MOLECULAR MECHANISMS OF BREAST CANCER INHIBITION BY VITAMIN E ANALOGUES

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Abstract

Over-expression of the receptor tyrosine kinase erbB2 makes cancer cells resistant to apoptosis. α-Tocopheryl succinate (α-TOS), a redox-silent vitamin E (VE) analogue, has been shown to kill many different types of cancer cells. We tested whether α-TOS and several novel VE analogues kill breast cancer cells over-expressing the anti-apoptotic receptor protein erbB2 (HER2). Our experiments revealed that VE analogues caused comparable levels of apoptosis in breast cancer cells expressing different levels of erbB2. To extend our understanding of the molecular mechanisms of cell death triggered by VE analogues, we investigated apoptotic pathways induced by the agents, one of which is the mitochondrial pathway. Generation of reactive oxygen species (ROS) preceded mitochondrial destabilization and execution of apoptosis, as evidenced by the anti-apoptotic effects of exogenous superoxide dismutase (SOD) and mitochondrially targeted coenzyme Q (MitoQ). Dissipation of the mitochondrial potential ($\Delta\Psi_m$) was followed by cytochrome c (Cyt c) and Smac/Diablo re-localization and caspase-dependent cleavage of death substrate. The other pathway studied was the survival (anti-apoptotic) Akt/NFκB signalling pathway. We document that α-TOS inhibited nuclear translocation of the subunit p65 of the transcriptional factor nuclear factor-κB (NFκB) in erbB2-over-expressing breast cancer cells, which is expected to result in inactivation of NFκB. A synergism of α-TOS and the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) in apoptosis induction was confirmed in experiments with breast cancer cells with high

and low expression of erbB2. To verify the efficacy of VE analogues in killing cancer cells *in vivo*, we also used the FVB/N *c-neu* transgenic mice with spontaneous breast carcinomas (high erbB2) and the human breast cancer MCF7 xenograft model (low erbB2) to investigate the effect of α-TOS on cancer progression. We show that α-TOS significantly inhibited tumour growth compared to the controls in both animal models.

We explored the premise that high level of erbB2 expression in cancer cells also allows their targeting by attachment of a specific peptide to an apoptogenic agent. Our group studied the possibility that the conjugate of the pro-apoptotic α-TOS and the heptapeptide LTVSPWY induces efficient apoptosis in erbB2-over-expressing breast cancer cells. The α-TOS-peptide conjugate induced more apoptosis in cells with high levels of erbB2 than did α-TOS only, while the opposite was observed for cells with low level of expression of the erbB2 protein. Apoptosis induced in erbB2-expressing cells by LTVSPWY-α-TOS was partially suppressed by erbB2 siRNA, as well as by inhibition of endocytosis or the lysosomal function. HPLC analysis revealed fast accumulation of α-TOS in cells with high level of erbB2 exposed to LTVSPWY-α-TOS. Finally, the peptide conjugate of α-TOS suppressed very efficiently breast carcinomas in the FVB/N *c-neu* mice. We conclude that conjugation of a specific peptide targets α-TOS to erbB2-expressing cancer cells, causing their rapid apoptosis, and propose an anti-tumour strategy that relies on targeting of anti-cancer drugs to malignant cells by their coupling to peptides recognized by receptors over-expressed by cancer cells.

Our data contribute to the knowledge of the molecular mechanism by which VE analogues kill breast cancer cells and support the notion that these agents are of clinical interest for treatment of breast carcinomas.

Statement of Originality

The work presented within this thesis was performed in the Apoptosis Research Group, School of Medical Science, Griffith University Gold Coast Campus. The research was carried out under the supervision of Professor Jiri Neuzil and Associate Professor Stephen Ralph.

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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This work would not be possible without the help from colleagues in different collaborating laboratories, who contributed as follows: Several new analogues of VE, such as α-tocopheryl maleate (α-TOM), α-tocopheryl maleyl amide (α-TAM) or α-tocopheryl succinyl amide (α-TAS) were synthesized by Dr. Marc Birringer (University of Jena, Germany) and Dr. Brian Salvatore (Louisiana State University, Shrevepoprt, LA, USA) following the design of these compounds together with my supervisor. EPR measurements were done by Dr. Paul Witting (University of Sydney, NSW, Australia), a co-author of several of our publications, with my assistance. The

mitochondrially targeted analogue of coenzyme Q (MitoQ) was kindly donated by Dr. Robin Smith (University of Otago, Dunedin, New Zealand). The peptide conjugate LTVSPWY-α-TOS was synthesized by Dr. Marc Birringer (University of Jena, Germany), a co-author of our paper (Cancer Res 67, 3337-3344, 2007). Marc Birringer initially synthesized a small amount of the conjugate for cell culture work. Larger amounts for *in vivo* experiments with the FVB/N *c-neu* transgenic mice were synthesized by Dr. Pavel Veprek and Dr. Miroslav Ledvina (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic), who also co-authored our recent paper (Cancer Res 67, 3337-3344, 2007). The idea to synthesize this conjugate follows from a previous report by Shadidi and Sioud (FASEB J, 17, 256, 2003) and was developed by my supervisor, who contacted the authors of the FASEB J paper for initial advice. The plasmid pcDNA3.1-Bcl-x_L was kindly donated by Dr. Richard Youle (NIH, Bethesda, MD, USA). Low-density lipoprotein was provided by Dr. Paul Witting, the plasmid harbouring wild type and dominant-negative (K44A) dynamin was donated by Dr. Kohji Takei (Juntendo University School of Medicine, Tokyo, Japan). The work was supported by grants awarded to Prof. Jiri Neuzil by the Australian Research Council, the Cancer Council Queensland and the National Breast Cancer Foundation.

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List of Publications

During the PhD stage, I have been involved in several projects in our laboratory, many of which resulted in publications in peer-reviewed journals, some even after I finished my PhD project. I am the first author on two original papers (papers 1 and 9 in the list below) and a review paper (paper 5). Paper 1 is the basis of Chapter 2 of the thesis, paper 9 is largely included in Chapter 3. Data in Chapter 4 are yet to be published (a manuscript will be submitted in near future). Besides the first authorship papers, I am a co-author on a number of peer-reviewed papers from our laboratory, due to involvement in the experiments (helping with cell culture, transfections, apoptosis assessment, western blotting or ultrasound imaging of experimental tumours).

1. **Wang XF**, Witting PK, Salvatore BA, Neuzil J (2005) α-Tocopheryl succinate induces apoptosis in HER2/erbB2-over-expressing breast cancer cells by signaling via the mitochondrial pathway. Biochem Biophys Res Commun 326, 282-289. (IF 2.75) *(Chapter 2 of this thesis is based on data published in this paper.)*
2. Neuzil J, **Wang XF**, Zhao Y, Wu K (2005) Vitamin E analogs and cancer. In Nutrition and Cancer Prevention. Awad AB, Bradford PG, eds. Marcel Dekker, New York, p. 111-137. Invited chapter.
3. Dong LF, **Wang XF**, Zhao Y, Tomasetti M, Wu K, Neuzil J (2006) Vitamin E analogues as anti-cancer agents: the role of modulation of apoptosis signaling pathways. Cancer Therapy 4, 35-46. Invited review.

4. Neuzil J, Dong LF, **Wang XF**, Zingg JM (2006) Tocopherol-associated protein-1 accelerates apoptosis induced by α-tocopheryl succinate in mesothelioma cells. Biochem Biophys Res Commun 343, 1113-1117. (IF 2.75)
5. **Wang XF**, Dong LF, Zhao Y, Tomasetti M, Wu K, Neuzil J (2006) Vitamin E analogues as anti-cancer agents: Lessons from studies with α-tocopheryl succinate. Mol Nutr Food Res 50, 675-685. Invited review. (IF 3.45)
6. Neuzil J, **Wang XF**, Dong LF, Low P, Ralph SJ (2006) Molecular mechanism of 'mitocan'-induced apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family proteins. FEBS Lett 580, 5125-5129. Hypothesis paper. (IF 3.26)
7. Neuzil J, Stantic M, Zobalova R, Chladova M, **Wang XF**, Dong LF, Prochazka L, Ralph SJ (2007) Tumour-initiating cells *vs.* cancer 'stem' cells and CD133: What's in the name? Biochem Biophys Res Commun 355, 855-859. (IF 2.75)
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10. Neuzil J, Tomasetti M, Zhao Y, Dong LF, Birringer M, **Wang XF**, Low P, Wu K, Salvatore BA, Ralph SJ (2007) Vitamin E analogs, a novel group of mitocans, as selective anti-cancer agents with multiple mode of action: The importance of being redox-silent. Mol Pharmacol 71, 1185-1199. Invited review. (IF 4.09)
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13. Neuzil J, Dong LF, Hahn T, **Wang XF**, Gold M, Chladova M, Prochazka L, Freeman RE, Zobalova R, Turanek J, Akporiaye ET, Dyason J, Ralph SJ (2007) Vitamin E analogues: a novel group of mitocans, anti-cancer agents that act by targeting mitochondria. Mol Aspects Med 28,607-645. Invited review. (IF 7.39)
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15. Dong LF, Low P, Dyason J, **Wang XF**, Prochazka L, Witting PK, Freeman R, Swettenham E, Valis K, Liu J, Zobalova R, Turanek J, Spitz DR, Domann FE,

Scheffler IE, Ralph SJ, Neuzil J (2008) α-Tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II. Oncogene 27, 4324-4335. (IF 6.44).

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Abbreviations

AIF	apoptosis-inducing factor
CDK	cyclin-cyclin-dependent kinase
CKI	CDK inhibitor
CII	complex II
Cyt c	cytochrome c
DCF	dihydrodichlorofluorescein diacetate
DcR	decoy receptor
DD	death domain
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbo-cyanine perchlorate
DISC	death-inducing signalling complex
DMPO	5, 5-dimethyl-1-pyrroline
DN	dominant-negative

DR	death receptor
EGFR	epidermal growth factor receptor
EPR	electron paramagnetic resonance
ER	estrogen receptor
EtBr	ethidium bromide
FADD	Fas-associated death domain
FITC-Tr	FITC-conjugated transferrin
FLIP	Flice-like inhibitory protein
GSK3β	glycogen synthase kinase-3β
HBSS	Hank's buffered saline solution
HER-2	human epidermal growth factor receptor-2
IAPs	inhibitors of apoptosis proteins
i.p.	intraperitoneal
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidoazolyl-carbocyanino iodide
LDL	low density lipoprotein
MFI	mean fluorescence intensity
MitoQ	mitochondrially targeted coenzyme Q
MOM	mitochondrial outer membrane
MM	malignant mesothelioma
MTT	methylthiazoletetrazolium
NFκB	nuclear factor-κB

NS	non-silencing
PH	pleckstrin domain
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
ROS	reactive oxygen species
s.c.	subcutaneous(ly)
siRNA	short-interfering RNA
SOD	superoxide dismutase
α-TAM	α-tocopheryl maleate amide
α-TAS	α-tocopheryl succinyl amide
TNF	tumour necrosis factor
α-TOM	α-tocopheryl maleate
mTOR	mammalian target of rapamycin
α-TOS	α-tocopheryl succinate
TRAIL	TNF-related apoptosis-inducing ligand
USI	ultrasound imaging
VE	vitamin E
VEGF	vascular endothelial growth factor
WT	wild-type
ΔΨm	mitochondrial inner trans-membrane potential

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Basic information about breast cancer

1.1.1. Epidemiology

Breast cancer is the most predominant tumour among women in industrialized countries. The incidence rate is 70-100 cases per 100,000 women per year and the mortality rate is 20-30 deaths per 100,000 women per year in Western countries, while this is about 5-fold less in Eastern Asia and Africa [Baselga et al., 2002; Key et al., 2001; Jemal et al., 2009]. The recognized risk factors for breast cancer are age, increased hormone exposure and genetic predisposition. Breast cancer is age-dependent, e.g. the incidence in North America and Europe is about 2.5% by the age of 55, 5% by the age of 65 and 7.5% by the age of 75 [Key et al., 2001]. Increased hormone exposure, such as early menarche, late menopause, oral contraceptive, or hormonal therapy together with alcohol consumption and obesity are all associated with increased risk. Breast feeding, early first pregnancy and physical exercise are associated with a reduced risk [Baselga et al., 2002; Key et al., 2001]. Majority of breast cancers arise sporadically. However, family history is responsible for about 2-5% of breast cancers. Genes involved in the hereditary forms of breast cancer include *BRCA1*, *BRCA2*, *p53*, *STK11/LKB1*, *PTEN* and *ATM* [Baselga et al., 2002; Key et al., 2001]. Other researchers also found that there are more risk factors acting separately and/or together to affect women health and possibly the risk of developing breast cancer. Moreover, stress in various forms also influences the incidence of breast cancer.

1.1.2. Biology of breast cancer

The mammary gland is a highly differentiated organ that is responsible for providing nutrition to the progeny. Mammary development starts during embryogenesis. In humans, males and females have similar rudimentary mammary gland at birth. Later mammary development is initiated with the beginning of female puberty and is dependent on the high level of estrogen produced by the ovary, as well as on the level of progesterone. After puberty, the mammary gland undergoes cycles of growth and involution regulated by the menstrual cycle, and cycles of pregnancy and lactation. Histologically, the mammary gland consists of a rudimentary branching duct system lying in a fat pad. Post-pubertal development leads to cyclical increase in ductal branching, resulting in a ductal tree that fills the fat pad. During pregnancy, further branching and end-bud development lead to an appearance that is reminiscent of bunches of 'grapes'. After weaning, mammary gland regression to a pre-pregnancy-like state is manifested by the apoptotic process.

The ductal structure consists of a continuous layer of epithelial cells responsible for milk synthesis and its release into the lumen. A second layer of myoepithelial cells contacts the basement membrane. The two cell layers, together with the surrounding tissue, form the basis of the ducts [Russo et al., 1987; 1994]. Epithelial cells are sites of estradiol action within the breast, comprising the epithelial cell estrogen receptor (ER) as documented by immunohistochemical analysis [Ricketts et al., 1991]. According to the literature, the luminal epithelial cells give rise to most breast

tumours.

One way of categorizing tumours is by means of staging using the tumour, nodes and the metastasis system, which then determines the treatment recommendations. Invasive breast cancer is divided into 4 stages, from small and localised (stage 1) to metastatic cancer (stage 4). Stage 1 tumours: these carcinomas are less than 2 cm in size. The lymph glands in the armpit are not affected and there are no signs that the cancer has spread elsewhere in the body. Stage 2 tumours: either they measure 2-5 cm or the lymph glands in the armpit are affected, or both symptoms occur simultaneously. However, there are no signs that the cancer has spread further. Stage 3 tumours: these tumours are larger than 5 cm and may be attached to the surrounding structures such as the muscle or the skin. The lymph glands are usually affected, but there are no signs that the cancer has spread beyond the breast or the lymph glands in the armpit. Stage 4 tumours: these tumours are of any size, but the lymph glands are usually affected and the cancer has spread to other parts of the body.

The grading of breast cancer refers to the appearance of the malignant cells and their differentiation status [Fisher et al., 1980]. The grade of breast cancer provides an idea of how rapidly the disease may develop. There are 3 grades: Grade 1 (low grade); Grade 2 (moderate grade); and Grade 3 (high grade). Low grade means that the cancer cells appear differentiated, like the normal epithelial cells of the breast. They are usually slowly proliferating and are less likely to spread. In the high-grade tumours

the cells appear low-differentiated. They are likely to proliferate faster and are more likely to spread.

1.1.3. Treatment recommendations for breast cancer

The treatment of breast cancer depends on several factors, such as: the stage of the disease, the age of the patient, the menopausal status, the size of the tumour and its grade, and the level of expression of receptors for ER and/or erbB2. In the earliest stages, surgery may be all that is needed, but surgery is often followed by radio-therapy of the remaining breast tissue to make sure that any surviving tumour cells are destroyed, particularly if only a part of the breast has been removed. Because there is a risk of cancer cells having escaped and being present elsewhere in the body, additional drug treatment (adjuvant therapy) might be necessary. Adjuvant therapy may consist of hormonal therapy or chemotherapy, or both. If the cancer has spread, it is usually treated with drugs (hormonal therapy, chemotherapy or monoclonal antibody therapy). Whether hormonal therapies or chemotherapies are used will depend on the organ that the cancer has metastasized to, how much time has passed since the original surgery, and whether or not the cancer cells express receptors for particular hormones or proteins on their surface. Several different hormonal therapies and many different types of chemotherapies can be used. Chemotherapies or hormonal therapies are sometimes used to shrink a large breast tumour before surgery. This approach is known as neo-adjuvant therapy.

Surgery: Several types of surgery are performed in order to remove breast tumours, such as lumpectomy (wide local excision), quadrantectomy (segmental excision) and mastectomy (whole breast removal) [Noguchi et al., 1994]. For many patients, mastectomy may not be necessary. It is now often possible to simply remove the area of cancerous tissue and some of the healthy surrounding tissue, and then apply radiotherapy to the remaining breast tissue. This is known as breast-conserving therapy. Research has shown that in early breast cancer, lumpectomy followed by radiotherapy is as effective at curing the cancer as mastectomy [Noguchi et al., 1994]. As a part of any surgery for breast cancer, the surgeon will usually remove the lymph glands from the armpit on the same side of the body. The lymph glands are then examined to check whether any cancer cells have spread into them from the original site of the tumour within the breast.

Radiotherapy: Radiotherapy, most often used after surgery, is applied to breast cancer patients, but is sometimes used before or instead of surgery. Two main types of radiotherapy are used to treat breast cancer: external radiotherapy and internal radiotherapy. If part of the breast has been removed (lumpectomy or quadrantectomy), radiotherapy is usually given to the remaining breast tissue to reduce the risk of recurrence [Noguchi et al., 1994]. The aim is to make sure that any remaining cancer cells are destroyed. If all the lymph glands have been removed from under the arm, radiotherapy to the armpit is not usually needed. If a few lymph glands have been removed and these contained cancer cells, or if no lymph glands have been removed,

radiotherapy may be given to the armpit to treat the lymph glands.

Chemotherapy: Chemotherapy is the use of cytotoxic drugs to destroy cancer cells. Chemotherapeutic drugs are sometimes given as tablets or, more frequently, applied intravenously. Chemotherapy is usually administered as an intermittent treatment for varying periods of time, and may last for less than one day or for a few days. This is followed by a rest period of a few weeks, which allows the patient's body to recover from any side-effects of the therapy. The number of courses the patient is given depends on the type of cancer the patient has and how well the tumour is responding to the drugs. Side-effects of chemotherapy include: anemia, nausea and vomiting, hair loss, lowered resistance to infections, and diarrhea. Anthracyclines (doxorubicin, epirubicin), taxanes (paclitaxel, docetaxel), cyclophosphamide, 5-fluorouracil and methotrexate are the most commonly used chemotherapeutic agents for breast cancer therapy [Crown, 1998]. Combinations of these agents have been used routinely for breast cancer treatment, most well known of which is the so called 'classical' CMF (cyclophosphamide, methotrexate and 5-fluorouracil) [Goldhirsch et al., 1998].

Hormonal therapy: Hormonal therapies can slow or stop the proliferation of breast cancer cells by either altering the levels of estrogens, which are naturally produced in the body, or preventing the hormones from being used by the cancer cells. There are many different types of hormonal therapy and each of them works in a slightly different way, so sometimes two different types of hormonal therapy may be given

together. Hormonal therapy may also be administered in combination with chemotherapy. Most common hormonal therapies are: anti-estrogen agents, agents that reduce estrogen production, progestogens, pituitary down-regulators, or ovarian ablation. Anti-estrogen agents work by preventing estrogen in the body from activating the ER and therefore halting proliferation induction of tumour cells. Tamoxifen is the most commonly used hormonal therapy for breast cancer [Haskell et al., 2003] and may be given in combination with other types of therapeutics known as aromatase inhibitors. The side effects which may be experienced by the patient include hot flushes and sweating, a tendency to put on weight, etc, but these side effects are usually mild. Rarely, it is possible for tamoxifen itself to cause endometrial cancer. Tamoxifen is commonly taken after surgery and for metastatic cancer, but if it is not effective in controlling the cancer, some of the other types of hormonal therapy may be used. A drug called toremifene (Fareston), which works in a similar way to tamoxifen, is occasionally used [Haskell et al., 2003]. Research and early tests suggest that it may carry less risk of endometrial cancer promotion than tamoxifen, and it may be less likely to cause hot flushes and sweating. However, the long-term effects of the drug are not yet known. At the moment, toremifene is only given to post-menopausal women. A group of agents called aromatase inhibitors work by blocking the production of estrogen in fatty tissues, in post-menopausal women. The commonly used aromatase inhibitors are anastrozole (Arimidex), letrozole (Femara), exemestane (Aromasin) and formestane (Lentaron). They generally do not cause many side effects, although they can cause hot flushes, feelings of nausea and joint pain. They are now

sometimes used instead of tamoxifen as the first hormonal therapy (first-line treatment) in post-menopausal women with metastatic breast cancer [Miller, 2003].

Trastuzumab (Herceptin): ErbB2 is amplified and over-expressed in up to 30% of breast cancers. Its amplification and over-expression have been associated with poor prognosis or low-level response to anti-cancer therapies. Therapy based on a humanized monoclonal anti-erbB2 antibody (trastuzumab/HerceptinTM) has been beneficial in metastatic breast cancer patients. Trastuzumab is the first monoclonal antibody with efficacy in breast cancer and the first oncogene-targeted therapy to yield a significant survival advantage in this disease. First-line trastuzumab in combination with chemotherapy resulted in a 25% improvement in overall survival compared with chemotherapy alone [Leyland-Jones, 2002].

1.2. VE analogues as potent pro-apoptotic and anti-cancer agents

1.2.1 VE analogues are redox-silent substances

Many anti-cancer drugs are non-specific, causing damage to normal cells and tissues, sometimes being converted to toxic products. Vitamin E (VE) is an essential micro-nutrient antioxidant. Experimental and epidemiological studies suggest that VE might reduce the risk of cancer, particularly lung and prostate cancer, although the evidence is not very convincing. α-Tocopherol (α-TOH) is the most prevalent chemical form of VE found in vegetable oils, seeds, grains, nuts, and other food. Albanes and colleagues examined the effects of α-TOH supplementation on the incidence of lung

cancer [Albanes et al., 1996]. They gave 50-69 year old smokers (>5 cigarettes per day) α-TOH (50 mg) and β-carotene (20 mg) daily for 5-8 years (median, 6.1 years). The results indicated no overall effect of α-TOH supplementation on lung cancer. Therefore supplementation with α-TOH does not prevent lung cancer in older men who smoke. But the idea has been attractive, as VE is a micronutrient with high antioxidant activity. Moreover, the level of VE can be manipulated, to certain level, by dietary supplementation. Neuzil and colleagues also showed that VE exerts little or no anti-cancer effect. The main reason may be due to its biological activity, since VE is a redox-active substance, which does not cause, unlike many anti-cancer agents, apoptosis of malignant cells [Neuzil et al., 1999]. However, α-tocopheryl succinate (α-TOS), a semi-synthetic analogues of VE, is redox-silent, and has been found to kill many types of cancer cells as documented in a number of reports [see *e.g*., Fariss et al.,1994; Neuzil et al., 1999, 2001c, 2007b; Pussinen et al., 2000; Yu et al., 2001].

1.2.2. α-TOS causes apoptosis in vitro

Malafa and Neitzel have demonstrated that α-TOS decreased cell viability in MDA-MB-231 and MCF7 human breast cancer cells [Malafa and Neitzel, 2000]. The effect of α-TOS on the proliferation and apoptosis of the B16F10 murine melanoma cell line was determined *in vitro*, using cultured cells. α-TOS decreased cell proliferation and increased cell apoptosis in a dose-dependent manner [Rama and Prasad, 1983; Prasad and Edwards-Prasad, 1982]. Barnett and colleagues treated CT26 colon cancer cells with α-TOS and verified that α-TOS inhibited their

proliferation and promoted their apoptosis [Barnett et al., 2002]. Gastric cancer is the eighth most common malignancy in males and the incidence continues to rise. Over 20,000 new cancer cases and over 13,000 cancer deaths are expected per year alone, with surgical resection remaining the only potentially curative modality. Even with operative intervention, 75% of patients experience recurrence following gastrectomy, and 5-year survival remains only 15-20% [Greenlee et al., 2000]. Rose and McFadden exposed gastric cancer cells KATO-III to α-TOS and performed a cell viability test. The results showed that α-TOS inhibited the growth of the cells in a dose- and time-dependent fashion [Rose and McFadden, 2001]. The anti-cancer efficacy of α-TOS most likely follows from its high pro-apoptotic activity. Therefore, α-TOS causes apoptosis in many malignant cells, while being non-toxic to normal cells and tissues [Neuzil et al., 2001b].

1.2.3. Pre-clinical studies on α-TOS

Thus far, there have been relatively few studies on the anti-cancer effects of VE analogues *in vivo*. This is rather surprising, since the toxic effects of α-TOS towards malignant cells have been known since the early 1980s [Prasad and Edwards- Prasad, 1982; Prasad et al., 2003]. Probably, the strongest evidence suggesting the potential use of VE analogues in the treatment of humans with neoplastic disease comes from the laboratory of Malafa. His team was the first to show that α-TOS inhibited the growth of MDA-MB-231 human breast cancer cells in nude mice [Malafa and Neitzel, 2000]. This is the first report on α-TOS inhibiting the growth of an established tumour

in vivo.

Melanoma is the leading cause of death from cutaneous malignancies [Greenlee et al., 2000]. Relapse after curative surgical treatment of melanoma remains a significant clinical challenge and accounts for most of the mortality from this disease [Balch, 1992; Balch et al., 2001]. In other studies, the B16F10 melanoma cells were allografted in nude mice and the effect of α-TOS on melanoma dormancy was measured by monitoring tumour volume. Tumour vascularity was quantified using CD31 immunostaining. The expression of vascular endothelial growth factor (VEGF), VEGF receptor 1 and VEGF receptor 2 in the tumours was assessed on the basis of the intensity of immunostaining. The results showed that α-TOS promoted melanoma dormancy and inhibited melanoma angiogenesis. α-TOS also suppressed the expression of VEGF, VEGF receptor 1 and VEGF receptor 2 in melanoma tumours [Malafa et al., 2002b; Malafa and Neitzel, 2000]. These findings support future investigations of α-TOS as a therapeutic agent against melanoma. The anti-cancer mechanism of α-TOS may involve inhibition of tumour angiogenesis via suppression of VEGF signalling. Similar suppression of experimental melanomas was observed by others [Kogure et al., 2003]. This notion follows also from findings that α-TOS causes apoptosis of proliferating but not growth-arrested endothelial cells [Neuzil et al., 2001a] by causing accumulation of high levels of reactive oxygen species (ROS) in the angiogenic cells [Dong et al., 2007].

Barnett and colleagues treated the CT26 colon cancer cells with α-TOS in an *in vivo* model of liver metastases. α-TOS promoted a 40% reduction of liver surface metastases. Five of the eight mice had an excellent response to α-TOS. Analysis of these five mice revealed a 75% reduction in the number of liver surface metastases [Barnett et al., 2002]. This is the first report of α-TOS inhibition of colon cancer metastases. Our group has shown that α-TOS suppresses colon cancer progression in a mouse xenograft model [Neuzil et al., 2001c]. The mechanism of α-TOS anti-tumour and anti-metastatic activity *in vivo* appears to involve both promotion of tumour apoptosis and inhibition of cell proliferation. These findings support further investigation of α-TOS as a drug to promote colon cancer tumour dormancy and to prevent metastases. Weber and colleagues have shown that VE analogues suppress colon cancer in an athymic mouse model much more efficiently than α-TOH, which exerted only minor, non-significant tumour suppression [Weber et al., 2002].

1.2.4. Pro-apoptotic/anti-neoplastic activity of VE analogues is structure-dependent

It has been proposed that a group of VE analogues may meet the criteria for an optimal anti-cancer drug [Neuzil, 2003; Neuzil et al., 2004]. Of these, α-TOS has been a focus of recent studies [Neuzil et al., 2007a, b]. Unlike VE, represented by α-TOH, α-TOS is redox-silent, due to esterification of its hydroxyl group (Figure 1). VE analogues share 3 major domains that determine their function, *i.e.* the *Hydrophobic Domain* that causes association of the compounds with biological

membranes and circulating lipoproteins; the *Signalling Domain* modulating certain signalling pathways, and the *Functional Domain*, which decides whether the compound is redox-active (and non-apoptogenic), as is α-TOH, or redox-silent and apoptogenic, as applies to α-TOS [Neuzil et al., 2007b].

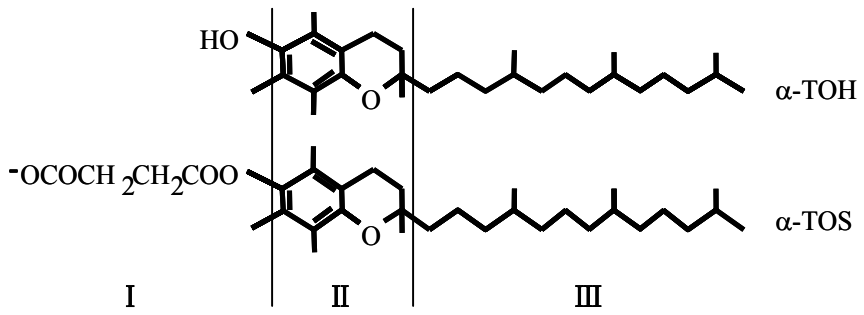

Figure 1. Basic structural features of α-TOH and α-TOS. The *Functional Domain* (I) decides whether the compound is redox-active (and non-apoptogenic), as in α-TOH, or apoptogenic, as in α-TOS; the *Signalling Domain* (II) modulates certain signalling pathways; and the *Hydrophobic Domain* (III) docks the compound in biological membranes and circulating lipoproteins.

The reason why α-TOS is pro-apoptotic, while α-TOH does not possess this activity (in fact, α-TOH is anti-apoptotic) has been investigated in considerable detail over the last few years. Importantly, Fariss and colleagues showed that the cell-killing activity of α-TOS requires an intact molecule of the agent [Fariss et al., 1994]. They reported that the ester, which can be hydrolysed by some cells to free VE, has identical activity as its ether counterpart that is not hydrolysable. Birringer and colleagues have explored in more detail the structure-function relationship of these agents and found

several interesting features [Birringer et al., 2003]. Firstly, the presence of the phytyl group was essential for the activity, since the succinyl ester of Trolox (a form of VE lacking the Hydrophobic Domain) was inactive. This can be explained by the fact that such a molecule is highly water soluble, therefore does not associate with lipidic biomembranes. Lowering the number of methyl substituents on the tocopheryl moiety decreased the pro-apoptotic efficacy of the compound. Replacement of the succinyl group by an uncharged group, for example acetate, or its methylation, obliterated the pro-apoptotic activity. On the other hand, esterification of α-TOH with other dicarboxylic acids modulated the apoptotic activity as follows: esters with higher number of carbons had lower activity than α-TOS, while esterification with unsaturated dicarboxylic acids, such as maleyl acid, highly enhanced the activity [Birringer et al., 2003].

Neuzil and colleagues reported that α-TOS selectively kills cells with a malignant or transformed phenotype, while being non-toxic to normal cells [Neuzil et al., 2001b]. This finding is complemented by reports which showed no toxic effect of α-TOS on normal fibroblasts [Jha et al., 1999] or prostate cells [Israel et al., 2000], and by a finding that α-TOS was toxic towards malignant but protective for normal stem cells [Gogu et al., 1991; Fariss et al., 1994]. We have recently found that α-TOS, when formulated in liposomes (a preferred formulation for clinical application), stimulated some 2-fold bone marrow proliferation reflected by an increase in GM-CFC progenitors [Turanek et al., 2009]. The unique structural features of agents like α-TOS

probably make them selective for malignant cells [Neuzil et al., 2001b]. While speculative at present, it is possible that the surprisingly high toxicity of α-TOS towards cancer cells and tissues is due to their persistence in such cells in the original form, while normal cells, including fibroblasts [Roberg et al., 1999], cardiac myocytes [Roberg and Ollinger, 1998], hepatocytes [Tirmenstein et al., 1999] and intestinal epithelial cells [Borel et al., 2001], are all capable of efficient hydrolysis of α-TOS to α-TOH. On the other hand, malignant cells, such as the human T lymphoma, malignant mesothelioma and colon cancer cells exert only a very low hydrolytic capacity towards α-TOS [Neuzil et al., 2001b; Stapelberg et al., 2005]. Another reason for the selectivity of α-TOS may result from its physicochemical properties. The VE analogue is a weak acid with a low pK_a value (5.6) [Neuzil et al., 2002b]. Therefore, at the neutral pH of normal tissue interstitium, majority of α-TOS exists in the charged, deprotonated state. Since there are no known transporters of α-TOS, it can be expected that it will cross the plasma membrane at a low rate via free diffusion. On the other hand, the acidic pH of the tumour interstitium causes protonation of a substantial portion of the total α-TOS, facilitating its free diffusion into the cell. In favour of this hypothesis, lowering the pH of the cell culture medium enhanced the killing of cancer cells by α-TOS, but not by γ-tocotrienol, an apoptogenic VE analogue that cannot be deprotonated [Neuzil et al., 2002b]. This is consistent with the idea that inducers of apoptosis that are weak acids may be selective anti-cancer agents due to their preferential uptake by tumour tissue cells [Gerweck et al.,1999]. This premise was documented, for example, for the weak acid chlorambucil, whose

anti-cancer efficacy increased in a model of experimental carcinomas upon injection of glucose into the bloodstream of the animals, which promotes glycolysis and, therefore, further acidification of the tumour interstitium [Kozin et al., 2001].

It is therefore possible to modify the structure of VE analogues so that agents with higher pro-apoptotic activity and selectivity for malignant cells are generated. For this reason, we studied a group of VE analogues in this present study, including the novel amides (see Figure 2 for their structures) [Birringer et al., 2003; Tomic-Vatic et al., 2005].

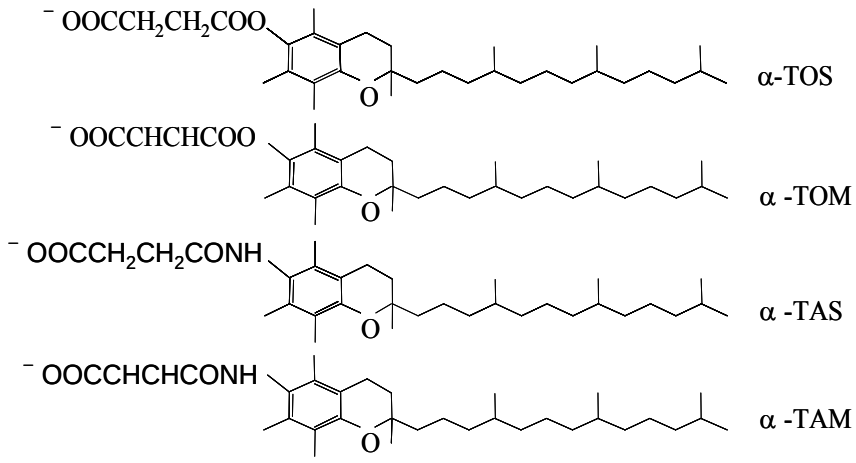

Figure 2. Structures of VE analogues used in this study. α-TOS, α-tocopheryl succinate; α-TOM, α-tocopheryl maleate; α-TAS, α-tocopheryl succinyl amide; and α-TAM, α-tocopheryl maleyl amide.

1.3. VE analogues as adjuvants for cancer immunotherapy

A commonly used practice in tumour treatment is to combine several anti-cancer agents. The reasons for this approach are two-fold: one is maximizing the apoptotic potential of the cells by overcoming resistance to one of the agents by utilizing complementing pro-apoptotic pathways; the other reason is to lower the doses of the individual drugs to suppress the potential secondary deleterious effects of the treatment. From this point of view, α-TOS has shown a substantial promise. There are several reports documenting that the VE analogue potentiates cancer cell killing by the immunological apoptogen Fas, *viz*. by mobilizing the Fas receptor from the cytosol to the plasma membrane; this has been shown for both prostate and breast cancer cells [Turley et al., 1997; Yu et al., 1999]. Our group has recently found that α-TOS causes increased expression of death receptors (such as the Fas receptor of the TRAIL receptors) (Neuzil et al., unpublished data). These findings suggest that α-TOS has the propensity to boost the immune system and to enhance cancer surveillance, as well as promote killing of cancer cells via autocrine signalling mechanisms. While the Fas ligand itself is highly cytotoxic, the TNF-related apoptosis-inducing ligand (TRAIL) is selective for cancer cells [Bonavida et al., 1999]. α-TOS can potentiate cancer cells to killing by TRAIL, which was shown for both colon cancer [Weber et al., 2002] and T lymphoma cells [Dalen and Neuzil, 2003]. One mechanism by which α-TOS sensitizes cells to killing by TRAIL is that the two agents use different pathways of apoptotic signalling. α-TOS activates the mitochondrial pathway, while TRAIL acts through the proximal, receptor-mediated route [Weber et al., 2002]. Moreover, the cooperative killing of colon cancer cells by

the two agents has been reported for the suppression of colon cancer in a pre-clinical model [Weber et al., 2002]. Another possible mechanism of synergism and/or cooperation between TRAIL and α-TOS is the inhibition of activation of the transcriptional factor nuclear factor-κB (NFκB). This factor controls the expression of a number of pro-survival genes and is activated in some cancer cells due to TRAIL cross-linking of one or more of its cognate receptors [Degli-Esposti et al., 1997]. α-TOS has been shown to suppress the activation of NFκB, and this may be due to the induction of pro-apoptotic signalling leading to caspase-3 activation that, in turn, cleaves the NFκB subunit p65 [Neuzil et al., 2001a]. A recent finding revealing that the suppression of NFκB activation by α-TOS, following exposure to TRAIL, sensitizes T lymphoma cells to killing by TRAIL [Dalen and Neuzil, 2003], may be of pharmacological importance.

1.4. Possible molecular mechanisms of α-TOS-induced apoptosis in cancer cells

1.4.1. The role of the mitochondrial pathway

Mitochondria are membrane-enclosed organelles distributed through the cytosol of most eukaryotic cells. They have an outer membrane that defines their structure and an inner membrane that encloses a fluid-filled matrix. The mitochondrial outer membrane (MOM) contains complexes of integral membrane proteins that form channels through which a variety of molecules and ions move in and out of the mitochondrion. The inner membrane contains 5 complexes of integral membrane

proteins: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II, CII), cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V). The structures of a typical eukaryotic cell and mitochondrion are shown Figure 3. From the functional point of view, mitochondria are indispensable for optimal life of most eukaryotic cells by mediating energy generation in the form of ATP.

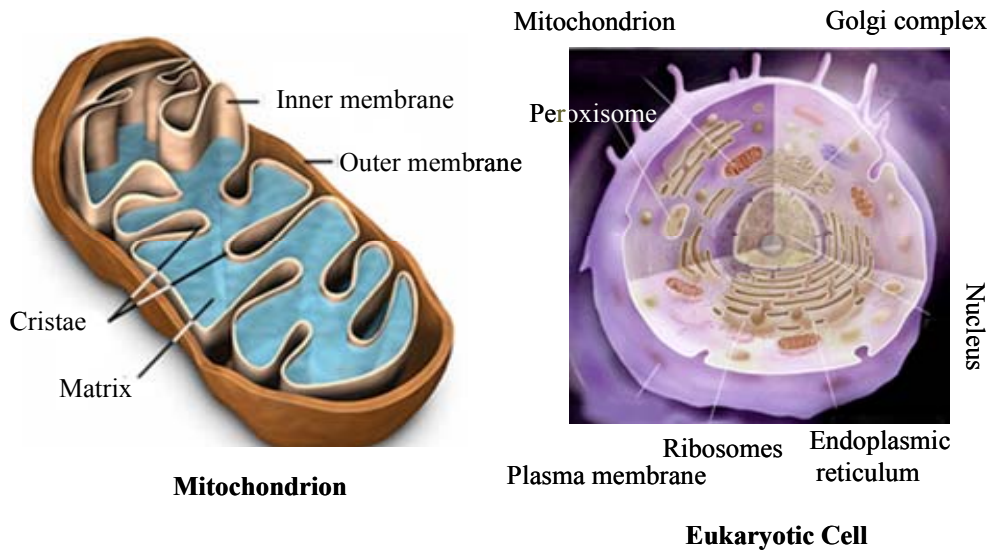

Figure 3. The structures of a mitochondrion and a eukaryotic cell

Recent research has demonstrated that mitochondria also play an important role in the programmed cell death [Green et al., 2004], and the role of mitochondria has also been unequivocally demonstrated for apoptosis induced by VE analogues [Neuzil et al., 2004, 2007a,b]. There is evidence that treatment of cells with α-TOS causes generation of ROS [Ottino and Duncan, 1997; Kogure et al., 2001, 2002; Weber et al.,

2003; Stapelberg et al., 2005]. However, the molecular mechanisms by which α-TOS kills cancer cells are not completely understood. It has been shown that this occurs by induction of apoptosis in malignant cells primarily due to mitochondrial destabilization [Alleva et al., 2001; Kogure et al., 2001; Neuzil et al., 2001d; Weber et al., 2003], a process amplified by the modulation of signalling pathways [Qian et al.,1997; Neuzil et al., 2001c; Yu et al., 2001]. Mitochondrial destabilization may be caused by the detergent-like activity of α-TOS [Neuzil et al., 2002], although recent data indicate the role of the mitochondrial complexes in promotion of apoptosis by VE analogues (see the Discussion part). α-TOS comprises a hydrophobic phytyl chain (Hydrophobic Domain) and the chargeable succinyl group (Functional Domain), separated by the bulky α-tocopheryl moiety (Signalling Domain) [Neuzil, 2003]. It is possible that α-TOS-induced apoptosis also involves lysosomal destabilization by the agent, an action running parallel to and/or amplifying mitochondrial disruption [Neuzil et al., 1999, 2002a]. We think that it is more likely that the drug primarily acts by destabilizing mitochondria and that the lysosomotropic effect is of auxilliary nature and may be dispensable [Neuzil et al., unpublished data]. α-TOS may signal along several pathways. It has been shown that, due to its α-tocopheryl moiety, the agent activates protein phosphatise 2A that, in turn, inhibits protein kinase c (PKC). This leads to hypophosphorylation of the anti-apoptotic protein Bcl-2, with ensuing mitochondrial labilization [Neuzil et al., 2001c]. This pathway itself does not cause apoptosis by VE analogues, but rather amplifies the succinyl moiety-mediated destabilization of mitochondria, since β-, γ- or δ-TOS lacking the PKC inhibitory

activity are less apoptogenic than the α-analogue [Neuzil et al., 2001c; Birringer et al., 2003]. Other signalling routes that are likely implicated in apoptosis induced by α-TOS include deregulation of the c-jun/AP-1 [Qian et al., 1997] and the TGF-β pathway [Turley et al., 1995], as well as inhibition of the cell cycle transition [Turley et al., 1997a,b].

Mitochondria are sites of mediators of apoptosis, whose re-localization relays further the upstream pro-apoptotic signals. In apoptosis induced by VE analogous, such downstream events following mitochondrial destabilization comprise mobilization of apoptotic mediators, which include cytochrome c (Cyt c), the apoptosis-inducing factor (AIF) and Smac/Diablo [Neuzil et al., 2004]. Cyt c, upon cytosolic translocation, forms a ternary complex with Apaf-1 and pro-caspase-9, leading to activation of the initiator caspase-9 with ensuing activation of the effector caspase-3, -6, or -7. At this stage, the cell enters the 'point of no return', *i.e.* the irreversible phase of the apoptotic pathway [Yamamoto et al., 2000; Weber et al., 2003]. It is now clear that this particular pathway is critically important in apoptosis induced by α-TOS in a variety of cancer cells [Neuzil et al., 2004]. Smac/Diablo is an important agonist of the caspase-dependent apoptotic signalling, since it antagonizes the caspase-inhibitory members of the family of inhibitors of apoptosis proteins (IAPs), including c-IAP1, c-IAP2, X-IAP and survivin [Du et al., 2000; Verhagen et al., 2000]. The expression of IAPs is under the control of the transcriptional factor NFκB, whose activity is inhibited by α-TOS [Erl et al., 1997; Neuzil et al., 2001a; Dalen and

Neuzil, 2003]. Cytosolic translocation of the Smac/Diablo protein may therefore promote inhibition of the survival pathways in apoptosis induced by α-TOS, which could maximize the apoptogenic potential in resistant cancer cells [Neuzil et al., 2003].

Another mitochondrial protein amplifying apoptosis in cells exposed to VE analogues is AIF [Weber et al., 2003] that translocates directly into the nucleus, thereby bypassing the caspase activation cascade [Susin et al., 1999]. AIF, upon translocation to the nucleus, triggers cleavage of chromatin in a caspase-independent manner [Cande et al., 2002]. AIF can therefore avoid mutations in the caspase-dependent signalling or situations where IAP proteins are over-expressed, that can render the cancer cell resistant, and may mediate α-TOS-induced apoptosis in cells resistant to conventional anti-cancer drugs that rely solely on caspase activation [Neuzil et al., 2004].

The mitochondrial pro- and anti-apoptotic proteins, including Bax, Bcl-2, Mcl-1 and Bcl-x_L, are important factors related to mitochondrial apoptotic signalling pathways [Cory et al., 2003]. Generation of the mitochondrial permeability transition pore has also been suggested in cells exposed to α-TOS [Yamamoto et al., 2000]. It is likely that this is modulated by a cross-talk between the mitochondrial pro- and anti-apoptotic proteins [Yamamoto et al., 2000; Weber et al., 2003]. Over-expression of Bax sensitized cells to α-TOS-induced apoptosis, whereas over-expression of Bcl-2 or

Bcl-x_L protected them from the VE analogue [Weber et al., 2003; Yu et al., 2003]. This was not observed when truncated proteins lacking the mitochondrial-targeting terminus were used for transfection of the cells [Weber et al., 2003]. Similarly, downregulation of Bcl-2 by anti-sense oligodeoxynucleotide treatment sensitized cells to the VE analogue [Neuzil et al., 2001b,c; Weber et al., 2003]. Finally, transfection with a gain-of-function mutant of Bcl-2 protected from while a loss-of-function mutant of the protein sensitized cancer cells to α-TOS [Neuzil et al., 2001c]: in these mutant versions of Bcl-2, Ser-70 was replaced with glutamine and alanine, respectively. This observation can be explained by PKC-dependent phosphorylation of Ser-70 that plays a role in mitochondrial docking of Bcl-2 [Ruvolo et al., 1998]. The major possible pathways of apoptosis induction by VE analogous are suggested in Figure 4.

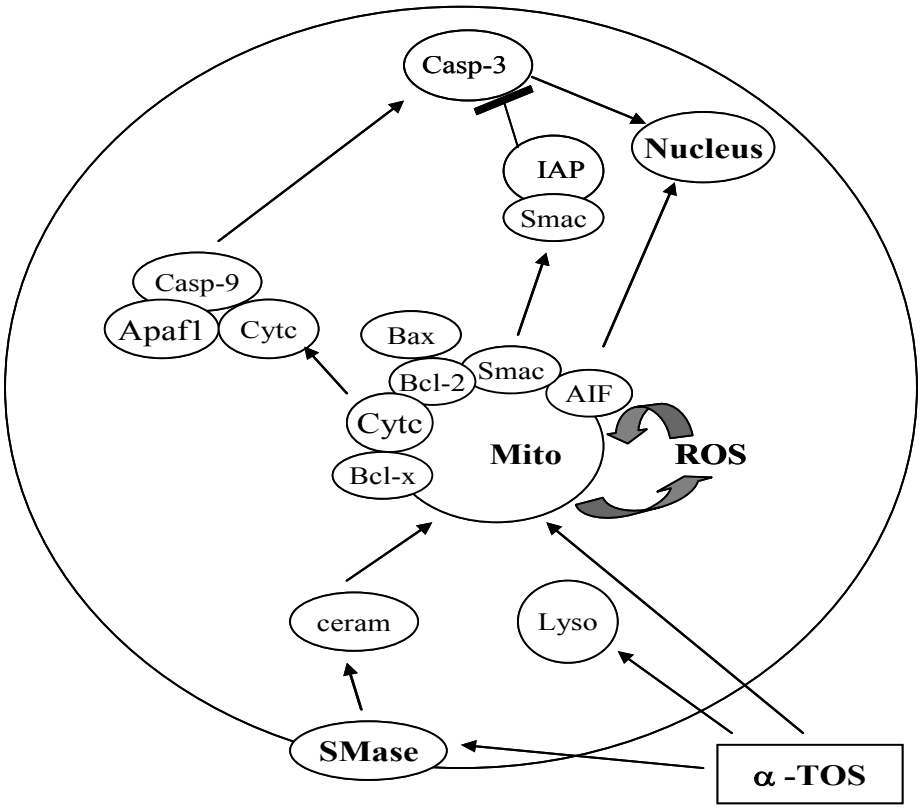

Figure 4. **Possible pathways of apoptosis induction by VE analogues.** α-TOS translocates to cells, where it causes ROS generation and MOM destabilisation. This, in turn, results in cytosolic re-localization of pro-apoptotic factors (such as Cyt c or Smac/Diablo) that can be regulated by the Bcl-2 family proteins. Cyt c, Apaf-1 and pro-caspase-9 form an apoptosome, a ternary complex resulting in activation of the initiator caspase-9 that then activates effector caspases. Smac/Diablo may amplify this process by suppressing the caspase-inhibitory activity of the IAP family proteins, while AIF transmits the mitochondrial destabilization directly to nuclear apoptotic events, bypassing the caspase cascade. The scheme also shows possible effects of α-TOS on lysosomes and the sphingomyelin metabolism.

1.4.2. Activation of death receptors by VE analogues

TRAIL/Apo2L has attracted much interest for being toxic to tumour cells rather than

to their normal counterparts [Pitti et al., 1996; Ashkenazi et al., 1999; Shi et al., 2003]. TRAIL has been proven relatively safe in *in vivo* studies of rodents and primates compared with other death receptor ligands, TNFα and the Fas ligand, which induce significant inflammation and tissue injury [Ashkenazi et al., 1999]. Two unique characteristics of TRAIL have been identified: firstly, TRAIL can selectively induce apoptosis in tumorigenic or transformed cells, but not in normal cells, highlighting its potential application in cancer treatment. Second, in contrast to other members of the TNF family, whose expression is tightly regulated and which is often only transiently expressed on activated cells, TRAIL mRNA is expressed continuously in a wide range of tissues [Wiley et al., 1995].

TRAIL is a type II membrane protein or is secreted in soluble form, which binds to its cognate death receptors (DR), DR4 and DR5, inducing their trimerization and intracellular recruitment of the adaptor protein Fas-associated death domain (FADD) [Schneider et al., 1997]. The death domain (DD), in turn, recruits pro-caspase-8 into a death-inducing signalling complex (DISC) that triggers autocatalytic cleavage and activation of caspase-8, which then leads to activation of the effector caspase-3 (type I pathway). Alternatively, the apoptotic pathway can be further amplified by involvement of mitochondrial signalling (type II pathway) [Scaffidi et al., 1998]. In this case, TRAIL-activated caspase-8 generates a truncated form of the protein Bid, which triggers the release of Cyt c from mitochondria, leading to the assembly of the apoptosome (Cyt c, Apaf-1, pro-caspase-9). Formation of apoptosome then results in

activation of caspase-9, which in turn activates effector caspases [Zou et al., 1999]. Type I cells exert DISC-activated caspase-8, which activates downstream effector caspases and triggers execution of apoptosis. However, in the majority of cancer cells (Type II), TRAIL-induced activation of caspase-8 is insufficient to kill the cells without employing the mitochondrial apoptotic programme, further documenting these organs as central purveyors of apoptotic cell death.

A number of proteins are involved in regulation of the TRAIL apoptotic pathway. The FLICE-like inhibitory protein (FLIP) contains two DDs that can bind to DDs of FADD and inhibit recruitment of pro-caspase-8 to DISC [Irmler et al., 1997]. IAP family proteins are characterized by the presence of one to three baculoviral IAP repeat (BIR) domains that bind to caspases [Verhagen et al., 2001]. Binding of IAPs to caspases can be inhibited by several proteins released from mitochondria. The second mitochondria-derived activator of caspases (Smac, also known as Diablo), which directly binds IAPs, is located in the inter-membrane space of mitochondria and is released into the cytosol upon changes in the mitochondrial membrane permeability [Verhangen et al., 2000]. Smac/Diablo facilitates apoptosis by liberating caspase-3 or -7 from inhibition mediated by the IAP family proteins. Tumour cells may avoid TRAIL-mediated killing by downregulation of DRs (extrinsic resistance). Besides the importance of the balance of DRs and decoy receptors (DcRs), which lack the functional cytoplasmic DD, the ratio between DR4 and DR5 plays a role in determining sensitivity to TRAIL. In addition, in order to induce apoptosis, DR4

equally responds to cross-linked TRAIL (membrane bound protein) and non-cross linked TRAIL (soluble protein), whereas DR5 signals only in response to non-cross linked, soluble TRAIL [Wajant et al., 2001]. It was observed that low expression of DRs on the cell surface is responsible for cellular resistance to TRAIL-induced cytotoxicity in human colon cancer cells [Jin et al., 2004]. Anti-cancer drugs have been shown to sensitize TRAIL receptor-negative cells to TRAIL-mediated apoptosis by inducing expression of DRs on the cell surface [Arizono et al., 2003]. Combination of TRAIL with other drugs therefore resulted in a cooperative or synergist effect [LeBlanc et al., 2002; Weber et al., 2002; Wang and EI-Deiry, 2003].

A synergistic and cooperative effect was observed when TRAIL was combined with α-TOS in malignant mesothelioma (MM) cells and the effect was selective for cancer cells [Tomasetti et al., 2004]. MM is a fatal type of neoplasia with poor therapeutic prognosis, largely due to resistance to apoptosis [Robinson et al., 2005]. Impaired apoptotic pathways render MM cells rather resistant to TRAIL-induced apoptosis. Sublethal doses of α-TOS significantly decrease the high IC_{50} values for TRAIL by the factor of ~10-100 [Tomasetti et al., 2004].

The observation that α-TOS and TRAIL synergize in p53-wt MM but not in the p53-null cells suggests a role of p53 in trans-activation of the pro-apoptotic genes involved in drug synergism [Tomasetti et al., 2006]. The p53 protein is a key component of the cellular ‘emergency-response’ mechanism [Levine, 1997; Sionov

and Haupt, 1999]. A variety of stress-associated signals activate p53 that induces growth arrest or apoptosis, thereby eliminating damaged and potentially dangerous cells [Lane, 1992]. The p53 apoptotic target genes can be divided into two groups; the first group encodes proteins that act through receptor-mediated signaling, the second group codes for proteins involved in regulation of the apoptotic effector proteins. α-TOS has the propensity to induce apoptosis in a p53-independent manner [Weber et al., 2002]. However, at low concentrations the VE analogue induces expression and activation of p53. It was observed that induction of p53 in cells exposed to α-TOS was concomitant with the increased expression of both DR4 and DR. Notably, such expression of DRs did not occur in the p53-null MM cells. Further, studies using siRNA directed at p53 revealed that the p53 protein contributes significantly to the expression of TRAIL's DRs. We propose therefore that p53-dependent upregulation of DR4 or DR5 in response to α-TOS is a basis for sensitization of MM cells to TRAIL. Additionally, the presence of redox environment efficiently contributes to enhanced expression of DR4 and DR5 via p53 in MM cells treated with VE analogues [Tomasetti et al., 2006]. Regulation of the activity of many transcription factors by redox modulators was previously described [Sun and Oberley, 1996]. Accordingly, a novel mode of action of α-TOS has been unveiled: reduction of the redox-sensitive amino acid residues on the p53 protein leads to an increase in the efficiency of TRAIL's DR expression, sensitizing MM cells to the immunological apoptogen. MM cells express both DR4 and DR5 on their surface, and their upregulation by α-TOS could facilitate activation of caspase-8 and cleavage of Bid. Kinetic analysis of

TRAIL-induced signalling revealed a transient activation of caspase-8, which resulted in induction, albeit low, of apoptosis. Caspase-8 activation was less pronounced in the presence of TRAIL and α-TOS. Under this setting, activation of the mitochondria-dependent apoptotic pathway, including Bid cleavage, Cyt c cytosolic mobilization and, finally, caspase-9 activation, was observed [Tomasetti et al., 2004]. Bid cleavage may lead to mitochondrial translocation of Bax, as shown for α-TOS in other cancer models [Weber et al., 2003, Yu et al., 2003]. It therefore appears that the elevation of p53 in response to α-TOS could facilitate TRAIL-induced apoptosis by releasing both Bid and Bax from their sequestration by Bcl-x_L, promoting mitochondria-dependent apoptosis.

1.5. ErbB protein family and cancer

The proto-oncogenic type I receptor tyrosine kinases of the erbB family include four members named for their homology to the *v-erbB* oncogene: erbB1 (EGFR, HER1), erbB2 (HER2/Neu), erbB3 (HER3) and erbB4 (HER4). The genes are considered oncogenes since they may give rise to cancer and since they are over-expressed in a significant percentage of breast cancer patients. The proteins feature the molecular weight of 170-185 kDa and share two structural aspects by which they can be distinguished form other receptor tyrosine kinases: two cysteine-rich clusters in the extracellular region and an uninterrupted tyrosine kinase domain in the cytoplasmic part [Carpenter and Cohen, 1990; Ullrich and Schlessinger, 1990].

The erbB1 protein was the first described member of the erbB family. It was also the first receptor for which ligand-dependent activation was demonstrated. The erbB1 gene is located on chromosome 7p13-q22 and codes for a protein of 1,210 amino acids which, when glycosylated, has the size of 170 kDa. Ligands which bind to the EGFR represent a family of growth factors, called the EGF family. The erbB1 protein has been reported to be internalized in clathrin-coated pits upon ligand binding, receptor dimerization and activation. Internalization is followed by lysosomal degradation [Baulida et al., 1996] and partial inactivation through phosphorylation of serine and threonine residues within the intracellular domain of the protein [Downward et al., 1985; Wiley et al., 1991]. The erbB1 protein is expressed in a variety of normal tissues, including breast tissue. Its importance has been supported by findings that its knock-out is lethal in mice. A large number of deletion variants of *erbB1* mRNA have been observed in various types of cancer, including breast cancer [Wikstrand et al., 1995] and ovarian cancer [Moscatello et al., 1995]. These deletions are the result of genomic re-arrangements, resulting in alternative splicing of *erbB1* mRNA. They are found both in the mRNA sequences coding for the extracellular as well as intracellular regions of the erbB1 protein [Sugawa et al., 1990], giving rise to truncated and often constitutively active, oncogenic receptors [Gilmore et al., 1985; Kris et al., 1985].

The *erbB2* (*c-neu*) gene is located on chromosome 17q21 and encodes a protein of 1,255 amino acids with the size of 185 kDa. The human *erbB2* gene was cloned by

homology screening with the *v-erbB* gene [Coussens et al., 1985] and has the highest homology to the *erbB1* gene among the erbB recetor protein family members. The highest homology to the erbB1 (HER2) protein is within its kinase domain (82%), and the protein is most distinct form erbB1 in the C-terminus, which contains most of the autophosphorylation sites. HER2 is the only orphan receptor of the erbB family, since no ligand for the receptor has been found to date. Activation of the erbB2 protein is therefore highly dependent on the expression of other family members, to which it is recruited as a preferred heterodimeric partner [Karunagaran et al., 1996]. Over-expression and/or mutation of *erbB2* are thought to lead to spontaneous dimerization and activation of the receptor in a ligand-independent manner [Ben-Levy et al., 1992; Stancovski et al., 1992; Sternberg and Gullick, 1989]. The erbB2 protein is amplified and/or over-expressed in a number of human cancers including gastric, esophageal, salivary, colon, bladder and lung cancers [Eccles et al., 1994; Jardines et al., 1993], and its over-expression correlates with tumour progression and aggressiveness, poor prognosis and elevated metastatic potential.

The erbB3 and erbB4 genes were both found by homology screening, and are located on 12q13 and 2q33, respectively. They code for proteins of 1,342 and 1,308 amino acids, whose size, when glycosylated, is 180 kDa [Kraus et al., 1989; Plowman et al., 1993]. The expression of the erbB3 protein is, in general, different from that of the erbB1 and erbB2 proteins, since it is frequently expressed in differentiated cells. It is important in the peripheral nervous system and in neuromuscular synapse formation

[Carroll et al., 1997; Marchionni et al., 1996; Riethmacher et al., 1997]. The erbB4 protein is expressed in several adult tissues, including kidney, brain and skeletal muscle [Kraus et al., 1989]. ErbB4 knock-out mice die at embryonic day 10-11, featuring severe cardiac and neural defects [Gassmann et al., 1995]. Over-expression of the erbB4 protein has been observed in 10-20% carcinomas of the breast, colon, ovary, prostate and endometrium, whereas its 'under-expression' was found in 40-80% of the malignancies, reaching 100% downregulation in squamous cell carcinomas of the head and neck [Srinivasan et al., 1998].

1.6. Akt signalling and cancer

The major complication associated with erbB2 over-expression is linked to auto-activation of downstream signalling pathway(s), in particular phosphatidylinositol 3-kinase (PI3K) activation leading to activation of Akt [Zhouet al., 2000; Vivanco and Sawyers, 2002], a serine/threonine kinase (also known as protein kinase B, PKB) that is a pivotal promoter of cellular survival [Dudek et al., 1997]. Akt causes activation of the transcriptional factor NFκB [Kane et al., 1999] that, in turn, leads to its trans-location to the nucleus and binding to promoter regions of a variety of specific pro-survival genes, such as those coding for the IAP family proteins [LaCasse et al., 1998]. Therefore, agents that inhibit NFκB activation in cancer cells over-expressing erbB2 are of clinical interest.

1.6.1. Structure of Akt

Akt is regulated by upstream second messengers as well as other enzymes (PI3K). For Akt, this activating process involves multiple inputs that strictly control the location, duration and power of the response. In mammals, there are three isoforms of Akt: Akt1, Akt2 and Akt3 (PKBα, PKBβ, PKBγ) (see Figure 5). All three isoforms share a high degree of amino acid homology and are composed of three functionally different regions: the N-terminal pleckstrin homology (PH) domain, the central catalytic (kinase) domain, and the C-terminal hydrophobic motif. This general structure is conserved across species (including *Drosophila melanogaster and Caenorhabditis elegans*), suggesting that regulation of Akt appeared early during the evolution.

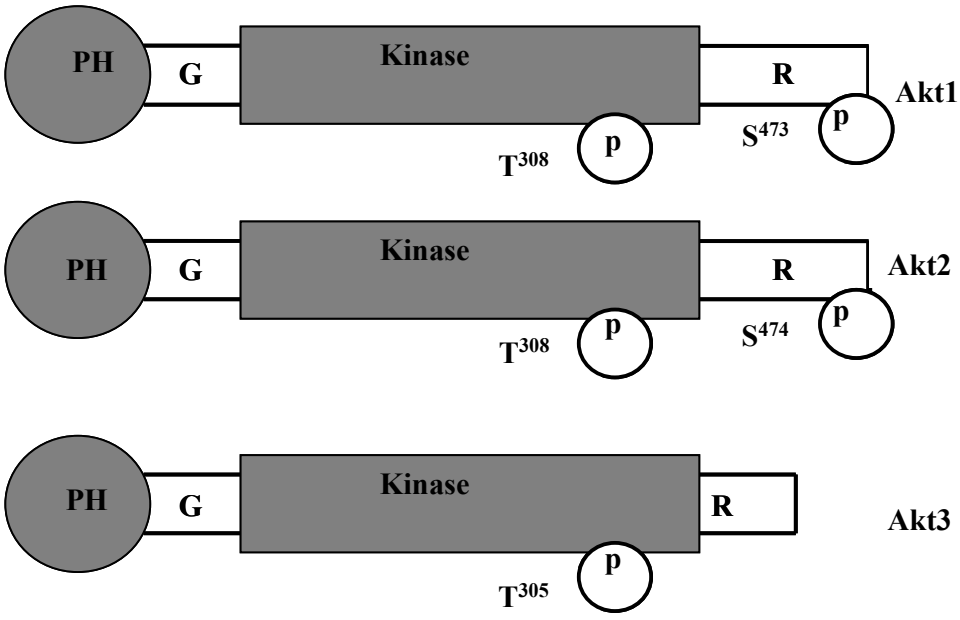

Figure 5. **Schematic organization of Akt isoforms.** All three Akt isoforms possess the kinase domain in the central region of the molecule. The PH domain acts as a phosphoinositide-binding module. The hydrophobic regulatory (R) motif is located at the carboxyl terminus adjacent to the kinase domain.

1.6.2. Phosphorylation of Akt

Akt is activated by, and dependent upon multistage phosphorylation. The main site of phosphorylation is within the activation T-loop at Thr308 (for Akt1). Phosphorylation of Akt on Thr308 causes a change in its conformation allowing for substrate binding and for highly elevated rate of catalysis. It has been shown that there is low level of phosphorylation of the activation loop in resting cells, and this phosphorylation increases rapidly upon agonist stimulation. The phosphorylation of Thr308 strictly regulates the activation of the Akt protein, and its mutation to alanine impairs the kinase activity [Alessi et al., 1996]. It has been established that Thr308 is phosphorylated by the 3-phosphoinositide-dependent kinase-1 (PDK1) [Alessi et al., 1997; Stephens et al., 1998]. PDK1 phosphorylates Akt *in vitro*, and over-expression of PDK1 in cells also leads to elevated Thr308 phosphorylation in the absence of natural agonists. PDK1 contains a C-terminal PH domain, and the rate of Akt phosphorylation by PDK1 is significantly increased *in vitro* by the addition of phosphotidyltrisphosphate or phosphatidylbisphosphate, which both recruit Akt to the plasma membrane [Alessi et al., 1997]. In cells in which the expression or activity of the PDK1 protein is impaired, Akt is unresponsive to mitogenic stimulations as a result of the loss of Thr308 phosphorylation [Williams et al., 2000; Flynn et al., 2000].

Additional level of Akt regulation is provided by phosphorylation of the C-terminal hydrophobic domain, namely that of Ser473 (for Akt1). The mechanism of Ser473 phosphorylation is not completely understood, although there is evidence suggesting

both autophosphorylation [Toker and Newton, 2000] and phosphorylation by distinct serine kinases, including the integrin-linked kinase [Persad et al., 2001].

1.6.3. Function of Akt

The main biological functions of Akt activation can be grouped into three categories: survival, proliferation and cell growth.

Survival: Apoptosis is a normal cellular function that controls cell number by eliminating 'excessive' cells. Cancer cells are endowed with several mechanisms to inhibit apoptosis and promote their survival. Akt functions in an anti-apoptotic pathway, since dominant-negative mutants of Akt block survival that is mediated, e.g., by an insulin-like growth factor [Dudek et al., 1997]. The mechanism by which Akt prevents cell death is multifactorial, because Akt directly phosphorylates several components of the apoptotic system. For example, the protein Bad is a pro-apoptotic member of the Bcl-2 family of proteins that promotes apoptosis by forming a non-functional heterodimer with the anti-apoptotic protein Bcl-x_L, and phosphorylation of Bad by Akt prevents this interaction [Datta, et al., 1997], restoring the anti-apoptotic function of Bcl-x_L. Akt inhibits the catalytic activity of the pro-apoptotic protease caspase-9 by its phosphorylation [Cardone et al., 1998]. Further, phosphorylation of members of the forkhead family of transcription factors (FoxO proteins) by Akt prevents their nuclear translocation and activation of gene targets [Brunet et al., 1999], which include several pro-apoptotic proteins. Akt can also influence cell survival by

means of indirect effects on two central regulators of cell death, NFκB [Kane et al., 1999] and p53 [Mayo and Donner, 2001; Zhou et al., 2001].

Proliferation: The cell cycle is regulated by the coordinated action of complexes of cyclins and the cognate cyclin-dependent kinases (CDK) and CDK inhibitors (CKIs). Cyclin D1 levels, which are important for the G1/S phase transition, are regulated at the transcriptional, post-transcriptional and post-translational level by distinct mechanisms. Akt has an important role in preventing cyclin D1 degradation by regulating the activity of the cyclin D1 kinase, glycogen synthase kinase-3β (GSK3β). After phosphorylation by GSK3β, cyclin D1 is targeted for degradation by the proteasome. Akt directly phosphorylates GSK3β and blocks its kinase activity, thereby allowing cyclin D1 to accumulate and promote the cell cycle progression [Diehl et al., 1998]. Akt can suppress the expression of cyclin kinase inhibitors, such as p27 and p21 [Graff et al., 2000], and can also modulate p21 and p27 activity by affecting their phosphorylation either directly or through an intermediate kinase. The functional importance of the relationship between Akt and the cell-cycle are supported by experiments showing that the blockade of PI3K or Akt activity leads to cell-cycle arrest [Vemuri and Rittenhouse, 1994; Castoria et al., 2001].

Cell growth: In addition to its role in proliferation, there is increasing evidence that Akt also affects cell growth. A central regulator of cell growth is the mammalian target of rapamycin (mTOR), a serine/threonine kinase that serves as a molecular

sensor regulating protein synthesis on the basis of the availability of nutrients. mTOR acts by activating the p70 S6 kinase, which enhances the translation of mRNAs with 5' polypyrimidine segments, and by inhibiting a translational repressor of mRNA that bears a 5' CAP structure. mTOR is a direct target of Akt [Nave et al., 1999]; however, it is still unclear how or whether phosphorylation of mTOR by Akt is the mechanism of its activation. Although pharmacological studies with the mTOR inhibitor rapamycin indicate that the Akt pathway regulates cell growth through mTOR, the PI3K-Akt pathway is unlikely to be the only stimulus that leads to mTOR activation in cancer cells. Since the VE analogue α-tocopherylbutyrate has been previously suggested to disrupt the erbB2-induced signalling pathway [Akazawa et al., 2002], they may be expected to negatively modulate the Akt-dependent pathways, whereby promoting apoptotic killing of cancer cells.

1.7. Targeting peptides may improve the efficacy and selectivity of VE analogues

Cancer therapy is currently limited by the difficulty of achieving efficient and specific delivery of drugs into cancer cells. During the past years, effort has been made to design optimal carrier vectors for efficient drug delivery, including cationic lipids and peptides [Hurford et al., 1995]. Although they deliver therapeutics into human cells, specific targeting of malignant cells is not provided. In order to increase this specificity, various cell surface receptors have been considered as targets, including erbB2. Small peptides would represent important delivery agents because of their

excellent tissue penetration and relatively easy synthesis as well as conjugation to drugs. Shadidi and Sioud have explored the possibility of selecting small peptides that bind to breast cancer cell lines [Shadidi and Sioud, 2002]. They reported that the LTVSPWY peptide sequence exhibited high level of binding to breast cancer cells rather than human primary cells (*e.g*. epithelial, endothelial, and hematopoietic) since this sequence exerts high affinity for the erbB2 receptor over-expressed in the responsive cancer cells. Due to the fact that the erbB2 protein is over-expressed in high percentage of breast cancer patients (up to 30%), it may be possible to use the targeting peptide to efficiently deliver drugs, including α-TOS (following its conjugation with LTVSPWY) to such breast cancer cells. It can be expected that VE analogue modified in this was will then be delivered specifically to cancer cells via the erbB2 receptor, which can be expected to enhance apoptosis induction in erbB2-over-expressing cells.

1.8. The FVB/N *c-neu* transgenic mouse model

Many murine models of experimental carcinomas have been in use in various laboratories. Of these, the FVB/N *c-neu* transgenic mouse carries the rat *HER2/neu* proto-oncogene driven by a mouse mammary tumour virus promoter [Muller et al. 1996; Boggio et al., 1998]. The product of *HER2/neu* is the protein erbB2, a 185 kDa receptor tyrosine kinase involved in growth signalling (see above). Its over-expression promotes constitutive growth-stimulating activity regardless of the extracellular regulatory mechanisms. As a result, spontaneous ductal mammary carcinomas appear

in the animal model at ~7 months in >70% of female mice, with metastases evident at ~10 months [Kurt et al., 2000; Nanni et al., 2000].

This is a very good model for studying the effects of inducers of apoptosis on breast carcinomas for several reasons. First, breast carcinomas in the FVB/N *c-neu* mice develop in locations similar to that in human patients. Second, the FVB/N *c-neu* mice are not immunocompromized, unlike the nude or NOD/SCID mice used in most cancer studies, therefore the tumour cells interact with the immune system of the animal. For these reasons, we decided to investigate the effect of α-TOS on the development of breast cancer in the FVB/N *c-neu* mice. We used this model together with immunocompromized mice that were xenotransplanted with MCF7 cell, characterised by low level of expression of the erbB2 protein.

1.9. Hypothesis and Aims

Hypothesis:

We propose that vitamin E analogues with apoptogenic activity efficiently kill breast cancer cells regardless of the level of expression of erbB2 and, in particular when efficiently delivered into the mouse model, suppress experimental breast carcinomas with both low and high expression of the oncogenic, pro-survival receptor tyrosine kinase erbB2.

Aims:

1. We will test whether different levels of expression of the erbB2 receptor tyrosine kinase affects induction of apoptosis in breast cancer cell lines by VE analogues.

2. We will study the molecular mechanisms of apoptosis induced by VE analogues in breast cancer cell lines with high and low level of expression of erbB2.

3. We will test whether VE analogues suppress the progression of experimental breast carcinomas using the FVB/N *c-neu* mice and human breast cancer MCF7 xenograft mouse models differing in the level of expression of the erbB2 protein.

4. We will study whether addition to the VE analogue α-TOS of a peptide targeting the erbB2 receptor promotes cellular delivery of the drug in cells with high level of expression of erbB2 that would be expected to enhance the apoptogenic activity of the

drug in such cells. If so, we will study the molecular mechanism of delivery of such peptide-α-TOS conjugate and its effect on breast carcinoma progression in the FVB/N *c-neu* mouse.

CHAPTER 2

VITAMIN E ANALOGUES INDUCE APOPTOSIS IN erbB2-OVEREXPRESSING BREAST CANCER CELLS VIA THE MITOCHONDRIAL PATHWAY

2.1 Summary

α-TOS is a redox-silent VE analogue with high pro-apoptotic and anti-neoplastic activity. In this part, we tested whether α-TOS and several novel VE analogues (some with higher apoptogenic activity than the prototypic α-TOS) kill breast cancer cells over-expressing the receptor tyrosine kinase erbB2. The results showed that VE analogues caused comparable levels of apoptosis in cells with both high and low level of the erbB2 protein. To extend our understanding of the molecular mechanism of cell death triggered by VE analogues, we investigated several pathways of apoptosis induced by VE analogues, of which the major one is the intrinsic, mitochondrial pathway. We found that generation of ROS preceded mitochondrial destabilization and execution of apoptosis, as evidenced by the anti-apoptotic effects of exogenous superoxide dismutase (SOD) and mitochondrially targeted coenzyme Q (MitoQ). Dissipation of the mitochondrial inner trans-membrane potential ($\Delta\Psi_m$) was followed by cytosolic re-localization of Cyt c and Smac/Diablo and by caspase-dependent cleavage of the death substrate. Resistance to apoptosis of the corresponding ρ^o (mtDNA-deficient) cells confirmed the critical dependence of the induction of apoptosis in breast cancer cells exposed to VE analogues on mitochondria and links it to the generation of radicals as judged by the delayed accumulation of ROS in the ρ^o cells.

In this chapter, we also used the FVB/N *c-neu* mice with spontaneous breast carcinomas and human breast cancer MCF7 mouse xenograft model to

investigate the efficacy of α-TOS on cancer progression. We showed that α-TOS significantly inhibited tumour growth compared to the controls in both animal models.

These data provide an insight into the molecular mechanism by which VE analogues kill breast cancer cells, independent of their erbB2 status. A group of novel analogues of VE may present the long sought-after therapeutic agents against breast cancer.

2.2 Key words

VE analogues; apoptosis; erbB2; mitochondria; breast cancer; FVB/N *c-neu* mice; breast cancer xenografts.

2.3 Introduction

A problem encountered in breast cancer management stems from over-expression of, or mutations in, receptor tyrosine kinases, in particular the proto-oncogene erbB2, a product of the *c-neu* gene [Roskoski, 2004; Slamon et al., 1989]. This tyrosine kinase trans-membrane protein is over-expressed in up to 30% of primary breast cancers [Kupryjanczyk et al., 2004]. Over-expression of the erbB2 oncogene correlates with poor prognosis in the patients because it enhances the metastatic potential of cancer cells [Slamon et al., 1987; Hung et al., 1992].

VE analogues, a novel class of compounds with strong pro-apoptotic activity, epitomized by α-TOS [Neuzil et al., 2004], induce apoptosis in a variety of malignant cells while being largely non-toxic to normal cells, as shown in both *in vitro* [Neuzil et al., 2001b; Jha et al., 1999] and *in vivo* models [Weber et al., 2002]. α-TOS also efficiently kills cancer cells deficient in tumour suppressor genes, including p53 and $p21^{Waf1/Cip1}$ [Weber et al., 2002]. The major pro-apoptotic signalling pathway operational in apoptosis induced by α-TOS is linked to mitochondria [Yamamoto et al., 2000; Weber et al., 2003]. Interestingly, an analogue of VE, α-tocopheryl butyric acid, has been shown to suppress the activation of erbB2, although the mechanism was not studied in much detail [Akazawa et al., 2002].

In this chapter, we studied the susceptibility of breast cancer cell lines expressing low and high levels of erbB2 to VE analogues. We show here that the agents induced similar apoptosis extent regardless of the erbB2 status of the cells, thereby efficiently bypassing the pro-survival effect of the erbB2-promoted pathway, and that apoptosis was mediated via the mitochondrial pathway. We also used FVB/N *c-neu* transgenic mice and the human breast cancer MCF7 xenograft mouse model to investigate the efficacy of α-TOS on growth of breast carcinomas differing in the level of expression of erbB2. The results show that α-TOS remarkably inhibited the growth of tumours in both animal models.

Our data reveal that VE analogues may be useful for treatment of breast cancers

with both low and elevated expression of the proto-oncogenic receptor tyrosine kinase erbB2.

2.4 Materials and methods

2.4.1. Cell culture and treatment

Human breast cancer cell lines MCF7 with low and MDA-MB453 with high expression of erbB2, and a murine breast cancer cell line N2O2 with low and NeuD12 with high expression of erbB2 were used in this study. Cells were routinely cultured in DMEM with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. For experiments, cells were plated and allowed to reach approximately 70% confluence, and were treated separately with up to 50 μM α-TOS (Sigma), α-TOM [Birringer et al., 2003], α-TAM, and α-TAS [Tomic-Vatic et al., 2005]. Analogues of VE are stereospecific, and we used the *all-rac* form of all agents. In some cases, cells were co-treated with 750 U/ml of SOD (PEG-tagged SOD; Sigma) or pre-treated (1 h prior to the addition of VE analogues) with 2 μM MitoQ [Kelso et al., 2001]. VE analogues and MitoQ were added to cells as stock solutions in EtOH at 1:1,000 (v/v). Maximal concentration of added EtOH in the media was <0.1% (v/v), which had no measurable effect on the cultured cells (data not shown).

2.4.2. Biochemical assays

2.4.2.1. Assessment of apoptosis

Apoptosis was quantified using the annexin V-FITC method, which detects phosphatidylserine externalised in the early phases of apoptosis as described elsewhere [Neuzil et al., 2001c]. Briefly, cells were plated at 10^5 per well in 24-well plates, and after overnight incubation treated with VE analogues. Floating and attached cells were collected (after trypsinisation of the adherent cells), washed twice with PBS, re-suspended in 0.2 ml binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4), incubated for 30 min at 4°C with 2 μl annexin V-FITC (Pharmingen), supplemented with 200 μl of propidium iodide (PI:50 μg /ml), and analysed by flow cytometry (FACS Calibur, Becton Dickinson) using channel 1 for annexin V-FITC binding and channel 2 for PI staining. Cell death was quantified as the percentage of cells with green (annexin V binding) plus red fluorescence (PI staining).

2.4.2.2. Assessment of IC_{50}

Cells were plated in 96-well plates at $2.5x10^3$ per well (200 μl complete DMEM medium), allowed to attach overnight, and treated with VE analogues. Cell viability was estimated using the methylthiazoletetrazolium (MTT) assay. Briefly, following exposure of cells to the agents, 10 μl of MTT (5 mg/ml in PBS) was added, and after incubation for 4 h at 37°C, the medium was removed and combined with 200 μl of 1% SDS. Absorbance was read at 550 nm using an

ELISA plate reader and the total absorbance (a surrogate for cell survival) was expressed relative to that obtained from control cells (designated as 100%). The IC_{50} value for each of the VE analogues tested was then estimated from the survival curves generated in this fashion.

2.4.2.3. Assessment of protein expression

Cells were treated as indicated and harvested by trypsinization. Following fixation with 2% formalin in PBS and permeabilization with 0.2% saponin in PBS containing 2% FBS (both at room temperature for 1 h), the cells were reacted with the primary antibody against Bcl-2 (Boehringer), Bcl-x_L, Mcl-1, or Bax (all Santa Cruz) followed by incubation with a secondary antibody conjugated to FITC (both at room temperature for 1 h). The level of protein expression was estimated by analysing the cells in a flow cytometer.

2.4.3. Assessment of $\Delta\Psi_m$

Dissipation of the mitochondrial inner trans-membrane potential ($\Delta\Psi_m$) was assessed using the polychromatic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetra-ethylbenzimidoazolyl-carbocyanino iodide (JC-1; Molecular Probes). JC-1 shows orange-red fluorescence in energized mitochondria, where it accumulates at the potential of -110mV or greater (in negative values), since its concentration increases and molecules of the dye form the so-called J-clusters, which exert red fluorescence. In mitochondria with dissipated $\Delta\Psi_m$,

the concentration of JC-1 is low, since it 'leaks' into the cytosol, emitting green fluorescence; this is suggestive of mitochondrial dysfunction. In brief, cells were treated as required, after which they were incubated for 10 min with 10 μM JC-1. The cells were then washed, harvested, and analyzed by flow cytometry. Since appearance of green fluorescence precedes disappearance of red fluorescence, the population of cells with low $\Delta\Psi m$ was evaluated on the basis of cells exhibiting increased green fluorescence.

2.4.4. Assessment of ROS production

To monitor the intracellular production of ROS, we utilized two different methods. First, we used a cell-permeable probe, dihydrodichlorofluorescein diacetate (DCF), to monitor cellular production of ROS. The probe is oxidized by cells to yield high intensity of fluorescence in the presence of simple (hydrogen peroxide) and more complex, related peroxides, although the specificity of the oxidizing species is rather broad. Therefore, DCF fluorescence reflects the amount of ROS formed (accumulated) and can be used as a very convenient method to detect accumulation of ROS in cells exposed to VE analogues. In brief, following treatment, cells were harvested, washed twice with PBS, and re-suspended in serum-free medium. The cells were then incubated with 5 μM DCF for 2 h at 37°C, washed with ice-cold HEPES/PBS and placed on ice. Fluorescence was assessed by flow cytometry. As a positive control, cells were treated with 100 μM H_2O_2 for 2 h.

ROS generation was also assessed directly by electron paramagnetic resonance (EPR) spectroscopy using the radical trap 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Sigma), essentially as described elsewhere [Weber et al., 2003]. In brief, cells were plated in T25 flasks, allowed to reach 60-70 % confluence (~$5x10^6$ cells per flask), washed and overlaid with modified Hanks-buffered saline solution [Thomas et al., 2002]. The washed cells were then loaded with 10 mM DMPO and incubated further in the presence or absence of 50 μM α-TOS. After various times, the cells and conditioned media were harvested, transferred into a quartz flat cell (Wilmad), and analyzed for the accumulation of DMPO spin-conjugates using the Bruker EMX bench-top spectroscope set at 293 K. The detection limit of the stable nitroxide (TEMPO) under identical conditions was ~50 nM.

2.4.5. Cell cycle analysis

Cells were plated at 10^5 per well in 24 well plates, allowed to attach overnight, and then incubated with α-TOS. Floating and attached cells were harvested, washed with PBS, re-suspended in 1 ml of ice-cold 70% ethanol and kept for at least 1 h at -20°C. The cells were spun down, re-suspended in 200 μl of a solution containing RNAse A (50 μg/ml; Sigma), and incubated in the dark with PI (5μg/ml in PBS) for 10 min on ice. Finally, the nuclear suspension was filtered through a 60 μm mesh and analysed by flow cytometry.

2.4.6. Western blotting

2.4.6.1. Preparation of cytoplasmic and mitochondrial fractions

Mitochondrial and cytoplasmic fractions were obtained using a kit (Pierce). Briefly, cells were supplemented with 800 µl of the Mitochondrial Isolation Reagent A, vortexed at medium speed for 5 s and incubated on ice for 2 min. 10 µl of the Mitochondrial Isolation Reagent B were added and the suspension vortexed at high speed for 5 s. The tubes were incubated on ice for 5 min, with vortexing at high speed every minute for 5 s. 800 µl of the Mitochondrial Isolation Reagent C were added and the tubes were gently mixed several times, and then centrifuged at 700 rpm for 10 min at 4°C. The supernatant was then centrifuged at 12,000 rpm for 15 min. The resulting supernatant represents the cytoplasmic fraction, the pellet contains isolated mitochondria.

2.4.6.2. Immunoblot analysis

Cytoplasmic proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes, which were blocked with 1% w/v skim milk, incubated with primary antibodies (all from Santa Cruz) and incubated further with HRP-labelled secondary IgGs. The blots were developed using the ECL kit (Pierce) and exposed to the X-Omat AR film. Alternatively, immuno-reactive proteins were visualized using the chemiluminescence protocol (ECL, Amersham) and the bands assessed using the Bio-Rad gel documentation

system. Anti-β-actin IgG (Santa Cruz) was used as a control for protein loading.

2.4.7. Preparation of ρ^o cells

The ρ^o phenotype refers to cells lacking mtDNA. Such cells are useful for studying the role of mitochondria in various processes, since their oxidative phosphorylation is impaired [Higuchi et al., 1997; Dey and Moraes., 2000; Park et al., 2004; Kwong et al., 2007]. To prepare such cells, the N2O2 and NeuD12 cells were treated with EtBr to remove mtDNA as reported elsewhere [Higuchi et al., 1997; Weber et al., 2003]. This method is based on exposure of cells to sub-lethal concentrations of EtBr, at which the agent preferentially intercalates into mtDNA that is not protected by histones and that contains virtually no non-coding regions. In brief, cells were cultured in 6-well plates and maintained in the ρ^o medium (DMEM, 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 3.5 g/l glucose, 50 mg/l uridine, 110 mg/l pyruvate phosphate) supplemented with EtBr (50 ng/ml). Cells were allowed to undergo 5-7 doublings before becoming mtDNA-deficient, as documented by lack of mitochondrial EtBr fluorescence. For experiments, ρ^o cells were placed in the ρ^o medium free of EtBr.

2.4.8. Mouse xenografts

Nude mice were inoculated subcutaneously (s.c.) with MCF7 cells ($2x10^6$ cells per mouse). After tumours reached the diameter of 3-7 mm (about 2 weeks after xenografting the mice), the mice were injected intraperitoneally (i.p.)

with 10 μmol α-TOS in DMSO every 3 days. Control mice were injected with an equal volume (100 μl) of DMSO only. Tumour volumes were estimated by measuring the maximal height, length, and width every 3 d using digital calipers. These experiments were carried out during the earlier stages of my PhD project, before our laboratory established the cutting-edge techniques of ultrasound imaging (USI) of tumours, which was applied for tumour evaluation in the FVB/N *c-neu* mice.

2.4.9. The FVB/N c- neu transgenic mice model

Female transgenic FVB/N *c-neu* mice bearing progressively growing tumours with mean volume of 25 mm^3 were randomly assigned to control or treatment groups. The effect of α-TOS on tumour progression was assessed by USI using the Vevo770 instrument (VisualSonics). The USI device is equipped with the RMV708 scan-head (VisualSonics) working at the frequency of 80 MHz and with resolution as high as 30μm. This allows for very precise visualization of tumours and accurate quantification of their volume in a non-invasive manner, even in the case of more deeply embedded carcinomas. For example, in some cases, carcinomas in the FVB/N *c-neu* mice are embedded so that only some 10% of the tumour mass is exposed above the surface of these animals. USI imaging allows for quantification of the complete tumour, while the use of callipers would vastly underestimate the tumour volume as well as the effects of anti-tumour drugs on the tumour progression. For α-TOS therapy, mice

received treatment with corn oil/4% ethanol (control) alone, or 15 or 5 μmol α-TOS in corn oil/4% ethanol administered i.p. every 3 d. All animal experiments were performed according to the guidelines of the Australian and New Zealand Council for the Care and Use of Animals in Research and Teaching and were approved by the Griffith University Animal Ethics Committee.

2.4.10. Statistical analysis

Between-group comparisons were made using mean ± S.D. and the unpaired Student's *t*-test. Differences in the mean relative tumour size (± S.E.M.) were examined using analysis of covariance (ANCOVA) with days as the covariate. Statistical analyses were performed using SPSS 10.0 analytical software. Statistical significance was accepted at $p<0.05$. At least three independent experiments were performed for majority of the studies, while at least 6 mice per group were used in the case of the *in vivo* studies. Images shown are representative of at least 3 independent experiments.

2.5 Results

2.5.1. VE analogues induce comparable levels of apoptosis in breast cancer cell lines with different expression of erbB2

Cancers over-expressing the receptor tyrosine kinase erbB2, in particular breast cancer, are rather resistant to established chemotherapy. For example, they are resistant to the combination of cyclophosphamide, methotrexate and 5-fluorouracil, one of the most commonly used regimens for patients with breast carcinomas [Yang et al., 2003]. We speculated that VE analogues might bypass the resistance to apoptosis in erbB2 over-expressing cells. We therefore tested whether α-TOS and several novel VE analogues, α-TOM, α-TAS and α-TAM induced apoptosis in breast cancer cell lines with low (N2O2, MCF7 cells) and high erbB2 expression (NeuD12, MDA-MB-453 cells), respectively. α-TOS is the prototypic VE analogue, while replacement of the succinyl moiety with the maleyl group enhances the apoptogenic activity of the agent [Birringer et al., 2003]. Attachment of the functional groups (succinyl and maleyl) to the VE structure via an amide bond further enhances the apoptotic efficacy of the comparable ester counterparts [Tomic-Vatic et al., 2005].

As documented in Figures 6 and 7, there was little difference in susceptibility of the two different levels of expressions of erbB2 lines to the VE analogue-induced apoptosis, with α-TAM showing the highest efficacy. This effect was observed for both the murine cell lines and the corresponding human breast carcinoma lines.

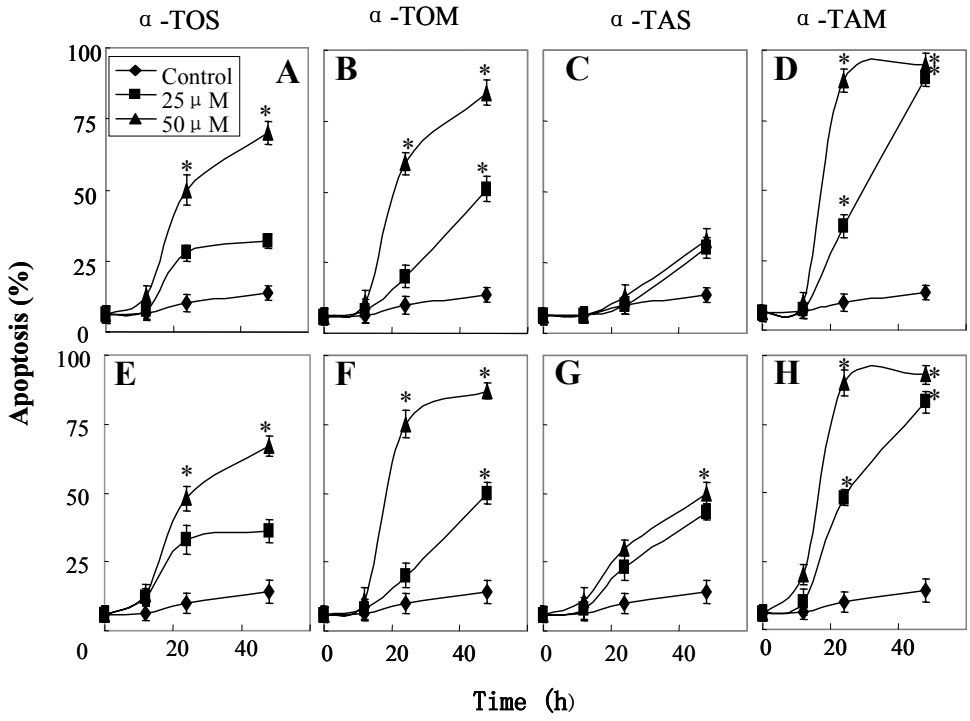

Figure 6. VE analogues induce apoptosis in mouse breast cancer cell lines with different expression of erbB2. N2O2 (A to D) and NeuD12 (E to H) cells were seeded in 24-well plates and allowed to reach 60-70% confluence. Following treatment with α-TOS, α-TOM, α-TAS and α-TAM at concentrations and for times indicated, apoptosis was assessed using the annexin V-FITC method and evaluated on the basis of percentage of annexin V-positive cells scored by FACS analysis. Data represent mean±S.D. (n=3). The symbol "*"denotes significant differences from the control (p<0.05).

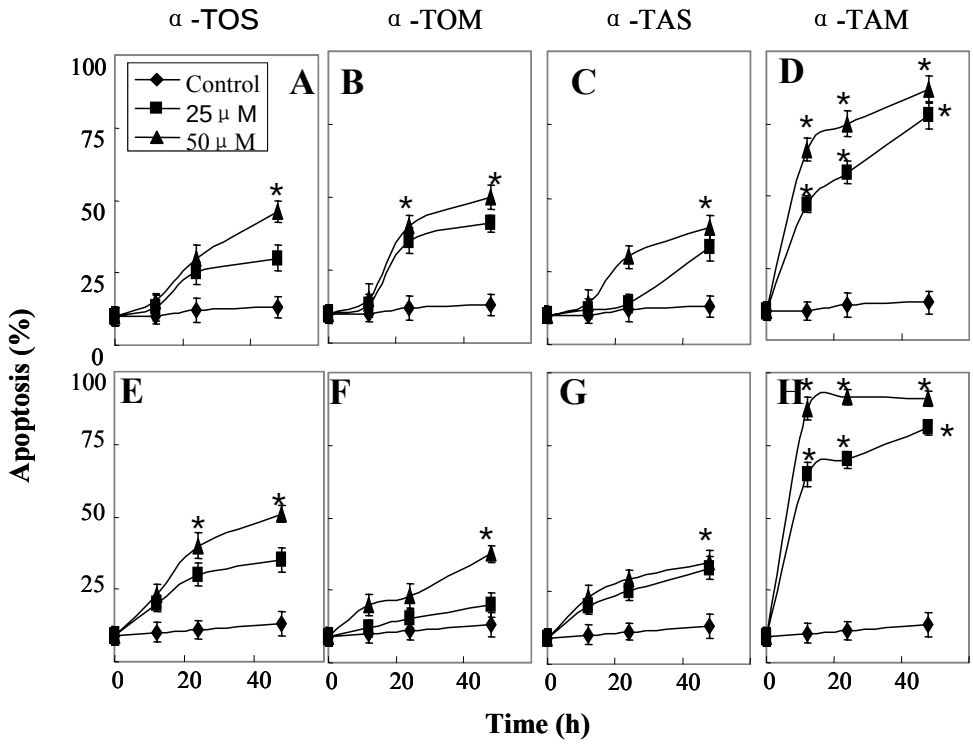

Figure 7. VE analogues induce apoptosis in human breast cancer cell lines expressing low and high levels of erbB2. MCF7 (A to D) and MDA-MB-453 (E to H) cells were seeded in 12-well plates and allowed to reach 60-70% confluence. Following treatment with α-TOS, α-TOM, α-TAS and α-TAM at concentrations and for times indicated, apoptosis was assessed using the annexin V-FITC method and evaluated on the basis of percentage of annexin V-positive cells scored by FACS analysis. Data shown are mean±S.D. (n=3). The symol "*" denotes significant differences from the control (p<0.05).

2.5.2. α-TOS causes increase in the sub-G_0G_1 population without cell cycle arrest

Assessment of N2O2 and NeuD12 cells revealed no appreciable differences in the distribution of the cells within its individual phases of the cell cycle between the cell lines exposed to of α-TOS (data not shown), suggesting that the VE analogue induces apoptosis in the breast cancer cells without causing cell cycle arrest. However, this analysis revealed that a significant percentage of cells accumulated in the sub-G_0G_1 region of the histograms upon treatment with α-TOS (Table 1). Overall, these data are completely consistent with the apoptosis data derived from annexin V binding (Figure 6).

Table 1. α-TOS causes an increase in sub-G_0 cell sub-population

Time (h)	N2O2 cells			NeuD12 cells		
	0 μ M	25 μ M	50 μ M	0 μ M	25 μ M	50 μ M
0	4.8±1.4	4.8±1.4	4.8±1.4	3.1±0.9	3.1±0.9	3.1±0.9
24	5.1±1.8	6.5±2.9	9.0±3.8	3.6±1.3	5.8±0.9*	8.1±2.6*
48	5.7±2.3	10.9±2.0*	26.6±5.8*	4.1±2.3	15.1 ±6.3*	34.2±6.8*

N2O2 and NeuD12 cells were treated with α-TOS as indicated. At the times shown, cell preparations were assessed for cell cycle distribution as detailed in the experimental section. Cells identified with DNA < 2n were considered as *sub-G_0G_1* population. Data shown are mean values±S.D. from three independent experiments. The symol '*' denotes a significant difference compared to the controls (p<0.05).

2.5.3. VE analogues show comparable IC_{50} values for N2O2 and NeuD12 cells

In the next experiments, we determined the IC_{50} values for the individual VE analogues for the murine cell lines with low (N2O2) and high level of erbB2 expression (NeuD12). We observed that there was virtually no difference for the two different cell lines (Table 2), which is consistent with the apoptosis data. Again, the amide α-TAM showed the highest activity.

Table 2. IC50 values of VE analogues

Analogues	N2O2 cells	NeuD12 cells
α-TOS	26.3	27.5
α-TOM	5.5	6.4
α-TAS	9.6	10.5
α-TAM	1.1	1.4

N2O2 and NeuD12 cells were treated with VE analogues at increasing concentrations for 24 h, and the IC_{50} values (μM) estimated from the MTT survival curves as detailed in the Experimental Section. Data shown are the mean values S.D. obtained from three to four independent experiments.

2.5.4. Effect of α-TOS on proliferation of MDA-MB-453 and MCF7 cells

We tested the anti-proliferative effects of α-TOS in the human cancer cell lines MDA-MB-453 and MCF7. As shown in Figure 8, the growth inhibitory effect of

α-TOS was observed in a dose-dependent manner after 48 h treatment. The maximal effects on proliferation inhibition were observed with 125 μM α-TOS, which inhibited proliferation in 79% of MDA-MB-453 cells and 52% of MCF7 cells. The IC_{50} values were 74.4 μM in MDA-MB-453 cells and 132.4 μM in MCF7 cells.

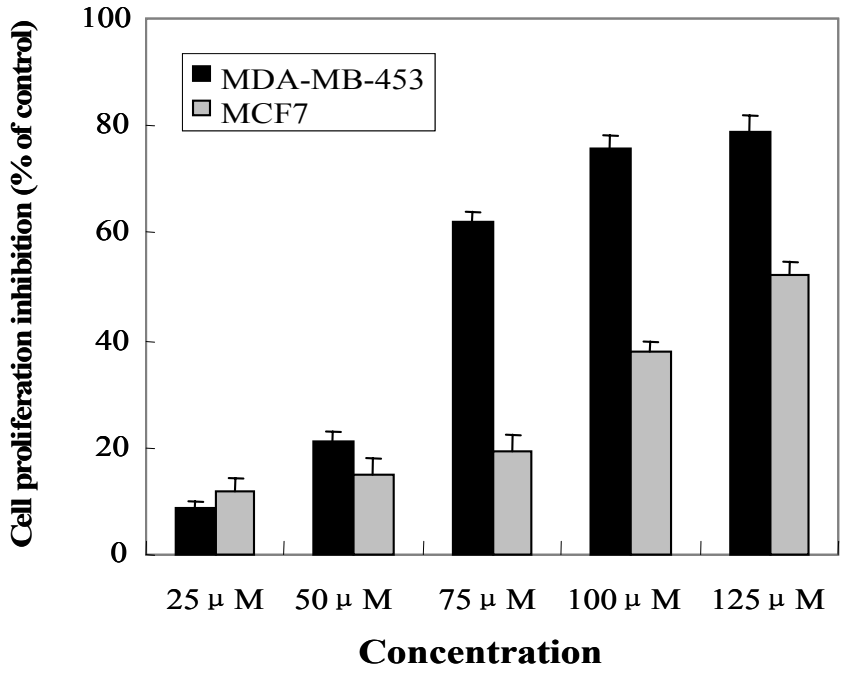

Figure 8. Effects of α-TOS on inhibiting the growth of MDA-MB-453 and MCF7 cells. Cells were seeded into 96-well plates and allowed to recuperate overhight. The cells were then incubated with the vehicle or different concentrations of α-TOS for 48 h. Cell proliferation was determined by MTT assay. Results are expressed as the percentage of cell proliferation relative to the proliferation of controls. Each value is the mean±S.D. of three independent experiments.

2.5.5. ROS are generated early in the N2O2 and NeuD12 cells exposed to α-TOS

Consistent with previously published data for other malignant cell lines [Weber et al., 2004], we observed early production of ROS in the NeuD12 and N2O2 cell lines exposed to α-TOS as documented in Figure 9. To do this, two approaches to ROS assessment were used. First, we investigated ROS production via an indirect method based on oxidation of the fluorescent probe DCF by cell-derived radicals and by their secondary oxidation products. Second, we directly evaluated ROS in cells pre-loaded with a radical spin trap, DMPO, using EPR spectroscopy. Thus, the time-dependent accumulation of oxidized DCF in both cell lines treated with α-TOS was monitored by flow cytometry as shown in Figure 9 A and B. Consistent with the notion that ROS production is increased upon treatment with VE analogues, independent EPR analysis of N2O2 and NeuD12 cells revealed an early increase in accumulated DMPO-OH radicals in the presence of the added α-TOS (4-line spectrum denoted with a circle in Figure 9 C (i)) that dissipated at later time points (Figure 9 D). In contrast, no DMPO-conjugate was detected in cultured cell preparations in the absence of the added α-TOS (Figure 9 C (ii)). Detection of DMPO-OH is a surrogate marker for cellular ROS production and may be formed through direct trapping of hydroxyl radicals or the superoxide radical anion that subsequently decomposes to yield DMPO-OH. Taken together, both the increased DCF fluorescence and enhanced ROS generation detected by EPR spectroscopy indicate that breast cancer cells respond to exogenously added α-TOS through a

rapid formation of ROS that subsequently act as mediators of down-stream pro-apoptotic events (see below).

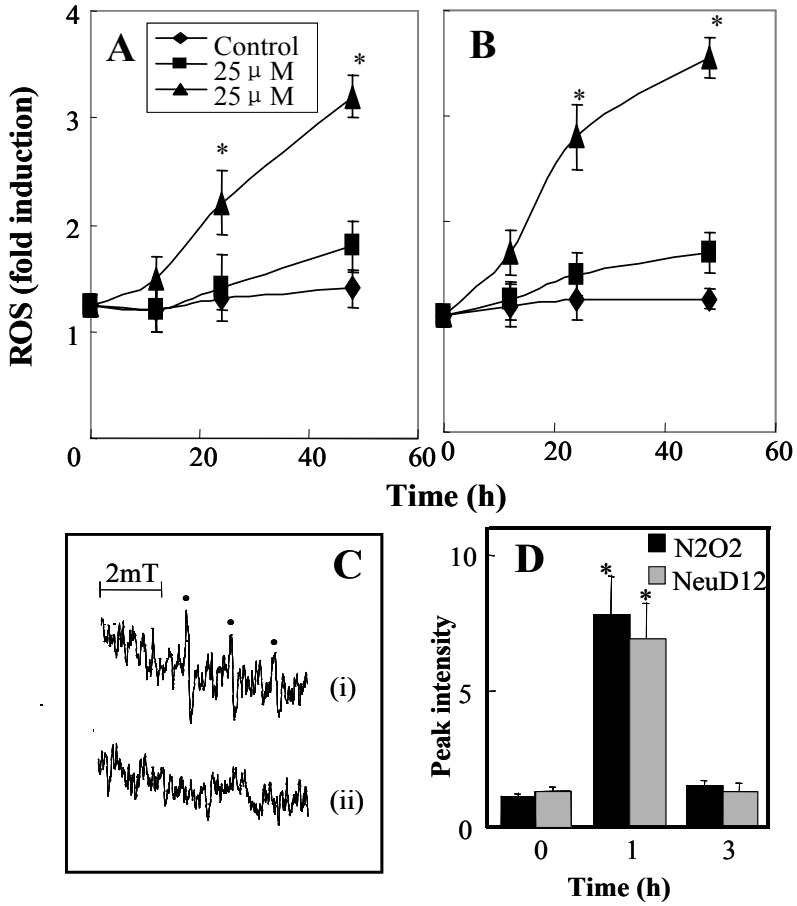

Figure 9. α-TOS induces generation of oxidative stress. Cells were exposed to α-TOS at concentrations and for time periods indicated, and generation of ROS was estimated by oxidation of DCF evaluated by FACS. Panel A shows results for N2O2 and B for NeuD12. Panel C shows generation of ROS in N2O2 cells as evaluated using EPR spectroscopy in the presence (i) or absence (ii) of α-TOS (50 μM, 1 h) as detailed in the Experimental Section. The dots in Panel C (i) denote the spectral lines characteristic of accumulated DMPO-OH. The EPR spectra in panel C are representative of three independent experiments. Panel D shows the DMPO-OH radical intensity in preparations of N2O2 and NeuD12 cells obtained through double integration of the low-field absorption using standard WINEPR software. Data represent mean values±SD (n=3). The symbol "*" denotes significant differences from the control (p<0.05).

2.5.6. ROS are essential for down-stream apoptotic signalling induced by VE analogues

That ROS are essential for down-stream apoptosis signaling induced by VE analogues is supported by decreased apoptosis in the presence of MitoQ as shown in Figure 10. MitoQ is a triphenylphosphonium (TPP) conjugate of ubiquinone-10 (coenzyme Q) that selectively accumulates in mitochondria, and is reduced to the corresponding analogue of ubiquinol-10 by the mitochondrial electron redox chain. Therefore, MitoQ acts as a very efficient, mitochondria-associated redox-active compound [Kelso et al., 2001]. Treatment of N2O2 and NeuD12 cells with α-TOS in the presence of MitoQ suppressed apoptosis induced by the VE analogue, strongly implicating ROS in the process. Further evidence for the role of ROS in apoptosis induction follows from experiments in which the cells were co-treated with exogenous SOD. As shown in Figure 10 B and D, supplementation with the enzyme completely prevented apoptosis in the two cell lines exposed to α-TOS, indicating superoxide as the ROS responsible for the downstream apoptosis induction.

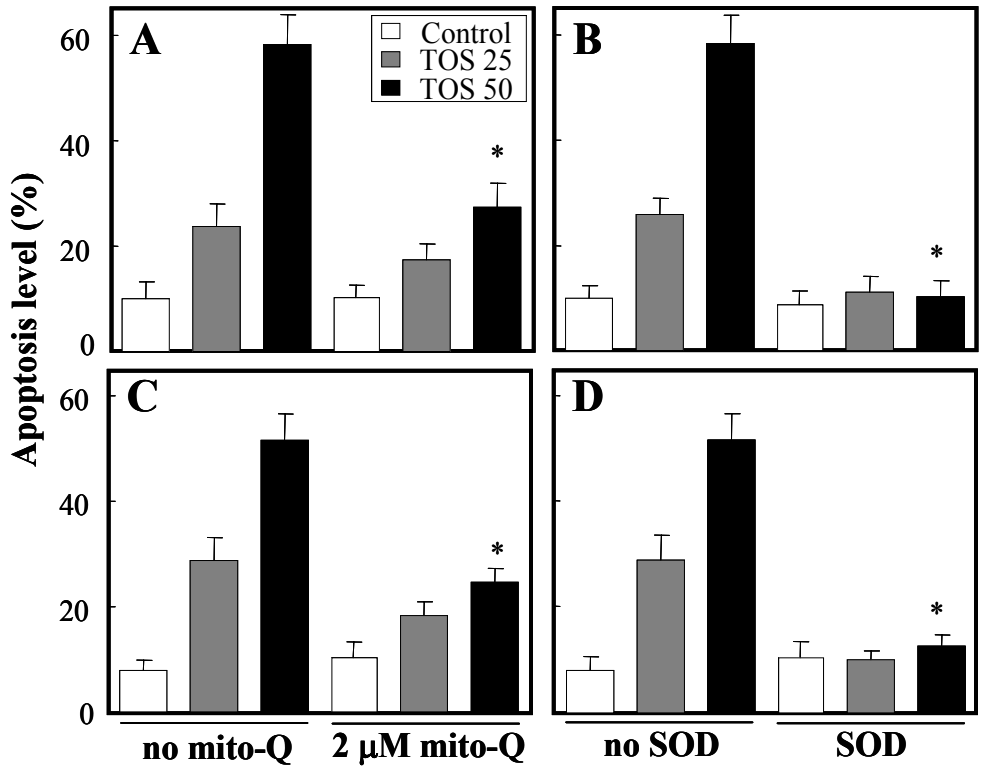

Figure 10. Inhibition of ROS generation suppresses α-TOS-induced apoptosis. N2O2 (A, B) and NeuD12 (C, D) cells were exposed to α-TOS at 25 or 50 μM for 24 h. Where indicated, cells were pre-treated for 1 h with 2 μM MitoQ (A, C) or co-treated with PEG-SOD (750 units/ml) (B, D). The cells were then assessed for apoptosis using the annexin V method, and the extent apoptosis was expressed as the percentage of annexin V-positive cells. Data shown are mean values±S.D. (n=3). The symbol "*" denotes significant differences from the experiment without MitoQ (A,C) or SOD (B,D) when cells exposed to α-TOS at 50 μM (p<0.05).

2.5.7. α-TOS causes a decrease in ΔΨm and cytosolic mobilization of Cyt c and Smac/Diablo in N2O2 and NeuD12 cells

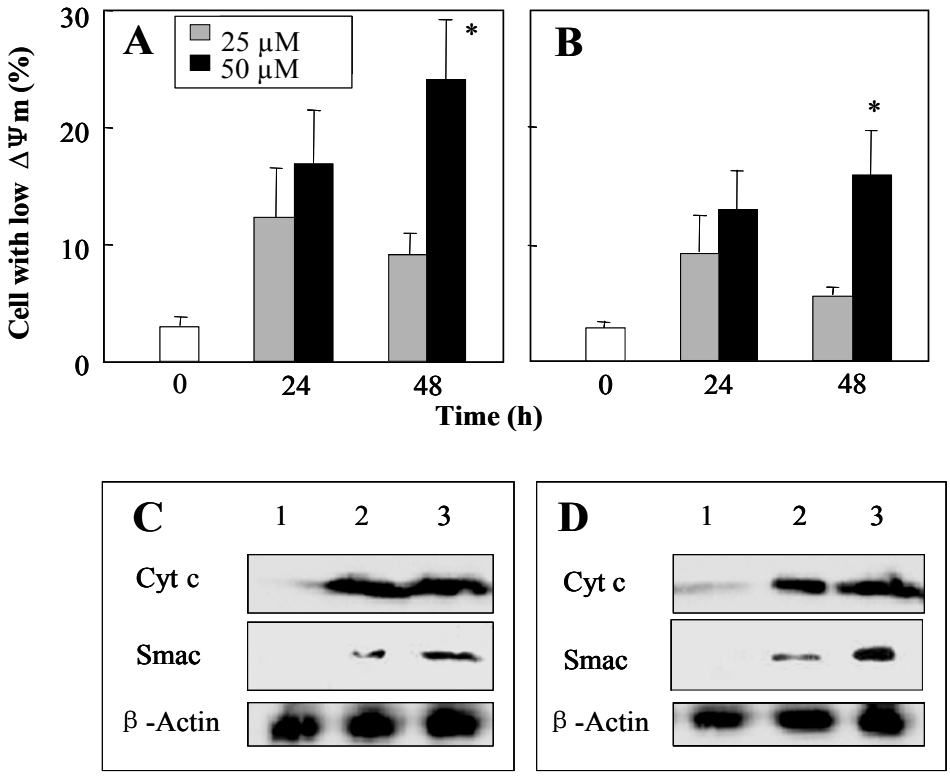

Figure 11. α-TOS causes mitochondrial destabilization and mobilization of pro-apoptotic factors. N2O2 (A, C) and NeuD12 (B, D) cells were exposed to α-TOS (25 μM or 50 μM) for the indicated times and assessed for ΔΨm dissipation by FACS analysis following staining with JC-1 (A, B). Control cells (1) or cells exposed to 50 μM α-TOS for 12 (2) and 24 h (3) were lysed and the cytosolic fraction probed by immunoblotting for the level of Cyt c and Smac/Diablo (C, D). Data shown are mean values±S.D. (n=3). Western blot images are representative of three independent experiments. The symbol "*" indicates statistical differences (p<0.05) between data acquired in cells treated for 24 h with 50 and 25 μM α-TOS.

Destabilization of mitochondria is frequently assessed on the basis of dissipation of the mitochondrial inner trans-membrane potential (ΔΨm) and the ensuing re-localization of mitochondrial apoptotic mediators. Data shown in Figure 11 indicate a decrease in ΔΨm in both cell lines exposed to α-TOS (A, B) and that this was accompanied by cytosolic mobilization of Cyt c, which is essential for formation of the apoptosome complex in the cytosol. We also observed cytosolic re-localization of Smac/Diablo in both cell types when treated with α-TOS (C, D), indicating that not only will the drug induce apoptosis in these cells by it will also prevent potential IAP-dependent suppression of caspase activation.

2.5.8. Deficiency in mtDNA confers resistance to α-TOS

We next sought a definitive evidence for the essential role of mitochondria in α-TOS-induced killing of the cells expressing low and high levels of erbB2. To test this, we prepared the corresponding 'modified' cells, *i.e.* their counterparts deficient in mtDNA (the ρ^o phenotype). This was done by long-term cultivation of the parental cells in the presence of sub-lethal levels of EtBr, which preferentially intercalates into the unprotected mtDNA, thereby preventing its replication. Data shown in Figure 12 document that, compared to the parental cells, the ρ^o cells were resistant to apoptosis induced by α-TOS, as judged by the relatively low annexin V binding. These findings are consistent with recent reports [Park et al., 2004; Higuchi et al., 1997] and provide direct evidence for the essential role of fully functional mitochondria in apoptotic killing of both N2O2 and NeuD12 cells.

Moreover, these data provide additional evidence for the importance of mitochondria-derived ROS in induction of apoptosis by α-TOS, since the ρ^o cells revealed low accumulation of ROS compared to the parental cells (Figure 12 C).

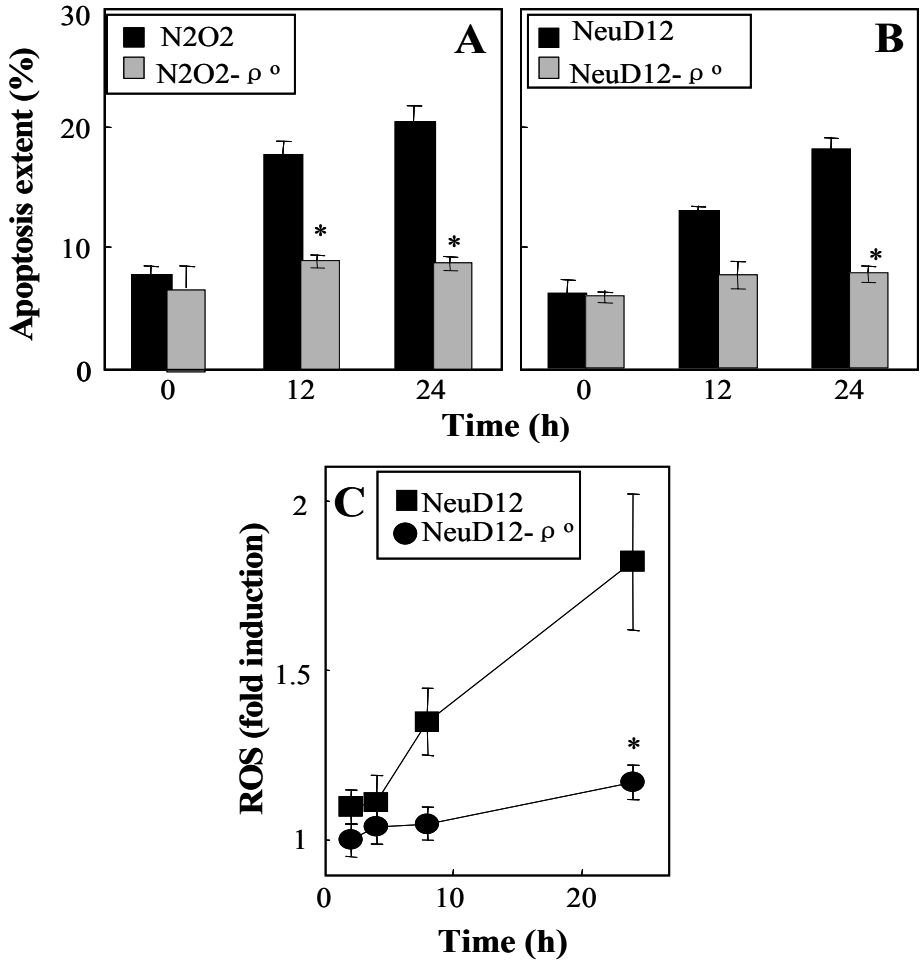

Figure 12. Deficiency in mitochondrial DNA confers resistance to α-TOS. N2O2 (A) and NeuD12 cells (B, C) were cultured in the presence of sub-lethal doses of EtBr to generate the ρ^o phenotype. Both the parental and ρ^o cells were exposed to 25 μM α-TOS for 12 or 24 h and assessed for apoptosis extent (annexin V binding) (A, B) and ROS generation (C). Data shown are mean values±S.D. (n=3). Compared to the parental cells, the ρ^o cellss were resistant to apoptosis induced by α-TOS. Moreover, ρ^o cells revealed low accumulation of ROS compared to the parental cells. The symbol "*" indicates significant differences between the ρ^o cells and the parental cells (p<0.05).

2.5.9. α-TOS inhibits tumour growth in athymic mice with human breast cancer cell xenografts

MCF7 cells were used for generation of xenografts in athymic (nude) immuno-compromized mice. The cells were injected s.c. on the back of each mouse. Once tumours were established (became palpable), animals received an i.p. dose of 100

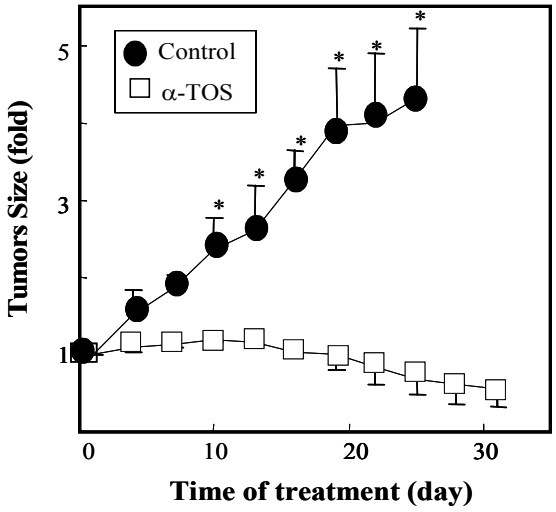

Figure 13. Effect of α-TOS on tumour size in nude mice. Male nude mice with tumours received α-TOS solubilised in DMSO or DMSO alone (control) via i.p. injection every 3 d. Tumour size was estimated using digital calipers. Irrespective of initial tumour size (3.0-7.0 mm in diameter), treatment with 100 μl of 100 μM α-TOS significantly reduced the size of tumours in nude mice compared to controls. Six animals were used in each group. The symbol '*' indicates significant differences between the control mice and mice treated with α-TOS (p<0.05). Differences in the mean relative tumour size (±S.E.M.) were examined using analysis of covariance (ANCOVA) with days as the covariate.

μl of 100 mM α-TOS (~100 mg α-TOS per kg, or 10 μmol α-TOS per animal per dose) dissolved in DMSO or the same volume of DMSO alone as control every third day. Tumour volumes were estimated by measuring the maximal length, width and height at the times indicated using digital callipers. The data obtained from this experiment revealed that α-TOS inhibited tumour growth in nude mice and caused a significant decrease in tumour volume compared to control animals (Figure 13).

2.5.10. α-TOS inhibits breast cancer growth in FVB/N c-neu mice

To determine the anti-cancer efficacy of α-TOS, transgenic mice were used that spontaneously form ductal breast carcinomas due to over-expression of erbB2 in the mammary epithelial cells [Guy et al., 1992]. Female mice that developed breast tumours were injected i.p. with α-TOS (5 or 15 μmol per mouse per dose), and treatment started when the tumours reached the volume of about 25 mm^3 as evaluated by USI). Before we started using the FVB/N *c-neu* transgenic mice (but after we finalised our experiments with the nude mice with MCF7 cell-derived xenografts), we established the cutting-edge method of tumour visualization and very precise quantification of their volume by means of USI. Figure 14 shows that treatment with 5 μmol α-TOS slowed the tumour progression by some 50%, while at 15 μmol, the VE analogue caused a significant reduction of the initial volume of breast tumour in the transgenic mice, indicating that the VE analogue also efficiently suppressed progression of breast carcinomas with high level of expression of erbB2, even causing

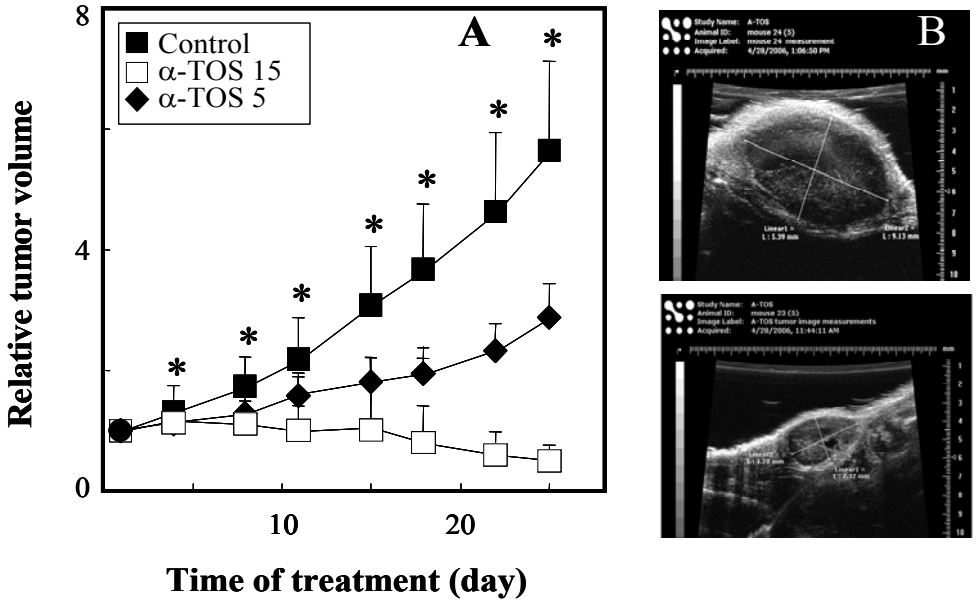

Figure 14. α-TOS inhibites breast carcinomas in *FVB/N neu* transgenic mice. Female FVB/N *c-neu* mice with small tumours were treated by i.p injection of 5 or 15 μmol α-TOS solubilized in corn oil/4% ethanol (n=11) or the excipient alone (control, n=8) once every 3 d. The tumour size was visualized and quantified using USI (A). B, representative images of tumours (d 18) treated with 15μmol α-TOS (lower image) and control (upper image) are shown. The data in panel A are mean values±S.E.M. The symbol "*"indicates significant differences between treated and the corresponding control mice (p<0.05). Differences in the mean relative tumour size (±S.E.M.) were examined using analysis of covariance (ANCOVA) with days as the covariate.

reduction of the carcinomas.

2.6 Discussion

In this chapter, we document that α-TOS and several novel VE analogues, *i.e.* α-TOM, α-TAS and α-TAM, induced apoptosis in breast cancer cell lines with

low (N2O2 and MCF7) and high (NeuD12 and MDA-MB-453) erbB2 expression. We found, as presented in Figures 6 and 7, there both the N2O2 and NeuD12 cell lines responded to the individual VE analogues in a similar manner and, as expected, α-TAM was the most efficient of them all. However, cells with lower level of expression of erbB2 appear to be slightly more susceptible toward the VE analogues, albeit this difference was not significant. Similarly, the VE analogues did not affect the cell cycle distribution of the breast cancer cell lines with high and low level of erbB2 expression. We did observe higher percentage of cells in the sub-G_0G_1 phase and, again, this percentage was similar for the cells with low and high level of erbB2, which corresponds to the apoptosis data derived from the annexin V assay. It is possible, though, that cell cycle arrest would occur with lower levels of α-TOS. We observed this earlier for mesothelioma cell lines, which underwent apoptosis at relatively high concentrations and cell cycle arrest at low levels of the agent (Tomasetti et al., 2004; Stapelberg et al., 2005). The IC_{50} values for the individual analogues of VE were completely consistent with the above data, *i.e*. they were very similar for the cells with low and high level of erbB2 expression. These results clearly indicate that over-expression of the pro-survival erbB2 protein does not compromise cultured breast cancer cells towards killing by VE analogues. Further experiments are necessary to unequivocally document whether the level of expression of erbB2 does play any role at all in susceptibility of breast cancer to VE analogues. Again, it cannot be excluded that at lower levels of the drugs, the erbB2-over-expressing cells may be less susceptible.

Previous reports strongly support the notion that mitochondria play a significant role as the major transducer of apoptosis induced by VE analogues, since they are a source of downstream apoptotic mediators and become destabilized as a result of α-TOS challenge [Weber et al., 2002; Yamamoto et al., 2000; Weber et al., 2003; Yu et al., 2003]. Therefore, we addressed the possibility that the intrinsic apoptotic signalling may be a mode whereby VE analogues bypass the anti-apoptotic effect of erbB2. It has been shown that generation of ROS is one of the early events in cells exposed to α-TOS [Weber et al., 2003; Ottino et al., 1997; Kogure et al., 2001]. As documented previously, early generation of ROS occurred in the two breast cancer cell lines tested, *i.e*. NeuD12 and N2O2 cells, as a result of their exposure to the VE analogue α-TOS. We employed here two independent methods of ROS assessment, one indirect, based on a fluorescent dye and flow cytometry, the other direct, making use of stable radical traps and EPR spectroscopy. The indirect method is useful since it allows for fast assessment of oxidative stress. However, it is not very specific, since the fluorescent probe DCF also detects secondary oxidation products. On the other hand, the direct method detects only the initially formed radical species and, moreover, spectroscopic analysis of the samples loaded with the radical trap provides information on the radical species formed, which follows from the spectrum detected.

The indirect method showed that radicals did form in the two cell lines exposed to α-TOS and that the level of radicals increased during the course of the experiment. However, as stated above, DCF and similar probes react in a rather non-specific

manner with secondary oxidation products, which explains the gradual increase in the fluorescence intensity. Therefore, we used the EPR spectroscopic method that revealed an early increase in the level of ROS (more specifically, superoxide), and this was followed by a decline in the EPR spectrum, probably due to relatively fast removal of superoxide by the endogenous MnSOD, clearly documenting generation of ROS as a rapid response to α-TOS exposure. The control EPR spectra suggest no presence of ROS, although it is known that cancer cells do produce low amounts of radicals that act as signalling molecules. The reason for not observing these ROS in the control cells is most likely due to the relatively high detection limit of the EPR spectroscopic method we used.

Based on this, we can propose that radicals are generated in the early phases of exposure of cancer cells to drugs, epitomized by α-TOS and that these species play an important role as signalling molecules triggering the apoptosis cascade. Recent results from our laboratory revealed that the target of α-TOS in cancer cells as well in other, rather specialized cells (angiogenic endothelial cells) is the mitochondrial complex II (CII; SDH, succinate dehydrogenase). More specifically, our group has shown that the VE analogue acts by displacing ubiquinone from its binding pocket in CII. Electrons that are generated by the SDH activity of CII (conversion of succinate to fumarate) cannot be intercepted by ubiquinone and interact with molecular oxygen to give rise to superoxide radicals [Dong et al., 2007, 2008, 2009], which consequently trigger the mitochondrial apoptotic pathway. EPR spectrum of breast cancer cells exposed to

α-TOS is typical for hydroxyl radicals (accumulating as DMPO-OH adducts). Therefore, the ROS generated as a response to α-TOS are either hydroxyl radicals, or, more probably, superoxide radicals (known to be produced by mitochondria [Adam-Vizi and Chinopoulos, 2006] that are converted to hydroxyl radicals.

Our data also clearly document that ROS, generated in breast cancer cells as an early response to α-TOS, are important for induction of apoptosis. This evidence is based, largely, on the finding that MitoQ, an analogue of ubiquinone modified by addition of a TPP group targeting it to mitochondria (more specifically, to the mitochondrial inner membrane, MIM), suppressed apoptosis induced in breast cancer cells by α- TOS as well as accumulation of ROS. It has been shown earlier that MitoQ prevents apoptosis involving generation of ROS as an early event [James et al, 2005], the reason being that the compound is kept in its reduced form (capable of scavenging ROS) via the mitochondrial redox chain along the MIM [Kelso et al., 2001; James et al., 2007]. Our findings with breast cancer cell lines shown here can be reconciled with studies documenting suppression of apoptosis by MitoQ induced in Jurkat cells by α-TOS [Alleva et al., 2001] and a recent report that MitoQ suppresses formation of radicals in cellular systems [Dhanasekaran et al., 2004]. We have also recently observed that MitoQ recovered SDH activity suppressed by α-TOS, most likely by having stronger affinity for the ubiquinone-binding site of CII than the VE analogue [Dong et al., 2008].

Additional data supporting the role of ROS in apoptosis induced by VE analogues come from studies showing suppression of ROS accumulation as well as induction of apoptosis following addition of SOD to the cells prior to their challenge with α-TOS. Moreover, the fact that SOD suppresses apoptosis supports the notion that the ROS generated in response to α-TOS is superoxide (more exactly superoxide anion radical). Collectively, these data present evidence for a crucial role of ROS in apoptosis induction by VE analogues and further suggest that superoxide radical anion is likely the species initially generated by the cells. Indeed, formation of superoxide radical anion was reported for smooth muscle cells as well as lung and glioblastoma cell lines exposed to α-TOS, which induced apoptotic cell death in these cell types [Kang et al., 2004]. Overall, our data provide strong support for mitochondria as both generators and targets of ROS in response to VE analogue exposure. As documented, accumulation of ROS is one of the first events in cancer cells exposed to α-TOS.

One of the early, ensuing events is dissipation $\Delta\Psi_m$ and cytosolic mobilisation of important apoptosis mediators downstream of mitochondria, in particular that of Cyt c and Smac/Diablo. We observed significant loss of $\Delta\Psi_m$ and cytosolic relocalization of both Cyt c and Smac/Diablo in breast cancer cell lines with both low and high expression of the receptor tyrosine kinase erbB2, N2O2 and NeuD12, respectively. It appears, though, that the level of dissipation of $\Delta\Psi_m$ was slightly lower for cells with higher levels of erbB2, perhaps indicating that some events accompanying apoptosis induction and progression may be affected by the level of expression of erbB2.

To account for potential differences in the expression of mitochondrial Bcl-2 family protein that may alter susceptibility of cells to VE analogues [Yu et al., 2003; Weber et al., 2003], we estimated the levels of expression of the Bcl-2, Bcl-x_L, Mcl-1 and Bax proteins in NeuD12 and N2O2 cells using flow cytometry. Overall, there was no appreciable difference in their expression in the cell lines expressing low and high levels of erbB2 (data not shown), consistent with a comparable susceptibility of the two cell lines to the VE analogues.

In the next experiments, we further investigated the role of mitochondria in apoptosis induced by α-TOS in breast cancer cells. We made use of the ρ^o phenotype of the cells, *i.e*. cells deficient in mtDNA. This phenotype can be relatively easily prepared from parental, mtDNA-proficient cells, by their maintenance for over some 5-7 doublings in the presence of sub-lethal EtBr. At this relatively low concentration, the dye intercalates preferentially into mtDNA, which, unlike genomic DNA, is not histone-protected. We then exposed the parental cells and the corresponding cells deficient in mtDNA to α-TOS and assessed them for ROS accumulation and apoptosis induction. We found that the mtDNA-deficient cells were resistant to α- TOS for both cell lines differing in the level of expression of erbB2. These findings are consistent with earlier reports [Weber et al., 2003; Higuchi et al., 1997; Dey et al., 2000; Park et al., 2004] and provide direct evidence for the essential role of fully functional mitochondria in apoptotic killing of both N2O2 and NeuD12 cells. More- over, these data provide additional evidence for the importance of mitochondria- derived ROS in induction of

apoptosis by α-TOS, since the breast cancer cell-derived ρ^o cells revealed low accumulation of ROS compared to their parental counterparts.

From the point of view of the molecular target of α-TOS, which is the ubiquinones site of CII, the data showing low level of apoptosis in the ρ^o cells may be counterintuitive, since none of the subunits of CII is coded for by the mitochondrial DNA, which codes at least one subunit of each of the other mitochondrial complexes (CI, CIII, CIV and CV). Therefore, ρ^o cells contain fully functional CII [Kwong et al., 2007]. However, ρ^o cells have been shown to be resistant to apoptosis due to low levels of accumulation of ROS, because they increase expression of the antioxidant enzymes like MnSOD and catalase as an adaptation to the stress imposed by EtBr exposure during preparation of the ρ^o cells [Park et al., 2004]. This may explain the resistance of ρ^o cells with functional CII to agents like VE analogues that trigger apoptosis via accumulation of ROS. Indeed, we now have preliminary data showing higher levels of MnSOD and catalase in ρ^o cancer cells and in cancer cells that acquire resistance to α-TOS by escalation of the dose of the agent (not shown).

One of the longer-term visions of our laboratory is to develop VE analogues into clinically attractive anti-cancer drugs. For this, we tested whether α-TOS has anti-cancer activity in two models of breast cancer. One model was based on xenografts derived from human breast cancer cells MCF7 with low level of erbB2, for the other model we used the FVB/N *c-neu* transgenic mouse that undergoes spontaneous

development of breast carcinomas due to high level of expression of the oncogenic erbB2. Importantly, we found that α-TOS was very efficient in both models of breast cancer. When applied at 10 μmol per dose per animal (low erbB2) or 15 μmol per dose per animal (high erbB2), the agent not only stabilized the tumours but caused their ~2-fold reduction. These data are extremely encouraging and indicate that VE analogues may become efficient drugs for therapy of breast cancer. In the future experiments, we will treat the animals till complete disappearance of the tumours (as evidenced using the highly sophisticated USI), and will then observe the mice for any possible recurrence of the carcinomas.

In conclusion, we show here that VE analogues have the propensity to efficiently kill breast cancer cells through apoptosis irrespective of their erbB2 status *in vitro* and suppress breast carcinomas in mouse models. We propose that the underlying mechanism is induction of apoptosis via the mitochondrial pathway. VE analogues can therefore be placed amongst a group of drugs targeting mitochondria (mitocans [Neuzil et al., 2007a,b]), an emerging paradigm for efficient anti-cancer strategy.

CHAPTER 3

A PEPTIDE CONJUGATE OF VITAMIN E SUCCINATE TARGETS BREAST CANCER CELLS WITH HIGH EXPRESSION OF ERBB2

3.1 Summary

Over-expression of the receptor tyrosine kinase erbB2 makes cancer cells resistant to apoptosis. We explored the premise that high level of erbB2 expression in cancer cells also allows their targeting by attachment of a specific peptide to an apoptogenic agent. Here we studied the possibility that the conjugate of the pro-apoptotic α-TOS and the heptapeptide LTVSPWY induces efficient apoptosis in erbB2-over-expressing breast cancer cells. Exposure of cells with low and high level of expression of erbB2 to α-TOS itself caused comparable apoptosis in all cell lines. The α-TOS-peptide conjugate induced more apoptosis in the erbB2-over-expressing cells than did α-TOS only, while the opposite was observed for cells with low erbB2. Apoptosis induced in the erbB2-over-expressing cells by LTVSPWY-α-TOS was partially suppressed by erbB2 siRNA, as well as by inhibition of endocytosis or the lysosomal function. HPLC analysis revealed fast accumulation of α-TOS in cells expressing high levels of erbB2 exposed to LTVSPWY-α-TOS. Finally, the peptide conjugate of α-TOS suppressed breast carcinomas in a transgenic mouse model with high level of erbB2. We conclude that conjugation of a specific peptide targets α-TOS to erbB2-expressing cancer cells, causing their rapid apoptosis, and propose an anti-tumour strategy that relies on targeting of anti-cancer drugs to malignant cells by their coupling to peptides recognized by receptors over-expressed in cancer cells. Such therapy will not only be efficient but will also be highly specific for cancer cells over-expressing receptors that are, as a rule, expressed at very low level in

non-malignant cells.

3.2 Keywords

α-TOS; peptide conjugate; erbB2; apoptosis; targeting; breast cancer

3.3 Introduction

Over-expression of receptor tyrosine kinases, such as that of erbB2 (a product of the *c-neu* gene), renders cancer cells resistant to apoptosis treatment and makes patients with erbB2 carcinomas a considerable therapeutic challenge [Roskoski, 2002; Slamon et al., 1989]. The major problem associated with high expression of erbB2 in cancer cells is linked to its spontaneous auto-phosphorylation and the ensuing activation of growth signalling pathways and pro-angiogenic and anti-apoptotic mechanisms.

ErbB2 is over-expressed in up to 30% of primary breast cancers. One of the major complications associated with erbB2 over-expression is linked to activation of Akt via the PI3K pathway [Zhou and Hung, 2003; Vivanco and Sawyers, 2002]. Akt is a serine/threonine kinase that promotes cellular survival [Dudek et al., 1997] due to phosphorylation of a variety of proteins including caspase 9 [Cardone et al., 1998], Bad [Datta et al., 1997], or Bax [Xin and Deng, 2005]. Akt also causes activation of NFκB [Kane et al., 1999], a pleiotropic transcription factor that controls expression of pro-survival genes, such as the

IAPs [LaCasse et al., 1998; Fukuda et al., 2002] and the caspase-8 inhibitor FLIP [Mitsiades et al., 2002].

We have studied the molecular mechanism of the pro-apoptotic and anti-neoplastic activity of VE analogues, epitomized by the redox-silent α-TOS [Neuzil et al., 2004, 2007b; Wang et al., 2006]. This agent has been proven efficient in selective induction of apoptosis in a variety of cancer cells in a selective manner [Neuzil et al., 2001b] and in suppression of cancer in several animal models [Malafa and Neitzel, 2000; Malafa et al., 2002]. We showed that α-TOS can bypass mutations or deficiencies in important tumour suppressor genes, such as p53 or $p21^{Waf1/CIP1}$ [Weber et al., 2002]. Recently, our results documented that α-TOS can also kill breast cancer cells over-expressing erbB2 [Wang et al., 2005]. Therefore, agents like α-TOS may prove efficient against breast cancer in a clinical setting, and this efficacy would be enhanced by selective delivery to malignant tissue.

A limiting point in efficient delivery of anti-cancer drugs is their uptake by malignant cells. Uptake of α-TOS is relatively slow because it, most likely, relies on passive diffusion. This follows from experiments, in which acidification of media enhanced uptake by cancer cells of α-TOS, a weak acid, because its free carboxyl group is protonated to a higher extent at lower pH, which is typical for tumour interstitium, while at the neutral pH of normal tissue interstitium α-TOS

is largely in the deprotonated (charged) form [Neuzil et al., 2002b]. We decided to explore the idea that targeting α-TOS to erbB2-over-expressing cells by generating a conjugate of the VE analogue with a specific peptide may be a way how to efficiently and selectively kill such breast cancer cells resistant to conventional treatment. We based our hypothesis on a paper by Shadidi and Sioud [Shadidi and Sioud, 2003], who, using the phage display approach, identified the heptapeptide LTVSPWY, which targeted the erbB2 anti-sense oligodeoxynucleotide to breast cancer cells and suppressed expression of the erbB2 gene. We show here that LTVSPWY-α-TOS efficiently kills breast cancer cells with high level of surface expression of erbB2. Our data suggest that peptides binding to receptors expressed on cancer cells coupled to inducers of apoptosis may efficiently target cancer cells.

3.4 Materials and Methods

3.4.1. Cell Culture

Human MCF7 breast cancer cells with low and MDA-MB-453 cells with high levels of erbB2 and MCF7/HER2 cells and MDA-MB-453_{K44A} cells stably transfected with an erbB2 vector [Shou et al., 2004; Kurokawa et al., 2000] were cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. MDA-MB-453_{K44A} cells contain the mutation in replacement of lysin 44 with alanin in the dynamin-2 protein. Function of dynamin-2 is essential for the clathrin-dependent endocytosis. Therefore, if you inhibit it, endocytosis is suppressed.

Dynamin-2 with the K44A mutation acts in a dominant-negative manner, that is it inhibits the function of the wild-type, endogenous dynamin-2 [Kashiwakura et al., 2004]. To inhibit the lysosomal function, cell medium was supplemented with 20 mM NH_4Cl that itself did not cause any cell death within the period of the experiment. In some cases, the cells were co-treated with the pan-caspase, caspase-8 or caspase-9 inhibitors, Z-VAD-FMK, Z-IETD-FMK, or Z-LEHD-FMK, respectively (all purchased from Calbiochem and used at 25 μM each).

3.4.2. Synthesis of α-TOS-peptide conjugate

The H-Tyr(t-Bu)-Trp(t-Boc)-Pro-Ser(t-Bu)-Val-Thr(t-Bu)-Leu-Rink amide was prepared according to the standard Fmoc protocol on a LIPS Vario Peptide synthesizer. All acylation reactions were carried out for 1 h using a 10-fold excess of Fmoc-amino acids activated with TBTU (1 equivalent) in the presence of DIPEA (2 equivalents) and NOBt (1 equivalent). The NH_2-terminal conjugation was carried out by activation of α-TOS with 1 equivalent of PyBOP in the presence of HOBt (1 equivalent) and DIPEA (2 equivalents). The conjugated peptide was cleaved from the resin using trifluoroacetic acid/triiso-propylsilane/water (95:2.5:2.5) for 2 h. The highly lipophilic peptide conjugate was extracted with diethylether and analysed by HPLC and electrospray-mass spectrometry (expected MH^+ ,1378.8, found MH^+ ,1378.7).

3.4.3.Assessment of apoptosis, ROS formation, and mitochondrial potential

The methods of assessment of apoptosis, ROS formation, $\Delta\Psi_m$ were the same as described in Chapter 2.

3.4.4.Western blotting

Cells were lysed in a buffer containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8), 0.1% Nonidet P-40, and a cocktail of protease inhibitors (2 μg/ml aprotinin, leupeptin and proteinin each, and 1 mM PMSF), and stored at -80°C until analysis. Protein levels were quantified using the bicinchoninic assay (Sigma). Protein samples (80 μg per lane) were boiled for 5 min and resolved using 10 % SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked (PBS containing 0.1% Tween and 5% skimmed milk) for 1 h, and incubated overnight with anti- dynamin-2 or anti- erbB2 IgG (both Santa Cruz). Anti-β-actin IgG (Santa Cruz) was used as a loading control. After incubation with an HRP-conjugated secondary IgG (Amersham), the blots were developed using the ECL kit (Pierce).

3.4.5. Cell transfection

The following plasmids were used: Bcl-x_L-EGFP [Wolter et al., 2004], and wild-type (WT) dynamin-2 and dominant-negative (DN; K44A) dynamin-2 [Kashiwakura et al., 2004]. In brief, MDA-MB-453 cells at 50-70% confluence

were supplemented with 5 μg DNA pre-incubated for 10 min with 10 μl Lipofectamine-2000 (Life Technologies) and 1 ml OptiMEM. After 3 to 4 h, cells were washed and supplemented with complete DMEM and left for 24 h, at which stage the cells were incubated with complete DMEM supplemented with the selection antibiotic G418. The cells were maintained in the selection medium for at least five passages, at which stage the cells were assessed for expression of Bcl-x_L by inspection for green fluorescence using a fluorescence microscope, showing at least 90% efficacy of transfection (not shown), or for expression of DN or WT dynamin-2 using western blotting.

3.4.6. RNA interference (RNAi)

ErbB2-specific and validated short-interfering RNA (siRNA) oligonucleotides and nonspecific scrambled siRNA were purchased from Ambion. Transfection of MDA-MB-453 cells with siRNA was performed using OligofectAmine (Invitrogen) and OptiMEM as reported elsewhere [Swettenham et al., 2005]. Briefly, cells were allowed to reach 50% confluence and supplemented with 60 pmol/l siRNA pre-incubated with OligofectAmine and overlayed with OptiMEM. Cells were washed 24 h later with PBS, overlayed with complete DMEM, cultured for additional 24 h, and the transgene expression confirmed by western blotting before using the cells for further experiments.

3.4.7. Mouse tumour experiments

Female transgenic FVB/N *c-neu* mice bearing progressively growing tumours with the mean volume of 25 mm^3 were randomly assigned to control or treatment groups. Tumour size was quantified by USI using the Vevo770 instrument and the 40 MHz RMV708 scan-head (both VisualSonics) allowing 30 μm resolution of individual scans. Mice received treatment with corn oil/4% ethanol alone (control), 5 or 15 μmol α-TOS, or 2.5 or 5 μmol LTVSPWY-α-TOS in corn oil/4% ethanol administered by i.p. injection every 3 d.

3.4.8. α-TOS analysis

To assess the intracellular level of α-TOS, a high performance liquid chromatography (HPLC) method described elsewhere [Fariss et al., 1989] was applied.

3.4.9. Endocytosis assay

Low density lipoprotein (LDL) uptake, mediated by clathrin-dependent endocytosis mediated by the LDL receptor, was assessed as reported [Jostarndt et al., 2004]. LDL was labeled with the fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylinedocarbocyanine perchlorate (DiI) for 8 h at 37℃. The MDA-MB-453 parental cells, and the WT and DN dynamin-2 transfectants at ~50% confluency were incubated with Dil-labeled LDL for 2 h at 37℃ and the level of internalized DiI-LDL estimated by flow cytometry. Endocytosis was also assessed using FITC-conjugated transferrin (FITC-Tr). In brief, FITC-Tr (Molecular Probes) at 0.1 mg protein per ml was added to cells at ~50% confluency, and the cells were

incubated in serum-free DMEM for 1 h at 37℃ and assessed for the level of fluorescence by flow cytometry.

3.4.10. Statistics

Same as Chapter 2.

3.5 Results

3.5.1. The LTVSPWY-α-TOS conjugate induces more efficient apoptosis in cells with high erbB2 than α-TOS

We studied the hypothesis that addition of a peptide, presumably recognized by the receptor tyrosine kinase erbB2, to α-TOS, targets the VE analogue to cancer cells over-expressing the receptor. To do this, we prepared a conjugate of α-TOS with the heptapeptide LTVSPWY by its addition to the free carboxylate of the succinyl domain of the VE ester. We found that cells with low and high expression of erbB2 were comparably susceptible to α-TOS itself, while the cells with low erbB2 were relatively resistant to the LTVSPWY-α-TOS conjugate, and the peptide conjugate of α-TOS was significantly more efficient in apoptosis induction in the cells with high erbB2 than was α-TOS, as documented for both human (Figure15) and mouse breast cancer cells (not shown). The LTVSPWY peptide itself did not induce any apoptosis discernible from the control (not shown). These results suggest that high level of expression of the erbB2 receptor tyrosine kinase leads to sensitisation of such cells to killing by the conjugate of

α-TOS and the heptapeptide LTVSPWY.

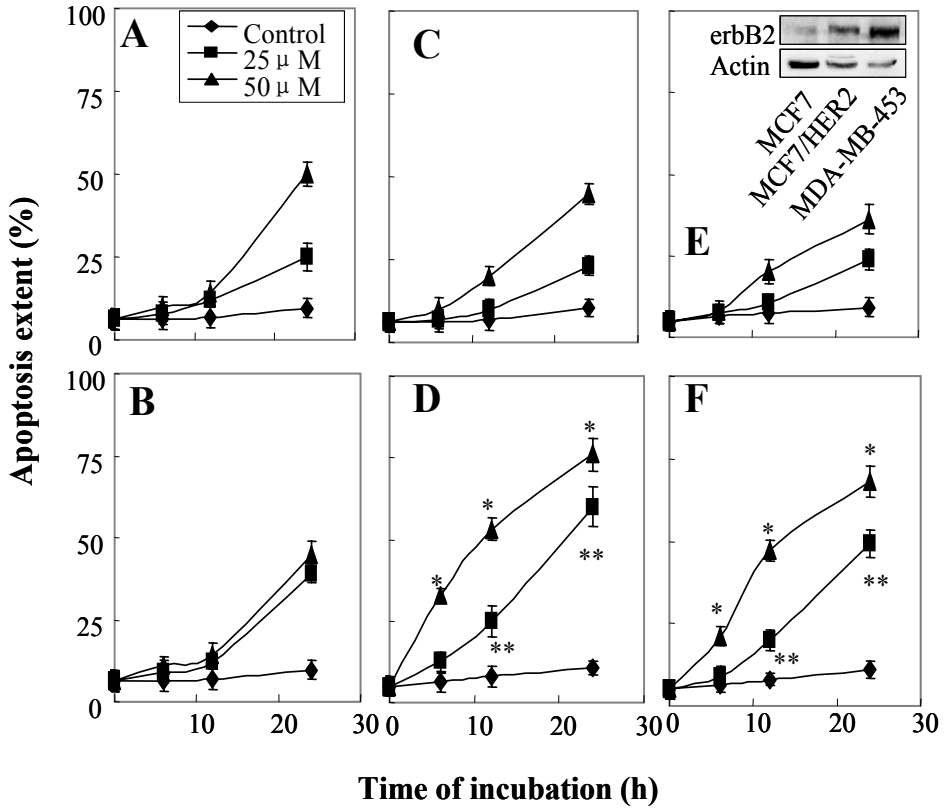

Figure 15. Induction of apoptosis in breast cancer cells exposed to α-TOS or α-TOS-LTVSPWY. MCF7 (A and B), MDA-MB-453 (C and D), and MCF7/HER2 cells (E and F) were incubated with α-TOS (A, C, and E) or LTVSPWY-α-TOS (B, D, F) at 25 or 50 μM for 6, 12, and 24 h and assessed for apoptosis using the annexin V method. Panel E, insert: level of expression of the erbB2 protein in MCF7, MDA-MB-453, and MCF7/HER2 cells were assessed by western blotting. The data are mean values±S.D (n=3). The symbol "*" indicates significantly different apoptosis in cells with low and high erbB2 exposed to α-TOS or LTVSPWY-α-TOS at 50 μM ($p<0.05$); the symbol **" indicate significantly different apoptosis in cells with low and high erbB2 exposed to α-TOS or LTVSPWY-α-TOS at 25 μM ($p<0.05$). Western blots are representative of three independent experiments.

3.5.2. Apoptosis induced by LTVSPWY-α-TOS is dependent on the intrinsic pathway

Previous reports showed that α-TOS induces apoptosis by the intrinsic, mitochondrial pathway [Neuzil et al., 2002a; Weber et al., 2003]. We tested whether mitochondria and the ensuing events are also involved in killing of MDA-MB-453 cells expressing high levels of erbB2 when exposed to LTVSPWY-α-TOS and compared the peptide conjugate with free α-TOS. We first studied the effect of LTVSPWY-α-TOS on generation of ROS and on dissipation of $\Delta\Psi_m$. Figure 16 A, B documents that the peptide conjugate caused accumulation of ROS and ΔΨm dissipation in MDA-MB-453 cells faster than did free α-TOS. The effect of LTVSPWY-α-TOS on mitochondrial destabilization and the subsequent events is due to mitochondrially derived ROS. This is supported by inhibition of ROS accumulation as well as of dissipation of ΔΨm by MitoQ (data not shown), an antioxidant targeted to the mitochondrial inner membrane that, subsequently, also inhibits apoptosis (data not shown).

We next tested whether apoptosis induced by LTVSPWY-α-TOS is dependent on caspases. Figure 16 C shows that the pan-caspase inhibitor Z-VAD-FMK as well as the caspase-9 inhibitor Z-LEHD-FMK suppressed apoptosis induction in MDA-MB-453 cells exposed to both LTVSPWY-α-TOS and α-TOS, while this was not observed when cells were co-treated with the caspase-8 inhibitor Z-IETD-FMK. This documents that caspases, activated downstream from

mitochondria, play an important role in the process.

Finally, we tested whether over-expression of the mitochondrial anti-apoptotic protein Bcl-x_L protects MDA-MB-453 cells from apoptosis induced by LTVSPWY-α-TOS. As documented in Figure 16 D, apoptosis was significantly inhibited in the erbB2-over-expressing cells transfected with Bcl-x_L when the transfectants were exposed to the peptide conjugate of α-TOS, similarly as when free α-TOS was used as an inducer of apoptosis. These data clearly document that mitochondria are important for apoptosis induction by the VE-peptide conjugate in the erbB2-over-expressing cells.

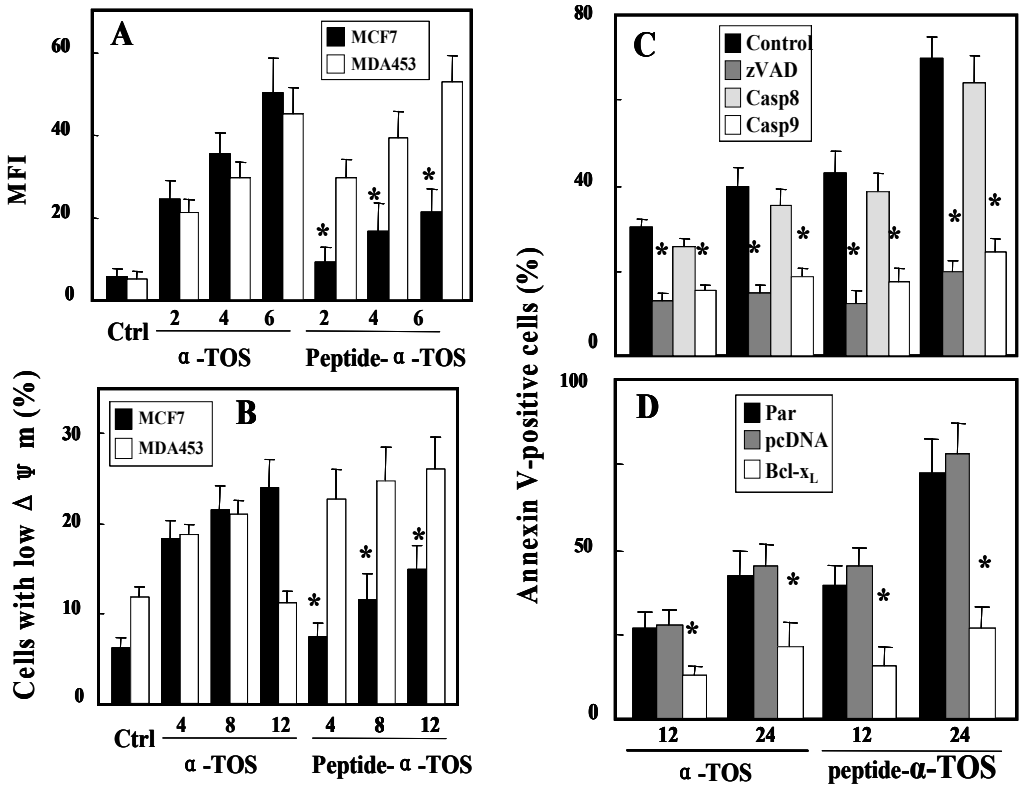

Figure 16. Apoptosis induced by α-TOS-LTVSPWY is mediated by the intrinsic pathway. MCF7 and MDA-MB-453 (MDA453) cells were exposed to 50 μM α-TOS or LTVSPWY-α-TOS for the periods (h) shown. The cells were evaluated for ROS generation using dihydroethidium (A) and for $\Delta\Psi_m$ using JC-1 (B). C, effect of the pan-caspase, caspase-8 (Casp8), or caspase 9 (Casp9) inhibitors (all 25 μM) on apoptosis induced by 50 μM α-TOS or LTVSPWY-α-TOS was evaluated by the annexin-V assay. D, effect of overexpression of Bcl-x_L on apoptosis induction by 50 μM α-TOS or LTVSPWY-α-TOS in MDA-MB-453 cells. Par and pcDNA are the parental and empty vector-transfected control cells. The data shown are mean values±S.D. (n=3). The symbol "*" indicates significantly different values obtained in MCF7 and MDA-MB-453 cells (A and B), or data significantly different from control values (C and D) (p<0.05). MFI, mean fluorescence intensity.

2.5.3. Knock-down of erbB2 impairs LTVSPWY-α-TOS-induced apoptosis

To find out whether high level of killing of MDA-MB-453 cells by LTVSPWY-α-TOS is specifically due to high expression of erbB2, we performed an experiment in which the receptor tyrosin kinase was knocked down by treatment of the cells with erbB2 siRNA. Figure 17 B shows that siRNA treatment for 48 h strongly suppressed expression of erbB2 protein in MDA-MB-453 cells. Cells with downregulated erbB2 as well as their parental counterparts were then exposed to α-TOS and LTVSPWY-α-TOS. Figure 17 A reveals that MDA-MB-453 cells pre-treated with erbB2 siRNA were more resistant to the LTVSPWY-α-TOS conjugate but not to α-TOS itself when compared to their parental counterparts. This finding documents that high level of erbB2 expression is important for efficient killing of cancer cells by the LTVSPWY conjugate of α-TOS but has no effect on apoptosis induced in the erbB2-over-expressing cells by free α-TOS.

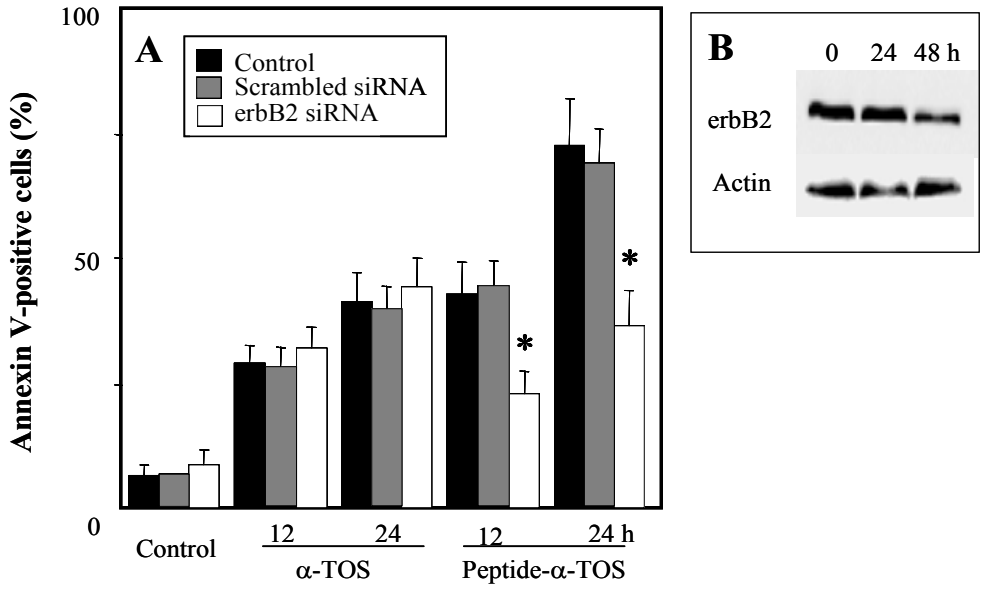

Figure 17. Knock-down of erbB2 suppresses apoptosis in MDA-MB-453 cells exposed to LTVSPWY-α-TOS. MDA-MB-453 cells were treated with the vehicle (control cells), or erbB2-specific or scrambled siRNA. A, after 24 and 48h, the cells were evaluated by western blotting for expression of the erbB2 protein (69±7% reduction in the expression of the erbB2 protein at 48 h). B, the cells exposed to erbB2 or scrambled siRNA for 48 h were treated with 50 μM α-TOS or LTVSPWY-α-TOS for 12 and 24 h, and assessed for apoptosis using the annexin V method. The data shown are mean values ± S.D. (n=3). The symbol "*" indicates statistically different data for siRNA-treated versus non-treated cells (p<0.05). Western blot images are representative of three independent experiments.

3.5.4. Apoptosis induced by LTVSPWY-α-TOS requires endocytosis and lysosomal function

A plausible explanation for more efficient apoptosis induced in erbB2-over-

expressing cells by LTVSPWY-α-TOS is that the conjugate is rapidly endocytosed by the receptor. To document this premise, we inhibited the endocytic activity of MDA-MB-453 cells by over-expression of DN dynamin-2 (MDA- MB-453_{K44A} cells) [Kurokawa et al., 2000]. Parental MDA-MB-453 cells as well as their counterparts transfected with WT dynamin-2 (MDA-MB-453_{WT} cells) or with DN dynamin-2 (MDA-MB-453_{K44A} cells) were exposed to α-TOS or LTVSPWY-α-TOS. Data in Figure 18 A clearly show that the transfected cells expressed higher levels of either the WT or DN dynamin-2, and that the resulting inhibition of endocytosis suppressed apoptosis induced in MDA-MB-453 cell by the α-TOS-peptide conjugate, while apoptosis triggered by α-TOS was comparable in the control and the endocytosis-inhibited cells.

Data in Figure 18 A also document that the erbB2-targeting peptide-α-TOS conjugate is internalised via clathrin-dependent endocytosis. This follows from the finding that DN mutations in dynamin-2, essential for completion of clathrin-dependent endocytosis, compromised the process. Two positive controls were used to support the notion that dynamin-2 is involved in clathrin-dependent endocytosis, *i.e.* cell expressing DN dynamin-2 failed to efficiently internalise both LDL and transferrin, both known to be endocytosed in a clathrin-dependent manner.

It is essential for induction of apoptosis that the peptide bond between the hepta-

peptide and α-TOS is hydrolyzed so that the VE analogue with the free carboxylate at the end of the succinyl moiety is liberated. This follows from our previous work, in which we showed that masking the free carboxylate of apoptogenic VE analogues by methylation obliterates their efficacy to kill cancer cells [Neuzil et al., 2001c; Birringer et al., 2003]. Since peptidases, mediating hydrolysis of peptide bonds, are abundant in lysosomes that form by maturation of endosomes, we tested if the lysosomal function is important for apoptosis in duction by LTVSPWY-α-TOS. A frequent way of inhibition of lysosomal activity is addition of ammonium chloride to cells that neutral-buffers the acidic pH of lysosomes, whereby rendering them non-functional. Figure 18 B reveals that inhibition of the lysosomal function resulted in suppression of apoptosis induced in MDA-MB-453 cells by LTVSPWY-α-TOS, while apoptosis induced by free α-TOS was only marginally affected. Ammonium chloride at 20 mM was non-toxic to the cells. These data provide evidence for the crucial role of lysosomes for conversion for the α-TOS peptide conjugate to the apoptogenic, free α-TOS.

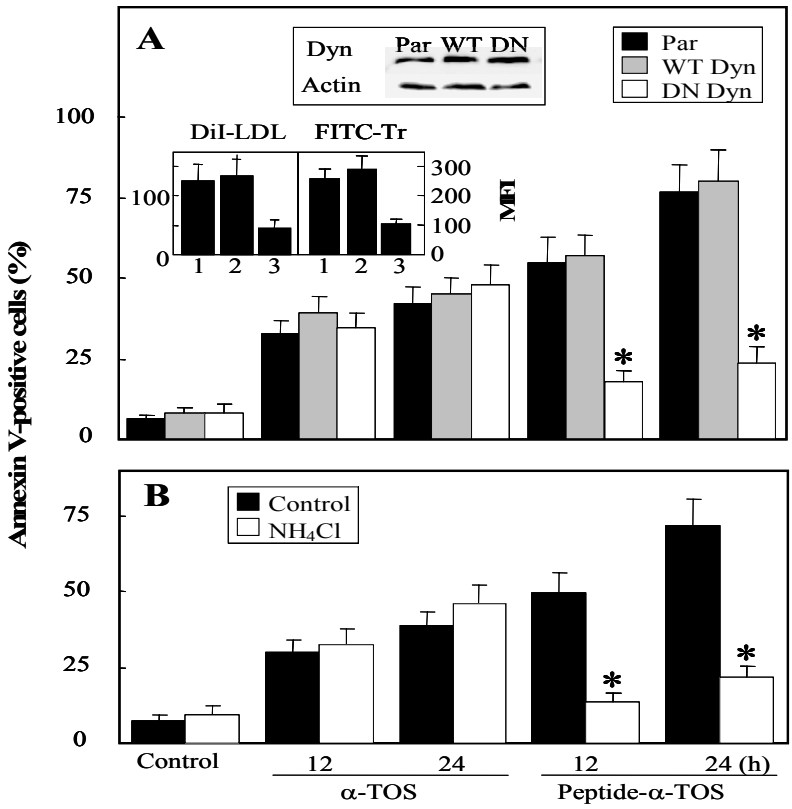

Figure 18. Inhibition of endocytosis and lysosomal function suppresses α-TOS-LTVSPWY-induced apoptosis in MDA-MB-453 cells. A, MDA-MB-453 cells or their counterparts overexpressing WT or DN dynamin-2 were exposed to α-TOS or LTVSPWY-α-TOS at 50 μM for various time intervals and evaluated for apoptosis using the annexin V method. Top inset, expression of dynamin-2 using western blotting in parental cells (Par), or cells overexpressing WT or DN dynamin-2. Bottom inset, endocytosis of Dil-labled LDL or FITC-Tr by parental MDA-MB-453 cells (column 1), or cells over-expressing WT (column 2) or DN dynamin-2 (column 3). B, MDA-MB-453 cells were either left in the original medium or medium with 20 mM NH_4CI. The cells were then exposed to the agents (50 μM) as indicated and assessed for apoptosis. The data are mean values±S.D. The symbol "*"indicates statistically different data obtained for cells with DN dynamin-2 *vs*. parental cells or cells with WT dynamin-2 *vs*. parental cells ($p<0.05$). Western blot images are representative of three independent experiments.

3.5.5. α-TOS rapidly accumulates in cells with high levels of erbB2 exposed to LTVSPWY-α-TOS

The above results strongly suggest that over-expression of erbB2 results in a fast uptake of LTVSPWY-α-TOS and in liberation of the apoptogenic α-TOS. To get more support for this notion, we evaluated the intracellular levels of free α-TOS in MCF-7 and MDA-MB-453 cells exposed to LTVSPWY-α-TOS and α-TOS, in some cases in cells with inhibited lysosomal activity or under conditions of inhibited endocytosis (using the MDA-MB-453_{K44A} cells). Figure 19 documents that MCF-7 cells accumulated α-TOS relatively fast when exposed to the free VE analogue but slowly when challenged with LTVSPWY-α-TOS. On the other hand, MDA-MB-453 cells exhibit fast kinetics of accumulation of α-TOS when exposed to LTVSPWY-α-TOS, while relatively slow accumulation of α-TOS occurred when the cells were incubated with free α-TOS. Very slow kinetics of α-TOS was observed when MDA-MB-453_{K44A} cells were exposed to LTVSPWY-α-TOS as well as when the cells were exposed to the peptide conjugate of the VE analogue in the presence of ammonium chloride.

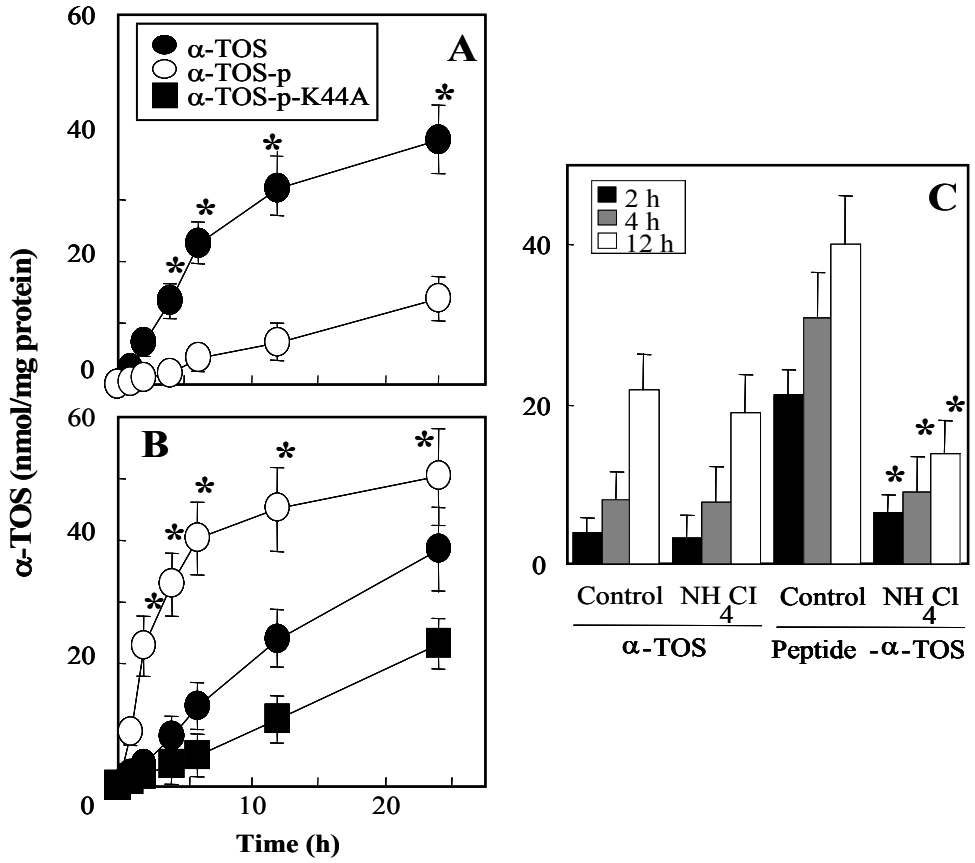

Figure 19. α-TOS rapidly accumulates in cells with high erbB2 exposed to LTVSPWY-α-TOS. MCF (A) and MDA-MB-453 or MDA-MB-453_{K44A} (B) cells were exposed to 50 μM α-TOS or LTVSPWY-α-TOS (α-TOS-p) for different periods and assessed for intracellular levels of α-TOS. C, effect of NH_4CI on α-TOS levels in MDA-MB-453 cells exposed to 50 μM α-TOS or LTVSPWY-α-TOS for indicated periods. The data shown are mean values ±S.D. (n=3). The symbol "*" indicates statistically different data of MCF7 cells exposed to α-TOS or LTVSPWY-α-TOS (A), MDA-MB-453_{WT} cells exposed to α-TOS or LTVSPWY-α-TOS, and MDA-MB-453_{K44A} cells exposed to LTVSPWY-α-TOS (B), and MDA-MB-453 cells exposed to LTVSPWY-α-TOS in the absence and presence of NH_4CI (C).

3.5.6. LTVSPWY-α-TOS suppresses breast cancer more efficiently than α-TOS in FVB/N c-neu transgenic mice

To test the anti-cancer efficacy of the peptide conjugate of α-TOS, transgenic mice were used that spontaneously form ductal breast carcinomas due to over-expression of erbB2 in the mammary epithelial cells. The mice were injected with α-TOS or LTVSPWY-α-TOS and the volume of tumours estimated using USI, which allows for precise visualisation and quantification of tumours even when embedded in the animal's tissue. The results in Figure 20 A document that the peptide conjugate of α-TOS suppressed the carcinomas with similar efficacy as the VE analogue itself did at a 3-fold higher dose (*c.f.* Figure 14). The effect of the LTVSPWY-α-TOS conjugate was significant, since we observed up to 5-fold reduction of the tumour volume over the 3 weeks of the experiment. Figure 20 B shows the results of the same experiment as in Figure 20 A when the tumour volume was estimated using callipers. Clearly, the USI approach is superior when compared with the manual assessment of tumour size using callipers, and provides data that clearly document the potential of the LTVSPWY-α-TOS conjugate as efficient agent to suppress, in a selective and efficient manner, breast cancer with high level of expression of the oncoprotein erbB2. The selectivity of the conjugate for cancer cells is supported by the observation that it had no adverse effect on the experimental animals.

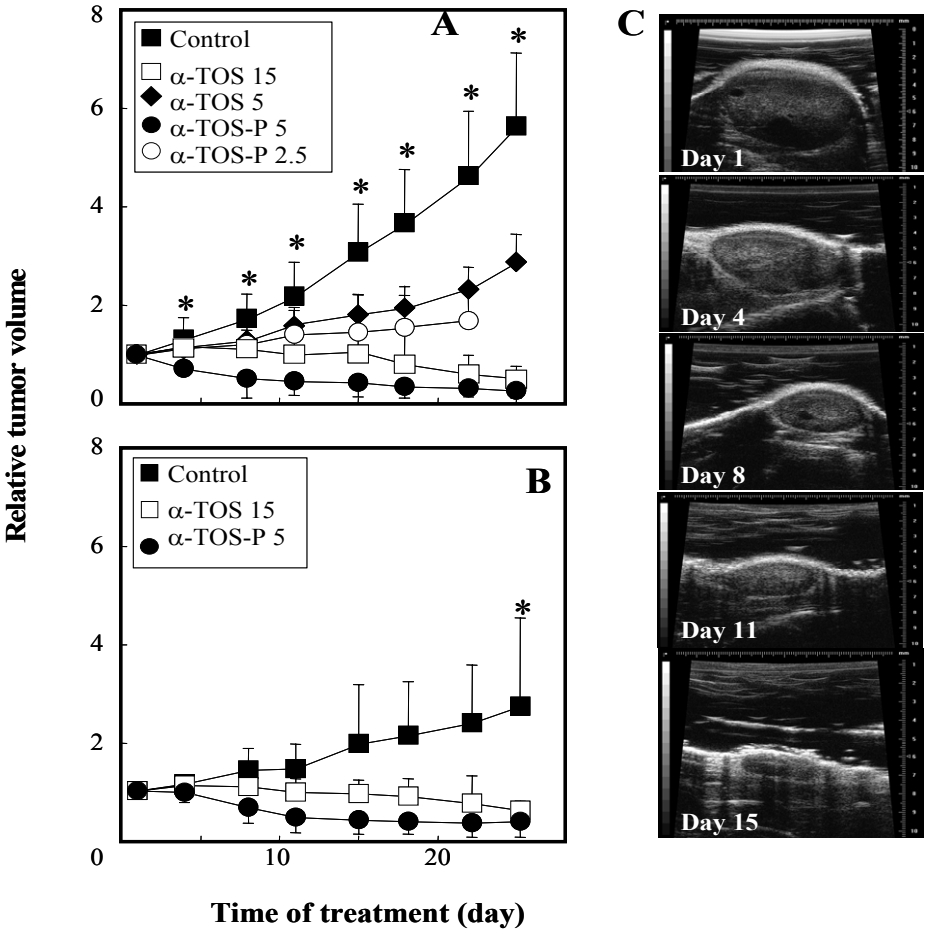

Figure 19. α-TOS-LTVSPWY suppresses breast carcinomas. FVB/N *c-neu* mice at 7 to 10 months of age with breast carcinomas detected by USI were treated by i.p injection of the vehicle, 5 or 15 μmol per mouse per dose of α-TOS, or 2.5 or 5 μmol per mouse per dose of LTVSPWY-α-TOS (α-TOS-P). The tumours were visualized and quantified using USI (A) or calipers (B), and are expressed relative to their volume at the onset of the treatment. C, representative images of a tumour from an animal treated with 5μmol α-TOS-P at the times shown. The data are mean values±S.D. (n=4-6). The symbol "*" indicates significant differences between the control animals and animals treated with α-TOS-P at 5 μmol per mouse per dose.

3.6 Discussion

In this Chapter, we have documented a paradigm, according to which modification of anti-cancer agents with peptides recognized by cell surface receptors, preferentially expressed by malignant cells, targets these agents to cancer cells, which makes the drugs more selective and efficient. This is exemplified here by modification of a strong apoptogenic compound from the group of VE analogues, α-TOS, with a peptide recognized by the tyrosine receptor kinase erbB2. We show that i) the conjugate of α-TOS and the heptapeptide LTVSPWY caused higher level of apoptosis in cells with high than low level of erbB2 expression; ii) downregulation of erbB2 partially inhibited LTVSPWY-α-TOS-induced apoptosis in cells with high erbB2; iii) apoptosis induced by LTVSPWY-α-TOS in cells with high erbB2 was dependent on both endocytosis and the lysosomal function; iv) cells with high erbB2 accumulated α-TOS at higher levels than when exposed to free α-TOS; and v) that LTVSPWY-α-TOS conjugate efficiently suppressed breast carcinomas in the *c-neu* transgenic mice.

When designing an approach how to efficiently and selectively target α-TOS to breast cancer cells with high level of expression of the receptor tyrosine kinase erbB2, we utilised results from the work by Shadidi and Sioud [Shadidi and Sioud, 2003]. These researchers identified that the heptapeptide LTVSPWY shows high affinity for breast cancer cells with higher affinity for breast cancer cells with increased expression of the erbB2 protein. The authors showed the selectivity of the heptapeptide for breast

cancer cells with high levels of erbB2 by attachment of GFP to the peptide and its internalization (as documented by intracellular green fluorescence) in cells with high erbB2. Also, they attached to the peptide erbB2 anti-sense oligodeoxynucleotides and managed to downregulate expression of the protein in breast cancer cells. This is a highly intriguing paradigm, since it can be used for targeting a host of divergent molecules to cells over-expressing specific cell surface receptors. Molecules delivered by this approach include antisense ODNs, ribozymes and siRNAs, targeting of all of which may be utilized in treatment of various pathologies without adversely affecting healthy tissues [Neuzil et al., 2001b; Tomic-Vatic et al., 2005]. There are reports that describe targeting of cytotoxic drugs to tumour cells encapsulated in carriers, including peptide-coated liposomes, as documented in both *in vitro* and *in vivo* models [Shadidi and Sioud, 2003; Stefanidakis and Koivunen, 2004].

Our results presented in this chapter are consistent with those of Song and colleagues [Song et al., 2005], who showed that an erbB2 single-chain antibody fused with protamine targeted erbB2 siRNA preferentially to erbB2-over-expressing breast carcinoma cells. Our data reveal that human and mouse breast cancer cell lines with high level of expression of erbB2 are more susceptible to killing by LTVSPWY-α-TOS than their counterparts with low levels of the protein. The difference in the level of the receptor tyrosine kinase determines to large extent susceptibility of the cells to the peptide conjugate of α-TOS. However, we also observed some, albeit lower level of apoptosis induced in breast cancer cells with low levels of erbB2 when

exposed to the LTVSPWY-α-TOS conjugate. This suggests that the heptapeptide is somewhat promiscuous, *i.e*. binding also to other receptors than erbB2, although with lower efficacy. Notwithstanding this notion, the level of expression of erbB2 does allow for at least some selectivity of killing by the peptide conjugate, and we would expect that normal cells, showing low erbB2 expression, will not be efficiently killed by the peptide-VE analogue conjugate.

Our results with cells in which endocytosis was blocked by over-expression of DN dynamin-2 suggest that internalisation of LTVSPWY-α-TOS by breast cancer cells is dependent on endocytosis via clathrin-coated vesicles, since dynamin-2 is essential for the formation and pinching off of a clathrin-coated vesicle and its conversion into an endosome [Kashiwakura et al., 2004]. Therefore, it can be postulated that after binding to erbB2 and/or another receptor, the peptide with the drug is endocytosed. In the acidic late endosome, the receptor is detached from the peptide-α-TOS conjugate, which travels to the lysosome, where acidic peptidases liberate α-TOS from its binding to the peptide. This is an important event in processing of the peptide-α-TOS conjugate by the cell, since free carboxylate is required for pro-apoptotic activity of α-TOS [Neuzil et al., 2001c; Birringer et al., 2003; Tomic-Vatic et al., 2005]. The free VE succinate then induces apoptosis by targeting mitochondria, leading to ROS generation as well as dissipation of $\Delta\Psi_m$, followed by activation of caspases and entry of the cell into the commitment phase [Weber et al., 2003; Yu et al., 2003; Wang et al., 2005].

Importantly, efficient uptake of the peptide conjugate of α-TOS by cancer cells resulted in pronounced suppression of breast carcinomas, as documented by experiments with the *c-neu* transgenic mice expressing high levels of erbB2 in the mammary epithelial cells [Guy et al., 1992]. Data in Figure 20 reveal that the peptide conjugate was some 3-fold more efficient than when free α-TOS was injected in the animals. This may be due to both more selective uptake by cancer cells with high erbB2 as well as due to higher efficacy of the conjugate to induce apoptosis. This result is suggestive of the pharmacological potential of this approach and, potentially, can be adapted to other peptides and cytotoxic agents.

In summary, we present in this chapter a paradigm, according to which an inducer of apoptosis, epitomized here by α-TOS, is modified by addition of the peptide LTVSPWY. The conjugate is rapidly endocytosed by the erbB2-over-expressing cells and internalised within endosomes. Their maturation into lysosomes results in hydrolysis of the peptide-α-TOS conjugate by the action of acidic peptidases. The liberated α-TOS compromises the mitochondrial function, which includes generation of ROS and dissipation of $\Delta\Psi_m$. This results in activation of the mitochondria-specific caspase-9 and entry of the cell into the apoptosis commitment phase.

To conclude, we present an approach useful for efficient cancer management, based on specific malignant cell targeting of anti-cancer drugs, as epitomised here by an apoptogenic VE analogue and breast cancer cells with high level of expression of

erbB2. Figure 21 shows the molecular mechanism of apoptosis induction in cancer with high level of erbB2 by LTVSPWY-α-TOS.

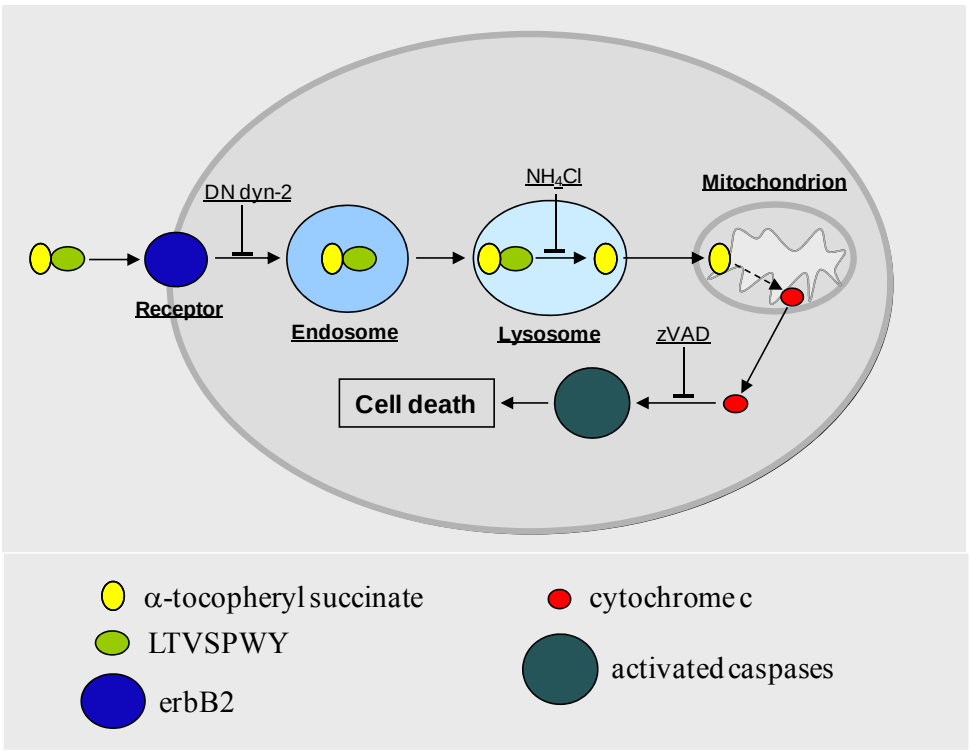

Figure 21. Molecular mechanism of apoptosis induction in cancer cells with high level of erbB2 by LTVSPWY-α-TOS. The conjugate of LTVSPWY-α-TOS binds to the receptor, presented in this case by the tyrosine receptor kinase erbB2. This results in endocytosis of the conjugate in clathrin-dependent vesicles, a step inhibited by over- expression of DN dynamin-2. Following maturation of the endosome into a secondary lysosome, the LTVSPWY-α-TOS is hydrolysed to yield free α-TOS, a step that is inhibited by neutralisation of the acidic milieu of the lysosome using, for example, NH_4Cl. The liberated α-TOS then destabilises mitochondria with ensuing cytosolic relocation of cytochrome c that, in turn, activates caspases (a step inhibited by the pan-caspase inhibitor zVAD), at which stage the cell enters the commitment phase.

CHAPTER 4

α-TOS SYNERGISES WITH TRAIL VIA THE NFκB PATHWAY IN KILLING ERBB2-EXPRESSING BREAST CANCER CELLS

4.1 Summary

The receptor tyrosine kinase erbB2 renders cancer cells resistant to apoptosis treatment and α-TOS has been shown to suppress several types of cancer. In this chapter, we document that α-TOS significantly inhibits nuclear translocation of the transcription factor NFκB in erbB2-over-expressing breast cancer cells, which is expected to result in inactivation of NFκB. We also show that α-TOS inhibits the expression of the anti-apoptotic molecules FLIP and c-IAP1 in breast cancer cells with high level of erbB2 expression. A synergism of α-TOS and TRAIL in inducing apoptosis was observed in breast cancer cells. Our results suggest that α-TOS mediates breast cancer cells apoptosis and synergies with TRAIL, which is likely associated with inhibition of activation of NFκB.

4.2 Keywords

erbB2; α-TOS; breast cancer; NFκB; TRAIL

4.3 Introduction

Over-expression of the erbB2 proto-oncogene correlates with poor prognosis in breast cancer patients [Slamon et al., 1987; Hung et al., 1992; Zhou et al., 2000]. The major complication associated with erbB2 over-expression is linked to activation of downstream signalling pathway and activation of Akt [Zhou et al., 2000; Vivanco and Sawyers, 200; Dudek et al., 1997]. Akt causes activation of the transcriptional factor NFκB [Kane et al., 1999]. In most non-transformed

cells, NFκB complexes (a heterotrimer composed of p50 and p65 subunits bound to an inhibitor subunit IκB) are largely cytoplasmic. Activation of NFκB leads its translocation to the nucleus and binding to promoter regions of specific pro-survival genes, such as those coding for IAPs [LaCasse et al., 1998], the caspase-8 inhibitor FLIP, or the TRAIL decoy receptor DcR1. Therefore, agents that inhibit NFκB activation in cancer cells with erbB2 over-expression are of clinical interest.

VE analogues, a novel class of compounds with strong pro-apoptotic activity, epitomized by α-TOS [Neuzil et al., 2004], have been shown to trigger apoptosis in a variety of cancer cells without toxicity to normal cells [Neuzil et al., 2001b; Weber et al., 2002]. We have recently found that α-TOS induces comparable apoptosis in breast cancer cells regardless of the erbB2 status [Wang et al., 2005].

TRAIL is a potent selective anti-cancer agent as shown using pre-clinical models. Some types of tumour cell lines possess intrinsic or acquired resistance to TRAIL. One reason why some types of cancer cells are resistant to TRAIL is that a transient NFκB activation is induced when the cells are exposed to the death ligand. A cooperative pro-apoptotic effect of α-TOS with TRAIL was observed *in vitro* and *in vivo* [Weber et al., 2002]. α-TOS, by inhibiting the TRAIL-induced transient NFκB activation, which in turn inhibits expression of pro-survival proteins (for example IAPs) that confer resistance of cells to TRAIL-induced apoptosis, may have a role in

adjuvant therapy of TRAIL-resistant cancers, and the agent was shown to synergize with TRAIL by promoting the mitochondrial apoptotic pathway [Weber et al., 2002]. α-TOS can also act by boosting the immune system, whose cells can very efficiently kill cancer cells by secretion of death ligands, including TRAIL [Tomasetti and Neuzil, 2006].

In this part of the work, we found that α-TOS inhibited nuclear translocation of NFκB in erbB2-over-expressing breast cancer cells. We also document here a synergism of α-TOS and TRAIL in the induction of apoptosis for breast cancer cells with both low and high expression of erbB2. Our data suggest that α-TOS mediates breast cancer cells apoptosis and synergies with TRAIL via inhibiting NFκB activation. α-TOS may therefore be useful for treatment of breast cancer cells with high expression of erbB2 or can be used for adjuvant therapy where the VE analogue is combined with the immunological apoptogen TRAIL, and both options are of clinical relevance.

4.4 Materials and methods

4.4.1. Cell culture and treatment

Human breast cancer cell lines MCF7 with low erbB2 expression and MDA-MB-453 with high erbB2 expression were routinely cultured in DMEM with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37^{o}C in a humidified atmosphere of 5% CO_2. At approximately 50-70% confluence, cells were treated with up to 50 µM α-TOS and/or 30 ng/ml human recombinant TRAIL [Weber et

al., 2002]. After exposure to the inducers of apoptosis for the required times, cells were harvested and assessed using different methods (see below).

4.4.2. Western blot analysis

4.4.2.1. Whole cell lysis

Cells were lysed in a buffer containing 250 mM NaCl, 25 mM Tris-HCl (pH 7.5), 5 mM EDTA (pH 8), 0.1% Nonidet P-40, and a cocktail of protease inhibitors (2 μg/ml aprotinin, leupeptin and proteinin each, and 1 mM PMSF), and stored at -80°C until analysis.

4.4.2.2. Nuclear and cytoplasmic fractionation

Cells were lysed with buffer A (10 mM HEPES pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) for 20 min on ice and then centrifuged at 14,000 rpm for 5 min. The supernatant was used as the cytoplasmic extract. After the pellet had been washed with buffer A, it was lysed with buffer B (50 mM HEPES pH 7.8, 50 mM KCI, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 25% glycerol) for 20 min and centrifuged at 14,000 rpm for 5 min. The supernatant was used as the nuclear extract.

4.4.2.3. Immunoblot analysis

Equivalent amounts of protein were resolved by 10% SDS-PAGE, and transferred to nitrocellulose membranes. After the membrane was blocked in Tris-buffer saline containing 0.05% Tween 20 (TBST) and 5% non-fat powdered milk, the membranes were incubated with primary antibodies (all from Santa Cruz). After washing three times with TBST for 10 min each, the membrane was incubated with HRP-labelled secondary antibody. The membranes were washed again, and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham, USA).

4.4.3. Assessment of Apoptosis

See Chapter 2.

4.4.4. RNA interference (RNAi)

Downregulation of erbB2 was performed as described in Chapter 3.

4.4.5 RT-PCR

Total RNA was extracted from cells by using Trizol Reagent (Invitrogen), and reverse transcription was carried out using AMV reverse transcriptase (Invitrogen) and oligo(dT)18 primer. 3 μl of the cDNA was used for amplification and PCR was run for 30 cycles. The PCR primers are as follows: c-IAP1 forward, 5'-GAA TAC TCC CTG TGA TTA ATG GTG CCG TGG-3'; reverse, 5'-TCT CTT GCT TGT AAA GAC

GTC TGT GTC TTC-3'; FLIP forward, 5'-CAT ACT GAG ATG CAA GAA TT-3'; reverse, 5'-GCT GAA GTC ATC CAT GAG GT -3'; β-actin forward, 5'-TGA CGG GGT CAC CCA CAC TGT GCC -3'; reverse, 5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG -3'.

4.4.6 Immunocytochemistry

Cells were placed on sterile coverslips in 12-well plates overnight, fixed with 100% methanol for 3 min, 1 % H_2O_2-methanol for 5 min, 95% methanol for 3 min and 50% methanol for 3 min. The cells were then exposed to anti-erbB2, anti-Akt and anti-pAkt IgG (all Santa Cruz) followed by biotinylated secondary antibody. After washing with PBS, the cells were incubated with streptavidin-HRP. Finally, the protein expression was detected by DAB solution.

4.5 Results

4.5.1. α-TOS inhibits NFκB nuclear translocation in breast cancer cells

We assessed the level of erbB2 and phosphorylated Akt (p-Akt) in MDA-MB-453 and MCF7 cells and documented that both cell lines express erbB2, pAkt and similar levels of Akt (Figure 22 A, B). This is consistent with the notion that expression of erbB2 translates to activated Akt. One of the results of Akt activation is activation of NFκB, which controls expression of a variety of anti-apoptotic genes. We found a decrease of the p65 protein (a subunit of NFκB) in the nuclear fraction and its

increase in the cytoplasmic fraction in both cell lines after their treatment with α-TOS (Figure 22 C). We therefore conclude that α-TOS inhibits translocation of NFκB from the cytoplasm to the nucleus in breast cancer cells with high erbB2 expression.

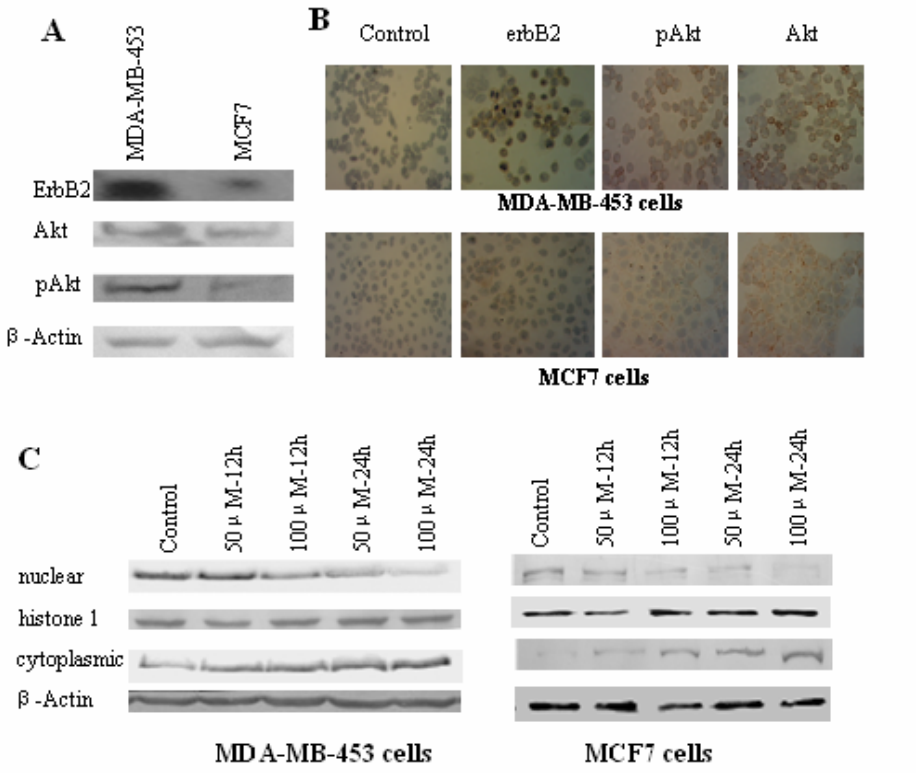

Figure 22. α-TOS inhibits p65 nuclear translocation in breast cancer cells. A. MDA-MB-453 and MCF7 cells were lysed and the whole cell lysate was probed by immunoblotting for the levels of erbB2, Akt and p-Akt. B. The expression of erbB2, Akt and p-Akt was detected by immunocytochemistry in MDA-MB-453 and MCF7 cells. C. Both cell lines were treated with α-TOS as indicated. At the times shown, nuclear and cytoplasmic fractionations (60 μg protein per sample) were resolved on a 10% polyacrylamide gel and probed by immunoblotting for the expression of p65. Histone 1 was used as a loading control for the nuclear fraction, actin for the cytosolic fraction. The images are representative of three independent experiments.

4.5.2. α-TOS suppresses c-IAP1 and FLIP expression in breast cancer cells

NFκB controls expression of pro-survival genes, such as members of the IAP family [LaCasse EC et al., 1998] and the caspase-8 inhibitor FLIP [Kim and Lee, 2005]. This prompted us to investigate the effects of α-TOS on expression of the IAP and FLIP proteins. We detected the expression of c-IAP1, c-IAP2 (both are members of the IAP family) and FLIP in the cells. Exposure to α-TOS suppressed the expression of c-IAP1 and FLIP (Figure 23), and had no effect on c-IAP2 expression (data not shown). Figure 23 A shows that α-TOS reduced the level of c-IAP1 mRNA in MDA-MB-453 and MCF7 cells in a dose- and time-dependent manner. Figure 23 B documents that α-TOS reduced the c-IAP1 protein expression in both cell lines. The decrease of FLIP mRNA amounts was also found in MDA-MB-453 (Figure 23 C) and MCF7 (data not shown) cells after treating with α-TOS. These data indicate that α-TOS suppresses the expression of c-IAP1 and FLIP in on the level of transcription.

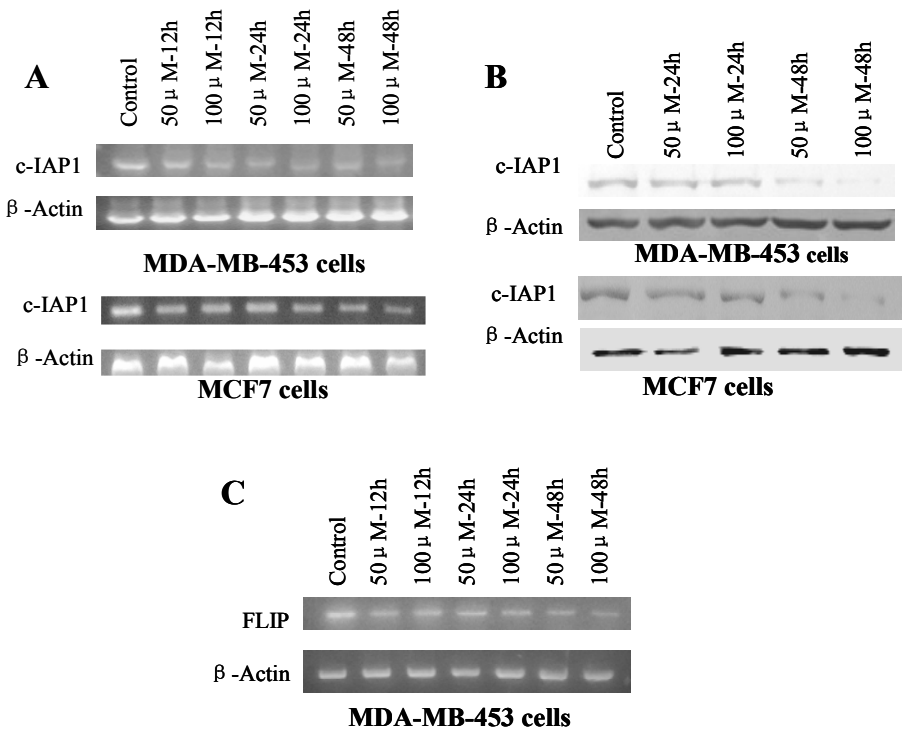

Figure 23. α-TOS inhibits c-IAP1 and FLIP expression in breast cancer cells. MDA-MB-453 and MCF7 cells (as shown) were exposed to a-TOS at times and concentrations shown and assessed for the expression of c-IAP1 mRNA by RT-PCR (A) and c-IAP1 protein by western blotting (B), and for the expression of FLIP mRNA by RT-PCR (C). The images are representative of at least three independent experiments.

4.5.3. Additive apoptosis effect of α-TOS and TRAIL

Although TRAIL is a potent anti-cancer agent, it is reported that breast cancer cells with erbB2 expression are resistant to TRAIL [Liu et al., 2008]. Figure 24 shows that MCF7 and MDA-MB-453 cells exerted low susceptibility to TRAIL-induced apoptosis, since both cell lines express erbB2 (albeit the level of erbB2 is relatively low in MCF7 cells). However, both cell lines are sensitive to α-TOS treatment. We

tested, whether treatment with TRAIL and a-TOS together was more efficient in killing the relatively TRAIL-resistant cells. Figure 24 documents an additive effect of α-TOS and TRAIL in both cell lines. We further investigated whether the expression of erbB2 ss the major reason for low susceptibility of the cells to TRAIL. We therefore downregulated the level of the protein using siRNA in MDA-MB-453 cells that express higher levels of erbB2 than MCF7 cells. Figure 24 C documents that knocking down the erbB2 protein sensitized the cells to TRAIL-induced apoptosis, which is consistent with the idea that erbB2 suppresses sensitivity to TRAIL killing.

4.6 Discussion

The transcriptional factor NFκB controls expression of pro-survival genes, such as the caspase inhibitors IAPs [LaCasse EC et al., 1998] and FLIP [Kim and Lee, 2005]. In non-malignant cells, NFκB complexes (a heterotrimer usually composed of the p50 and p65 subunits bound to the inhibitor subunit IκB) are largely cytoplasmic. Activation of NFκB results in proteasomal degradation of the inhibitory subunit IκB and nuclear translocation of NFκB. The activated NFκB then binds to the cognate promoters of genes controlled by the transcription factor, resulting in their expression. As mentioned, many of these genes express anti-apoptotic proteins, such as FLIP and the IAPs. Therefore, inhibition of NFκB activation may be of great benefit for efficient killing of resistant cancer cells, such as those exerting higher with erbB2 expression. In this regard, we hypothesized that α-TOS, may meet this premise. One of the reasons to believe so was the finding that α-TOS killed breast cancer cells

irrespective of their level of erbB2 [Wang et al., 2005] and that Akazawa and colleagues also documented that α-tocopheryloxybutyric acid, a compound analogous to α-TOS efficiently killed breast cancer cell with high level of erbB2 [Akazawa et al., 2002], although the mechanism was not clarified.

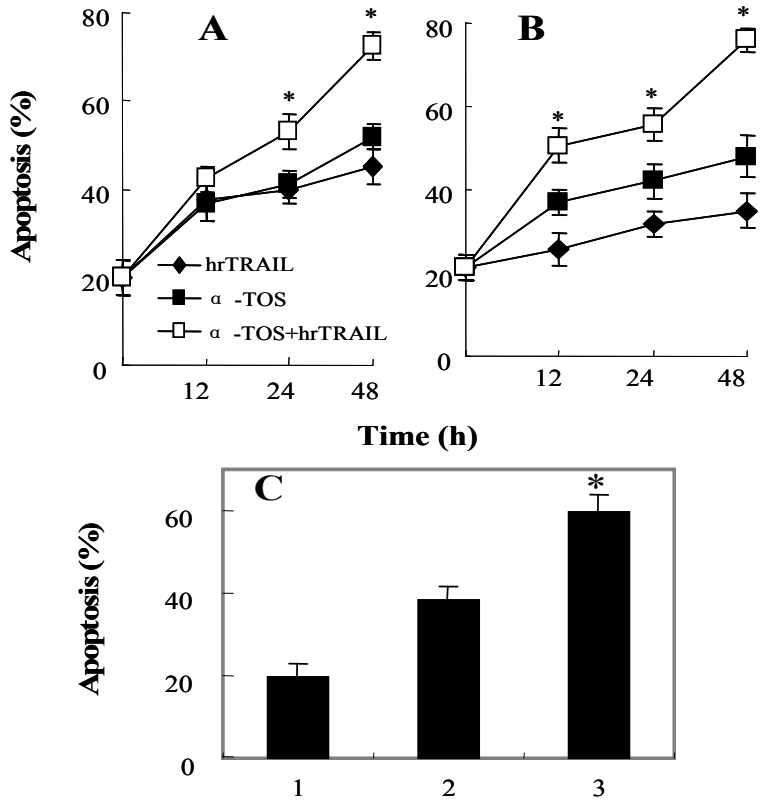

Figure 24. Additive effect of α-TOS and TRAIL in induction of apoptosis in breast cancer cells expressing erbB2. MCF7 (A) and MDA-MB-453 (B) cells were treated with TRAIL (30 ng/ml) and/or α-TOS (50 μmol/L) and for the times periods indicated. Apoptosis was assessed using the annexin V method and evaluated on the basis of percentage of annexin V-positive cells scored by FACS analysis. Data shown are mean values±SD (n=5-8). The symbol "*" documents significant difference in apoptosis in cells exposed to TRAIL + α-TOS *vs*. cells exposed to TRAIL or α-TOS (p<0.05). (C) Apoptosis induction in control MDA-MB-453 cells (1), and cells pre-treated with non-silencing (NS) (2) or erbB2 siRNA (3) and exposed for 48 h to TRAIL at 30 ng/ml. The data are derived from three independent experiments and presented as mean values±S.D. (n=3). The symbol "*" documents significant difference in apoptosis in cells pre-treated with erbB2 siRNA to TRAIL *vs*. cells pre-treated with NS siRNA (p<0.05).

We therefore decided to study whether α-TOS affects the localization of NFκB using antibody against p65, a compulsory subunit of NFκB. We found that exposure of MDA-MB-453 and MCF7 cells to α-TOS resulted in relative translocation of NFκB from the nuclear compartment to the cytosol. This indicates that α-TOS likely inhibits the activity of NFκB in the breast cancer cells with different levels of expression of erbB2, and this is reminiscent of a paper reporting on inhibition of activation of NFκB by α-TOS in the context of cardiovascular disease [Erl W et al., 1997]. Akazawa and colleagues suggested that α-tocopheryloxybutyric acid acted by suppressing auto-phosphorylation of erbB2 [Akazawa A et al., 2002]. We have now preliminary data that this could be also one of the mechanisms behind inhibition of NFκB in erbB2-expressing breast cancer cells. Further, we have preliminary data showing that α-TOS suppresses activation (phosphorylation) of PI3K and Akt in breast cancer cells. It ought to be stressed that more work needs to be done before we understand in detail the molecular mechanism, which underlies inhibition of NFκB activation in breast cancer cells with higher level of erbB2. Notwithstanding, a pattern is being formed suggesting that the level of inhibition of NFκB by α-TOS may depend on the level of erbB2 expression; that is, the agent appears more efficient in the cell lines that exert higher level of expression of the tyrosine receptor kinase.

TRAIL has been reported to induce apoptosis selectively *in vitro* and *in vivo* [Bonavida B et al., 1999; French and Tschopp, 1999]. A previous study showed that TRAIL-activated caspase-8 can generate the truncated Bid protein, which triggers the

release of Cyt c from mitochondria, leading to the assembly of the apoptosome with ensuing activation of effector caspases. TRAIL can also, following activation of caspase-8, induce direct activation of effector caspases [Zou et al; 1999]. In both cases, casepase-8 activation is inhibited when the cancer cells upregulate the expression of the protein FLIP. When mitochondria are involved in TRAIL-induced killing, the IAPs, often upregulated in cancer cells, counteract the apoptogenic signalling. Although TRAIL is a potent anti-cancer agent, it is known that certain tumour cells possess intrinsic or acquired resistance to the immunological apoptogen. Trauzold and colleagues demonstrated that treatment of cells with TRAIL resulted in strongly increased distant metastasis of pancreatic tumours *in vivo* [Trauzold A et al., 2006].

We show here that MCF7 and MDA-MB-453 cells were relatively resistant to TRAIL-induced apoptosis (Figure 24). Moreover, we document that α-TOS exerted an additive effect with TRAIL in both cell lines (Figure 24). Our results are consistent with previous studies in colon cancer cells [Weber et al., 2002], where synergy of TRAIL and α-TOS was reported. Similarly, a synergistic effect of TRAIL and α-TOS was observed in mesothelioma cells [Tomasetti et al., 2004]. In these cells, α-TOS caused increased expression of TRAIL's death receptors (both DR4 and DR5). The different mode of action for α-TOS in sensitizing the cells to TRAIL may be due to the fact that the mesothelioma cell lines used in the study expressed very low levels of erbB2. Therefore, sensitization of cancer cells to TRAIL-induced killing by α-TOS by

inhibition of activation of NFκB may be specific for cells expressing higher levels of erbB2. Our results indicating that α-TOS may sensitize breast cancer cells expressing relatively low or high erbB2 to TRAIL-induced killing by suppression of NFκB activation can be reconciled with a recent report by Dalen and Neuzil [Dalen and Neuzil J, 2003]. The authors showed in their paper that α-TOS suppressed transient resistance to TRAIL by interfering with NFκB activation [Dalen and Neuzil, 2003]. Another publication reported on an additive effect of α-TOS with TRAIL, the reason being that the two inducers of apoptosis utilise two different apoptotic signalling pathways, converging at the level of caspase-3 [Weber et al., 2002].

Data shown in this chapter, supported by other reports (see above), point to the following mechanism by which α-TOS can overcome resistance of cancer cells with higher level of erbB2 to apoptosis (Figure 25). The agent suppresses the erbB2-dependent activation of NFκB. At this point in time, it is not clear at which level this occurs: it could be via inhibition of erbB2 auto-activation as shown elsewhere [Akazawa et al., 2002]. This then would translate to inhibition of activation of the central pro-survival factor Akt and /or NFκB activation. Ultimately, this would relay to lower levels of expression of the caspase inhibitors FLIP and IAPs, and to opening of apoptotic pathways to α-TOS itself as well as sensitising the cancer cells to other inducers of apoptosis. Regardless of the precise mechanism, inhibition of NFκB activation by α-TOS has a pro-apoptotic effect, and can also be utilised in adjuvant cancer therapy.

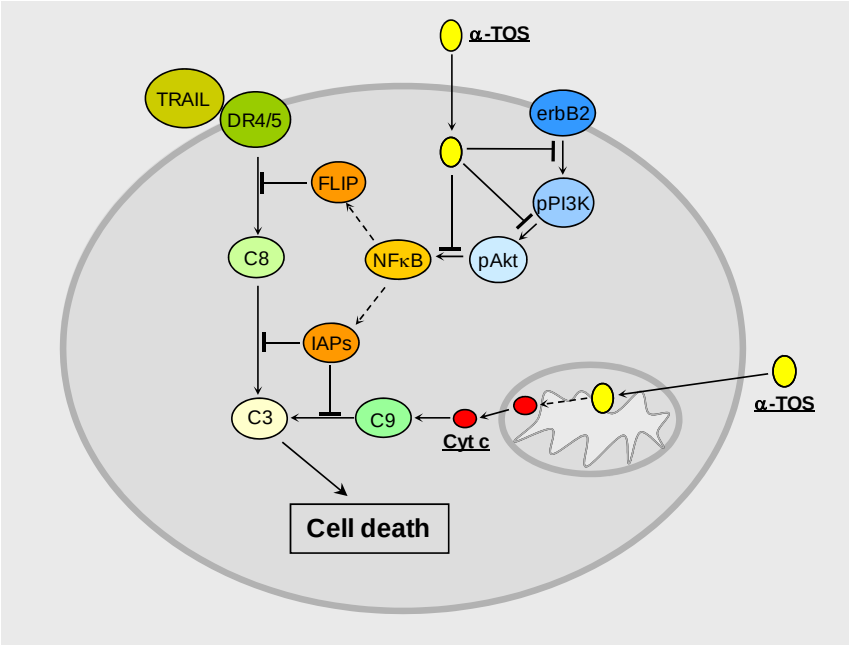

Figure 25. Possible molecular mechanisms by which α-TOS overcomes resistance of erbB2-over-expressing cells to apoptosis and sensitizes them to TRAIL. Expression of erbB2 leads to activation of the central survival protein Akt. The kinase activates a variety of substrates including NFκB that causes increased expression of caspase inhibitors FLIP and IAPs, which suppress both death receptor-mediated (extrinsic) and mitochondria-dependent (intrinsic) apoptosis. We propose that α-TOS suppresses activation of NFκB by a mechanism that may include inhibition of erbB2, PI3K or Akt activation. This results in lowering of the expression of FLIP and IAPs, and in opening the apoptotic signalling for α-TOS itself as well as sensitising the cells to killing by TRAIL. C3, C8 and C9: caspase-3, caspase-8 and caspase-9, respectively.

CHAPTER 5

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

The focus of this thesis was several fold:

1. Understand better the molecular mechanism of apoptosis triggered by VE analogues, epitomised by the redox-silent α-TOS and several novel VE analogues [Neuzil et al., 2007b] in breast cancer cell lines with low and high level of expression of erbB2.

2. Design a system for efficient and cancer cell-selective delivery of VE analogues, in particular α-TOS, into breast cancer cells with enhanced expression of erbB2.

3. Study a possible mechanism by which VE analogues could efficiently overcome resistance of erbB2-over-expressing breast cancer cells that would result in sensitisation of the cells to apoptosis inducers.

The data presented here answer, to large extent, the three major points above. We document that VE analogues trigger the mitochondrial (intrinsic) apoptotic pathway, which includes generation of ROS, dissipation of the mitochondrial inner trans-membrane potential, cytosolic translocation of apoptotic mediators (Cyt c, Smac/Diablo) and activation of caspases. Importantly, the data clearly show that the level of erbB2 protein, known to promote anti-apoptotic/pro-survival signaling, does not compromise the efficacy with which VE analogues kill breast cancer cells. In fact, we did observe slightly higher resistance to VE analogues in breast cancer cells with high levels of erbB2, although the difference was non-significant. It is possible that apoptosis in such cells is slightly delayed, since the high level of erbB2 causes spontaneous auto-phosphorylation of the receptor kinase, which translates into

activation of Akt and NFκB, followed by increased levels of the caspase inhibitors FLIP and IAPs. Since α-TOS suppresses activation of NFκB in erbB2-over-expressing cells, which results in lower expression of the anti-apoptotic proteins FLIP and IAPs [Neuzil et al., unpublished data], the drug opens the pathways for apoptosis it induces itself as well as it does sensitise such cells to other apoptogens, similarly perhaps as we found for the death ligand TRAIL. Moreover, the fact that VE analogues cause cytosolic translocation of Smac/Diablo, a protein that compromises the anti-apoptotic activity of IAPs, indicates further promotion of apoptosis induced by these agents.

Recent data from our laboratory clearly document that VE analogues trigger apoptosis in cancer cells by targeting the mitochondrial CII. More specifically, the drugs have the propensity to displace ubiquinone from its binding site in CII. By doing so, electrons that are generated during conversion of succinate to fumarate on CII cannot be intercepted by their natural acceptor, ubiquinone, and interact with molecular oxygen to give rise to superoxide [Dong et al., 2007, 2008, 2009]. This radical species then causes destabilisation of the MOM that leads to cytosolic translocation of apoptotic mediators Cyt c and Smac/Diablo. The mechanism of this destabilisation is the focus of ongoing experiments of our laboratory. We have recently proposed a hypothesis, based on preliminary data and work of others, how such destabilisation may be mediated [Neuzil et al., 2006]. According to this hypothesis (supported also by our preliminary data), superoxide causes dimerisation of the protein Bax by

catalysing a disulphide bond between its monomers, accompanied by exposure of the trans-membrane domain of the protein and its movement into mitochondria to form a channel in the MOM. Superoxide, generated in breast cancer cells in response to their challenge to VE analogues also causes FoxO-dependent transcriptional upregulation of the BH3-only protein Noxa that diverts the anti-apoptotic protein Mcl-1 from the pro-apoptotic protein Bak, which is thereby free to form a channel in the MOM, which then (together with the Bax channel) makes it possible to Cyt c or Smac/Diablo to translocate (Figure 26) [Ralph and Neuzil, 2009]. Recent data from our laboratory show that, at least in some cancer cell lines, α-TOS preferentially causes formation of the Bak channel [Prochazka et al., 2010], a process that involves transcriptional regulation by the FoxO1 factor [Neuzil et al., unpublished]. Further, we found that cancer cells that lack both Bax and Bak are relatively resistant to apoptosis induced by α-TOS, which is delayed by some 24 h, and probably proceeds in a mitochondria-independent manner, although the exact mechanism is unknown [Neuzil et al., unpublished data].

Notwithstanding, all (or vast majority) of results from our studies and work of others clearly point to mitochondria as very important organelles that are involved in initiation of apoptosis in (breast) cancer cells by analogues of VE. That CII is an excellent target for VE analogues to kill breast cancer cells can be exemplified by the findings that subunits of CII only rarely mutate. A relatively high frequency of mutations in CII have been described for neoplasias of the peripheral nervous system,

such as paragangliomas or phaeochromocytomas, while only 1 out of one million breast cancer patients carry a mutation in CII [Peczkowska et al., 2008]. We can therefore propose the importance of our research, highlighting the fact that analogues of VE, epitomised by the prototypic α-TOS, are excellent anti-cancer drugs, where a clinical trial is imminent.

The importance of our results can be further highlighted in the context of recent findings that individual types of cancer are complex and can differ considerably in their array of DNA mutations, harbouring different sets of genetic causes [Parsons et al., 2008; Jones et al., 2008]. This indicates that it will be very unlikely to cure cancer by drugs targeting only a few gene products or single pathways that are essential for tumour survival [Hayden, 2008]. What is needed then is an invariant target, common to all cells, but which is predominantly only affected by drugs when delivered inside the cancer cells. Such a target is presented by mitochondria that are essential for virtually all cells, including cancer cells (that rely for ATP generation partly on glycolysis, partly on mitochondria) [Gogvadze et al., 2008], and mitochondria are intimately involved in apoptosis induced by VE analogues. We have recently found that a novel class of VE analogues targeted directly to mitochondria by a specific chemical modification (addition of the triphenylphosphonium group) induced high level of apoptosis in breast cancer cell lines (by targeting CII) and very efficiently suppressed breast carcinomas in the FVB/N *c-neu* mouse [Dong et al., 2009], giving further support to the premise that mitochondria play a very important role as targets

for anti-cancer drugs, including VE analogues.

Results presented in the thesis can be reconciled with the fact that erbB2-positive breast cancer is rather recalcitrant to therapy [Nahta and Esteva, 2007] and that currently the only option to treat such breast carcinomas is using the humanised antibody Herceptin, which presents several problems: 1. it is rather cardiotoxic [Riccio et al., 2009]; 2. its application is very expensive (currently >$50,000 per patient per year) [Pearson et al., 2007]; 3. the outcome of the therapy is often rather dubious [Ross et al., 2003]. Therefore, it is imperative to design new drugs that may be both cancer cell-specific and relatively inexpensive; VE analogues, exemplified by the prototypic α-TOS, appear to meet these premises.

One of the most important results presented in this thesis is greatly relevant to the potential clinical use of VE analogues against breast cancer with high level of expression of erbB2. To this end, we show here that α-TOS rather efficiently and in a selective manner (with the experimental animals showing no signs of secondary toxicity) suppressed breast carcinoma progression in the transgenic FVB/N *c-neu* mice, which spontaneously develop breast cancer featuring over-expression of erbB2. This model is very relevant to the human disease: the animals feature a fully functional immune system and form ductal breast carcinomas, one of the prevailing types of breast cancer in human patients. Further, we designed, synthetised and verified an intriguing form of α-TOS, *i.e.* its conjugate with the heptapeptide

LTVSPWY. This peptide has relatively high efficacy for receptors expressed by breast cancer cells, with preference for erbB2. We found that the LTVSPWY-α-TOS conjugate is rapidly endocytosed by the erbB2-over-expressing cells and internalised within endosomes, whose maturation into lysosomes results in hydrolysis of the conjugate by the action of acidic peptidases. The liberated α-TOS then compromises the mitochondrial function of the target cells, resulting in their fast and efficient demise via apoptosis, which translates into very pronounced anti-tumour effect when applied in the FVB/N *c-neu* mice. Therefore, this approach presents an innovative way how to deliver anti-cancer drugs to malignant cells that express surface receptors expressed at relatively low levels by normal cells in an efficient and selective manner.

Thus far, very little is known about the effect of VE analogues on cancer in the case of human patients. One of the problems encountered is the process of administration of the agents. Most VE analogous with anti-cancer activity are hydrophobic esters that are completely hydrolysed upon intestinal uptake. Only several ether variants of VE analogues, such as the ether α-tocopheryloxyacetic acid can be administered orally, although it has thus far been tested (successfully) only in mouse models of breast cancer [Hahn et al., 2006]. Other means have to be used to deliver the drugs to the bloodstream, where these hydrophobic compounds associate with the circulating lipoproteins that deliver them to the peripheral cancerous tissue and, later on, clear them via the liver so that VE analogues will not accumulate over the course of the treatment period to reach levels potentially toxic for non-cancerous tissues [Neuzil,

2002, 2003]. The intraperitoneal or intravenous injection used in animal experiments is not readily applicable to humans since it may cause an additional complication, such as pronounced local inflammation. A plausible delivery of α-TOS and other analogues of VE with anti-cancer activity may be achieved by transdermal application of the drug, and we are now in the stage of planning a phase I/phase II trial, in which breast cancer patients will receive α-TOS and, potentially other, more apoptogenic VE analogues, via transdermal delivery, using a variety of nannocarrier systems (liposomes, nannofibers, etc). If successful, a very powerful therapeutic approach may be available to cure breast cancer patients, including those with high expression of erbB2, which can be currently treated only using the exceedingly expensive humanised antibody Herceptine that is also considerably cardiotoxic.

Future work should include more detailed investigation of the molecular mechanism which governs inhibition by VE analogues of the erbB2-triggered anti-apoptotic/pro-survival pathways operational in breast cancer patients with high levels of erbB2 that cause 'tribulations' in the therapy [Nahta and Esteva, 2007], and we are currently carrying out experiments to achieve this target. Work has been initiated by our group in collaboration with colleagues on a plausible formulation of VE analogues for transdermal delivery of the drugs, and the first results appear promising. In conclusion, more has to be done before VE analogues can be used for human therapy. Notwith-standing, the author of this Thesis believes that the results presented here have made a contribution towards the ultimate goal: the design and development of an efficient and

inexpensive drug against breast cancer, in particular the highly recalcitrant and recurrent form characterised by high level of expression of the receptor tyrosine kinase erbB2.

CHAPTER 6. REFERENCE LIST

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APPENDIX. PUBLICATIONS BY THE APPLICANT RELEVANT TO THIS THESIS

The results of this thesis are covered in Publication 1 (contents of Chapter 2) and Publication 2 (contents of Chapter 3), which are attached to the thesis. A manuscript related to Chapter 4 will be submitted in near future.