponding vehicle. The SR 48692 treated group, was force fed every day with 1 mg/kg SR 48692. Result shown is the ratio of tumor size compared to day 1. (E) Typical IHC performed on paraffin sections for NTS, NTSR1 EGFR and Phospho-EGFR labelling, 200× magnification.

total PKB levels remained unchanged. Chronic stimulation of PKB was observed in the NTSR1 overexpressing cells when cultured in 10% FBS medium (Fig. 4E and F).

NTS/NTSR1 complex enhances migration and invasion of HCC cells

We estimated the migration speed of two cell lines at the same time and their effect on the intact type I collagen matrix, over a period of 48 h. As shown in Fig. 5, HEP-R1a cells migrate at speed of $9.1 \pm 0.78 \mu\text{m/h}$, whereas HEP 3B cells were slower with a speed of $6.13 \pm 0.27 \mu\text{m/h}$ ($p = 0.002$). Addition of NTS or LF-NTS antibodies inhibited the acceleration of HEP-R1a cells, which returned to a migration speed similar to wild type cells (Fig. 5A). Similar observations were made for PLC-R1a and PLC-R1b cells, when exposed to SR48692, the speed was significantly decrease by 27% ($p = 0.02$ vs DMSO) and 33% ($p = 0.01$ vs DMSO), respectively. Nevertheless, the respective migration speeds were not significantly different for PLC/PRF5, PLC-R1a and PLC-R1b, with 5.63 ± 0.52 , 6.44 ± 0.93 , and $6.03 \pm 0.66 \mu\text{m/h}$, respectively.

In parallel, invasiveness characteristics were evaluated, on type-I collagen-coated inserts. Compared with HEP 3B cells, the number of invading cancer cells for HEP-R1a cells and HEP-R1b cells was increased by $211 \pm 19\%$ ($p < 0.01$) and $617 \pm 94\%$ ($p < 0.01$), respectively (Fig. 5C). When cells were exposed to NTSR1 antagonist, or NTS or LF-NTS antibodies the increase of cellular invasion

was either fully or partially abolished in HEP-R1a and HEP-R1b cells (Fig. 5D). In our hands, the PLC/PRF5 cells were highly invasive, overexpression of NTSR1 did not markedly change these characteristics. Collectively, these data suggest that the NTS and NTSR1 promote the invasiveness and migration of HCC cells, and corroborate the data previously published for HCC [32] and breast cancer [6].

NTS/NTSR1 enhance experimental HCC tumor progression through EGFR activation

To determine the contribution of NTS/NTSR1 complex on tumor progression, mice were implanted with HCC cell lines and NTSR1 overexpressing clones. HEP 3B and PLC/PRF5 cells showed similar growth rates, but the growth rate at day 42 was 2.9 and 2.08 fold higher for HEP-R1a and PLC-R1b as compared to respective parental cells (Fig. 6A). As shown in Fig. 6B the tumor weights are in correspondence with the tumor size.

When animals were treated with SR48692, no influence on the HEP 3B tumor growth rate was detected, but a twofold growth rate inhibition of HEP-R1a tumors was seen (Fig. 6C and D). The final tumor size of HEP-R1a tumors treated with SR48692 was similar to the untreated HEP 3B.

We confirmed the expression of NTS in the tumors of HEP 3B and HEP-R1a cells. In both tumors clusters of strong intra-

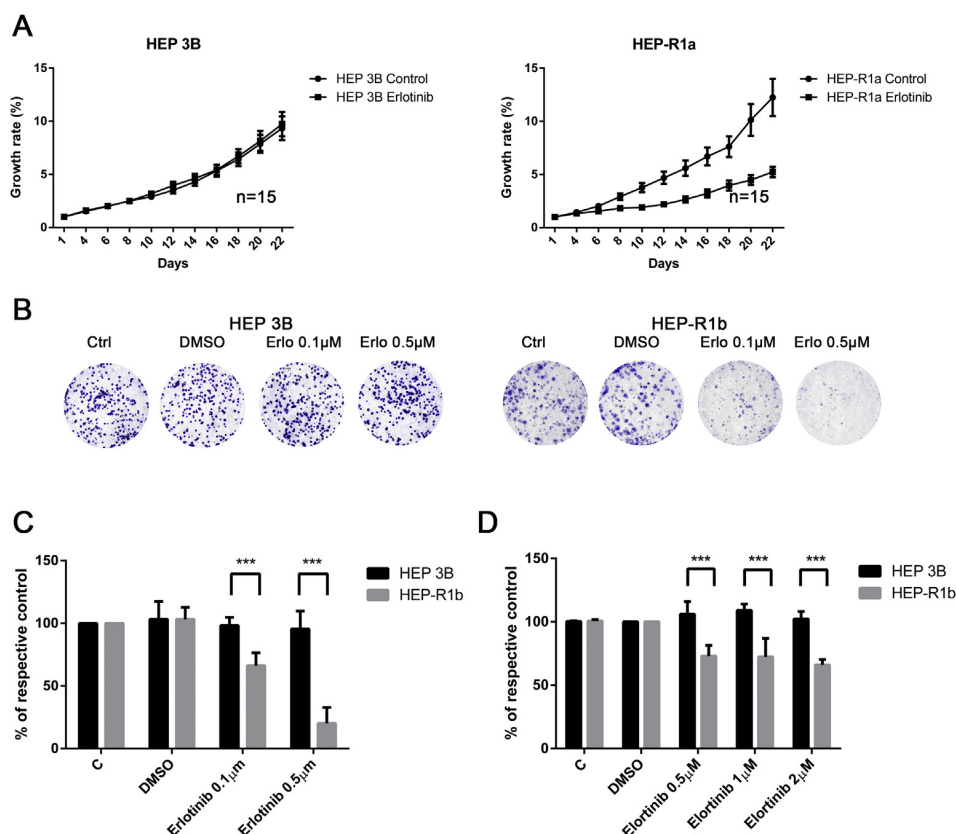


Fig. 7. Neurotensin regulation restores response to erlotinib. (A) Experimental tumors were generated from HEP 3B and HEP-R1a in the same mice. HEP 3B cells were injected 7 days before HEP-R1a cells. In the control group, the average HEP 3B tumor size was $168.2 \pm 38.1 \text{ mm}^3$ and the HEP-R1a tumor size was $79.7 \pm 16.4 \text{ mm}^3$ at day 1. The treated group carried HEP 3B tumors of $164.7 \pm 31.2 \text{ mm}^3$ and HEP-R1a tumors of $87.2 \pm 19.13 \text{ mm}^3$ at day 1. Mice were treated with 75 mg/kg erlotinib every day for 22 days, *per os*. Tumor size was measured every two days. Result shown is the ratio of tumor size compared to day 1. (B) Photos of clonogenic assays performed with HEP 3B and HEP-R1b cells treated with different doses of erlotinib after two weeks of treatment. (C) Clonogenic assay colonies were quantified using the Image J software. Results are the means of 6 independent experiments and expressed as percentage of respective control. (D) Cellular proliferation of HEP 3B and HEP-R1b cells using Prestoblu[®] cell viability reagent. Results are the means of three independent experiments and are expressed as percentage of respective control.

cytoplasmic labelling were seen distributed randomly on the slide (Fig. 6E a and e). The labelling of NTSR1 in HEP-R1a was mostly intra-cytoplasmic and weak. Nevertheless, cell clusters with very strong cytoplasmic and membrane expression were often seen on the slide (Fig. 6E b). NTSR1 labelling was not detected in HEP 3B tumors (Fig. 6E f). In the HEP-R1a tumors, the labelling intensity of phospho-EGFR was heterogeneous with a thin to thick line around the entire cell membrane. The strong labeling was often localized at the front of the tumor or close to the blood vessel. Examples of a

thin or strong labelling are shown in Fig. 6E c and d. In contrast, in HEP 3B tumors, phospho-EGFR labeling was absent or very weak as shown in Fig. 6E, G and H.

NTS/NTSR1 restores response to erlotinib

HEP 3B and HEP-R1a were xenografted on the same mice. The mice were randomly distributed into two groups based on the size of the HEP 3B tumors. The growth rate of the HEP-R1a tumors was

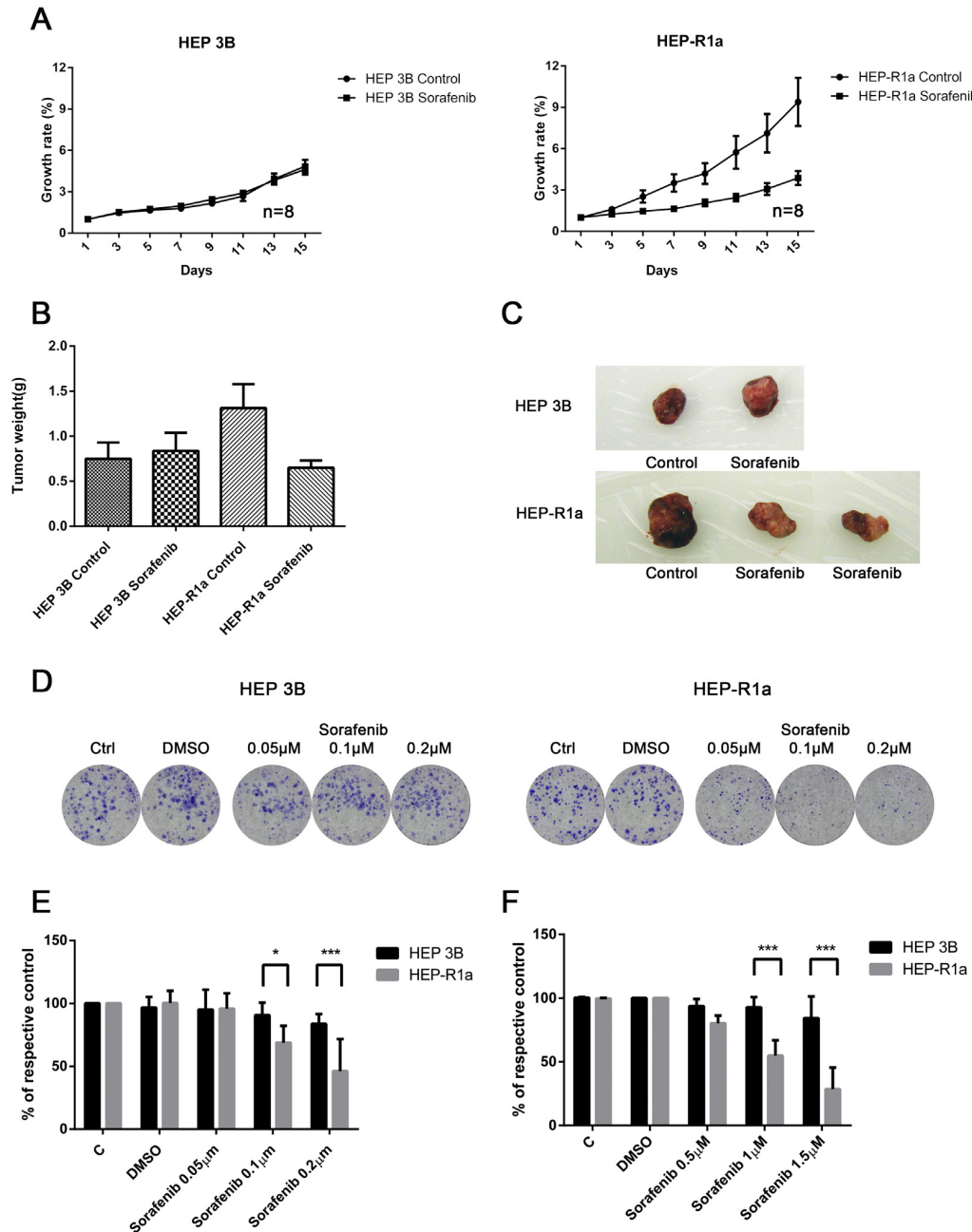


Fig. 8. Neurotensin regulation restores response to sorafenib. (A and B) Experimental tumors were generated from HEP 3B or HEP-R1a in different mice. When the tumor size reached 110 mm³, mice were randomly distributed in two groups for each tumor model. A control group was force fed with corresponding vehicle. The sorafenib treated group was force fed every day with 15 mg/kg sorafenib. Result shown is the ratio of tumor size compared to day 1. (C) Representative subcutaneous tumors at the end of the experiment. (D) Example of clonogenic assay performed with HEP 3B and HEP-R1a cells treated with different dose of sorafenib for two weeks. (E) Clonogenic assay colonies were quantified using the Image J software. Results are the means of six independent experiments. (F) Cellular proliferation performed with Prestoblu® assay on HEP 3B and HEP-R1a cells. Results are the means of six independent experiments.

drastically affected by the EGFR inhibitor, whereas HEP 3B tumors did not respond to erlotinib (Fig. 7A). *In vitro*, we confirmed the erlotinib response in cells overexpressing NTSR1 in clonogenic assays. As shown in Fig. 7B and C, the number of colonies formed by HEP-R1b cells was reduced in a dose-dependent manner when cells were exposed to erlotinib. In contrast, HEP 3B cells are not sensitive to erlotinib. Cellular proliferation tests provided a similar result. The cells overexpressing NTSR1 were more sensitive to erlotinib than HEP 3B cells (Fig. 7D).

NTS/NTSR1 restores response to the standard of care treatment of advanced HCC, sorafenib

We challenged HEP 3B or HEP-R1a tumors with sorafenib. As the treatment has no influence on the HEP 3B tumor growth rate, sorafenib inhibited the growth rate of HEP-R1a tumors by two fold (Fig. 8A). The final tumor size of HEP-R1a tumors treated with sorafenib was $400.3 \pm 59.9 \text{ mm}^3$ and remained similar to the untreated HEP 3B tumors $516.3 \pm 139.72 \text{ mm}^3$. In HEP-R1a tumors, sorafenib reduced tumor weight by two fold compared to the control group, whereas tumor weight was similar for HEP 3B treated or not with sorfenib (Fig. 8B and C).

In order to separate the angiogenesis inhibition effect of sorafenib, we performed clonogenic and proliferation assays *in vitro*. As shown in Fig. 8D and E, HEP-R1a cells show a remarkably dose-dependent effect of sorafenib in both assays. In contrast, HEP 3B cells did not respond to sorafenib (Fig. 8F). These results suggest that sorafenib reduced tumor progression by inhibiting cell growth in the NTSR1 expressing tumors.

Discussion

NTSR1 is weakly expressed (or absent) in normal tissues. However, NTSR1 is overexpressed in many types of solid cancers especially in cancers from the gastrointestinal tract. One known

mechanism for its abnormal expression is linked to the activation of the NTSR1 promoter by a dysregulated β -catenin pathway [24,30]. In our clinical data, NTSR1 was found positive in 56% of the patients, and in the TCGA data set, NTSR1 mRNA was found overexpressed in 13% of the specimens. Activation of the β -catenin pathway is generally found in 40–70% of HCC [12,14,15]. This pathway alters many different liver functions, and contributes to hepatocyte transformation by directly or indirectly regulating many genes at the transcription level including NTSR1. Interestingly, a recent study proposed that NTS/NTSR1 signaling enhanced epithelial/mesenchymal transition by activating Wnt/ β -catenin signaling [32]. Together the data from these two papers suggest a feed forward regulation of the NTSR1 signaling on abnormal β -catenin signaling.

In the liver, EGFR is known to be a target gene of the Wnt/ β -catenin pathway [26]. Here we confirmed that EGFR is a Wnt/ β -catenin targeted gene, the most deregulated in correlation with NTSR1 overexpression in HCC. In parallel, our *in vitro* results associated an increase in EGFR protein levels with its sustained activation. NTS/NTSR1 would therefore be expected to be a mediator between the Wnt/ β -catenin pathway and EGFR signaling. These regulations enhance invasive and migratory cellular effects and consequently metastatic processes in HCC cells, and are similar to results in breast, lung cancer, and neck squamous cell carcinomas [6,21,33]. Even the endpoint effects are similar between the carcinomas, though the mechanisms sustaining these effects are different, involving diverse metalloproteinases, epidermal growth factor receptors and their ligands [6,33]. These results argue that the NTS/NTSR1 complex is a possible regulator with an upstream position in the chain of cellular events involved in HCC progression.

As a consequence of sustained activation of the EGFR-ERK cascade by NTS/NTSR1, erlotinib is efficient on tumor growth rates. EGFR mutations are rare in HCC [25]. Clinical trials with erlotinib in HCC have not been conducted on a selected population according to their EGFR genetic profile and there are no encouraging results for the application of erlotinib in HCC [18,27].

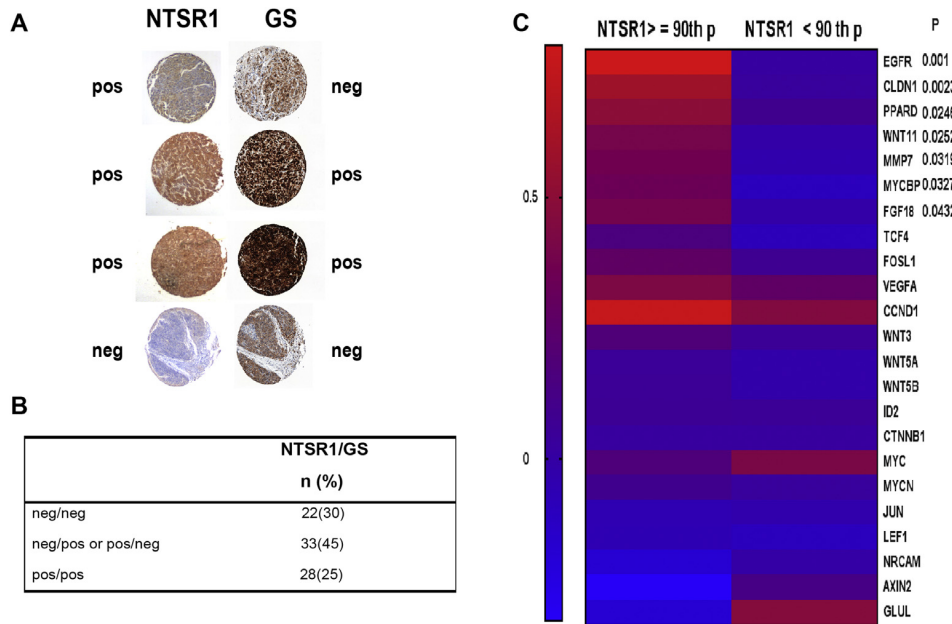


Fig. 9. Correlation between NTSR1 expression and Wnt/ β -catenin activated pathway. (A) Examples for negative and positive labelling for NTSR1 (left) and glutamine synthetase (right). (B) The number and proportion of HCC scored positive or negative for NTSR1 and glutamine synthetase within the 73 HCC tumor specimens. (C) Heat map of the gene expression profile for the major genes directly targeted by the β -catenin activated pathway (defined as those with Tcf binding sites) with the greatest differences in expression (red, high; blue, low) compared to NTSR1 expression ≥ 90 th p. vs NTSR1 expression < 90 th p. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sorafenib showed better success in HCC because it is a multi-kinase inhibitor, acting on tumor growth through the inhibition of the PDGFR and Raf, but also on angiogenesis through the inhibition of VEGFR. Sorafenib has slightly improved survival benefits of HCC patients [16], but the treatment is sometimes discontinued because of its toxicity. Our results suggest that the evaluation of NTSR1 in HCC could be a theranostic biomarker to select eligible patients for these inhibitors. The NTSR1 marker would identify a different patient subpopulation than the β -catenin biomarker, as only 25% of the tumors exhibit a positive labelling for NTSR1 and are also positive for glutamine synthetase, a marker for the activated β -catenin pathway used in diagnostic centers [3] (Fig. 9A and B). In addition, amongst the genes directly altered by the activated β -catenin pathway, only half are associated with high NTSR1 expression (Fig. 9C). Major wnt/ β -catenin targeted genes with a high impact on cell proliferation, such as c-Myc, cyclin D1, or c-Jun are not associated with NTSR1 overexpression.

NTSR1 activation by NTS secreted from HCC cancer cells, or from the hepatic portal vein, may contribute to cancer aggressiveness by enhancing concomitant expression and activation of the EGFR. Sustained activation of NTSR1 improves sorafenib and erlotinib response. We propose the evaluation of NTSR1 in HCC as a theranostic biomarker to select eligible patients for these inhibitors.

Conflict of interest

No conflict of interest

Acknowledgment

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.canlet.2016.11.032>.

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Résumé: Le cancer de l'ovaire est la huitième cause de décès par cancer chez la femme dans le monde. La majorité des cancers de l'ovaire est diagnostiquée à un stade avancé, car cette maladie n'a souvent pas de symptômes apparents pendant les premiers stades. Le traitement proposant la combinaison carboplatine-paclitaxel donne un taux de réponse complet dans 40 à 60 % des cas. Cependant, plus de 90 % des patientes récidive après 2 ans, et les patientes récurrentes dans la plupart des cas deviennent incurables en raison du développement de la chimiorésistance. Il a largement été montré que le complexe de neurotensine (NTS) et son récepteur à haute affinité 1 (NTSR1) favorise la progression du cancer par prolifération, survie, migration, invasion et néoangiogenèse *in vitro* et/ou *in vivo* dans de nombreux cancers. A ce jour, le rôle du complexe NTS/NTSR1 dans la réponse à la chimiothérapie à base de platine n'a pas été pris en compte. Dans ce travail, j'ai étudié si les inhibiteurs ce complexe pouvait améliorer la réponse à la chimiothérapie, et les mécanismes associés à cet effet. Dans une série de 46 patients du cancer de l'ovaire, NTS et NTSR1 ont été détectés respectivement dans 72 % et 74 % des cas. L'étude du transcriptome de cancer de haut grade a montré que l'expression du NTSR1 était liée à des stades plus élevés et au statut "résistant au platine". J'ai pu mettre en évidence la contribution de la voie NTS/NTSR1 à réponse la chimiothérapie en utilisant deux lignées cellulaires de cancer de l'ovaire. En présence d'un antagoniste spécifique du NTSR1, le SR 48692, des cellules cancéreuses de l'ovaire ou des tumeurs expérimentales ont montré une réponse amplifiée au carboplatine. En effet, la présence du SR 48692 diminue l'efflux de carboplatine des cellules, et ainsi augmente les dommages à l'ADN induit par le platine. L'apoptose a également été renforcée en présence de cet antagoniste. Ces résultats renforcent notre hypothèse selon laquelle le blocage de la voie NTS/NTSR1 améliore la réponse à la chimiothérapie et potentiellement sensibilise les cellules tumorales qui sont résistantes.

Mots clés: neurotensine, récepteur haute affinité de la neurotensine, cancer de l'ovaire, chimiothérapie à base de platine

Contribution of the complex neurotensin and its high affinity receptor to the response of chemotherapy in ovarian cancer

Abstract: Ovarian cancer (OC) is the eighth most common cause of cancer death in female worldwide. Because OC has often no apparent symptoms at the early stages, the majority is diagnosed at the advanced stages. The combination of carboplatin plus paclitaxel, results in a complete response rate in 40-60 % of the cases. However, more than 90% patients relapse after 2 years, and in most cases, recurrent patients becomes incurable due to the chemoresistance. The complex of neurotensin (NTS) and its high affinity receptor 1 (NTSR1) has been shown to promote cancer progression in many type of cancer, via proliferation, survival, migration, invasion cellular effects, and neoangiogenesis. To date, the role of the complex NTS/NTSR1 in the platinum-based chemotherapy has not been considered. I studied whether NTS/NTSR1 inhibitors could enhance chemotherapy and the related mechanism. In a series of 46 patients, NTS and NTSR1 were detected in 72% and 74% of cases, respectively. Transcriptome analysis in a large series of high grade OC showed that NTSR1 expression was correlated with higher stages and with platinum resistance. We studied the contribution of NTS/NTSR1 pathway to chemotherapy by using two OC cell lines. In the presence of NTSR1 specific antagonist, SR 48692, OC cells or experimental tumors showed an enhanced response to carboplatin. SR 48692 decreased the efflux of carboplatin and increase the DNA damage induced by the platinum. Apoptosis was also enhanced in the presence of NTSR1 antagonist. These results strengthen our hypothesis that the blockade of NTS/NTSR1 pathway enhances the response to chemotherapy and potentially sensitizes tumor cells resistant to platinum.

Key word: Neurotensin, High affinity receptor of neurotensin, Ovarian cancer, Platinum-based chemotherapy