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The cytotoxic effects of the anticancer drugs rviscumin and paclitaxel are dependent on cellular HER-2 (c-erbB2/neu) levels

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Recombinant Viscumin (rViscumin) is a new anticancer drug currently in clinical phase 1. It represents the pure, biochemically defined active component of plant derived mistletoe lectin and is a potent apoptosis-inducing compound with a unique mode of action leading to cell death through inactivation of translation of the target cell. Additionally, in low concentration ranges of rViscumin an immunomodulatory activity of the innate immunosystem is observed. Upregulation of HER-2 (c-erbB2/neu), a member of the HER receptor family of receptor tyrosine kinases, has been reported in 20-30 % of human adenocarcinomas of the ovary and has been linked to an unfavorable prognosis in these patients. The relationship between HER-2 receptor levels and drug sensitivity is of considerable interest since this molecular marker may allow to better predict response to chemotherapy. In the present study, we abrogated HER-2 expression in human SK-OV-3 ovarian cancer cells by ribozyme targeting and established stable cell lines with different residual HER-2 levels. In proliferation assays and in anchorage-independent soft agar assays, SK-OV-3 cells responded well to rViscumin. Interestingly, ribozyme-mediated down-regulation of HER-2 protein resulted in markedly decreased cellular sensitivities. This effect was comparable to Paclitaxel (Taxol) which is one of the most important cytotoxic drugs and widely used in cancer therapy. Although the mechanism of cytotoxicity of Paclitaxel is completely different, cellular sensitivity was similarly decreased upon ribozyme-mediated HER-2 depletion. Detailed analysis revealed that the HER-2 dependence of the cellular response to rViscumin or Paclitaxel is at least partially due to differential induction of apoptosis which is decreased in HER-2 depleted SK-OV-3 cells. Furthermore, the Paclitaxel-mediated inhibition of cell cycle was lost upon HER-2 reduction. Finally, downstream signal transduction pathways were analyzed. Western blotting revealed rViscumin-mediated, dose-dependent induction of p42/p44, p38 and SAPK/JNK which, again, was dependent on HER-2 expression levels. Our data introduce rViscumin as potential drug in ovarian cancer treatment. We also show that HER-2 expression levels display multiple effects on rViscumin and Paclitaxel cytotoxicity on molecular levels which may effect the clinical response to cancer chemotherapy.

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Activation of Fas-mediated apoptosis in 5-fluorouracil treated breast and colorectal cancer cell lines

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Fas (CD95/Apo-1) is a member of the TNF cell surface receptor family. Binding of Fas Ligand (FasL) causes trimerization of Fas and leads to the recruitment of the adaptor protein FADD (Fas-associated death domain), which in turn recruits caspase 8 (FADD-like IL-1-converting enzyme, FLICE) to form the death-inducing signalling complex (DISC). Caspase 8 molecules become activated at the DISC and in turn activate pro-apoptotic downstream molecules. c-FLIP (FLICE inhibitory protein) inhibits caspase 8 recruitment and processing at the DISC. We have found that the expression of Fas was up-regulated >10-fold in the p53 wild type MCF-7 breast cancer and HCT116 colorectal cancer cell lines in response to treatment with 5-Fluorouracil (5-FU), however, this did not result in activation of caspase 8. Although FasL expression was unaffected by 5-FU treatment, immunoprecipitation reactions demonstrated that the interaction between receptor and ligand was up-regulated. Analysis of c-FLIP expression in 5-FU treated MCF-7 cells demonstrated that its expression was up-regulated and revealed the presence of a truncated form of the protein that is generated during inhibition of caspase 8 activation at the DISC. MTT cell viability and clonogenic survival assays demonstrated a very strong synergistic interaction between 5-FU and the agonistic Fas antibody CH-11 in both MCF-7 and HCT116 cell lines (combination index <0.1). Cell cycle and PARP cleavage assays revealed that this synergy was due to activation of apoptosis. Furthermore, caspase 8 was activated following co-treatment with 5-FU and CH-11 in MCF-7 cells, but not by single treatment with either drug. In addition, c-FLIP expression was down-regulated prior to caspase 8 activation in 5-FU and CH-11 co-treated MCF-7 cells. We have also observed synergy between CH-11 and both the antifolate raltitrexed and the DNA damaging agent oxaliplatin in MCF-7 and HCT116 cell lines. However, synergy between CH-11 and 5-FU was not observed in p53 null MCF-7 and HCT116 daughter cell lines that fail to up-regulate Fas in response to 5-FU. Our results suggest involvement of c-FLIP in blocking Fas-mediated apoptosis following 5-FU treatment and raise the possibility of using Fas-targeted approaches to stimulate apoptosis in chemosensitised cancer cells.

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Cell cycle arresting and apoptosis-inducing activity of mycobacterial cell wall-DNA complex (MCC) towards human bladder cancer cells

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Intravesicle administration of MCC emulsion in patients with carcinoma in situ of the bladder who have failed BCG therapy and/or chemotherapy results in a significant number of complete clinical responses. The potential for MCC as a treatment for other types of bladder cancer has been examined by determining its activity towards a panel of human bladder cancer cell lines with known defects in cell cycle/apoptosis regulators. The human bladder cancer cell lines RT4 (benign papilloma), Hs 172.T (carcinoma, fibroblast), SCaBER (squamous cell carcinoma), UMUC-3 (TCC), SW 780 (grade 1 TCC), TCC SUP (grade 3 TCC), HT 1197 (grade 3 TCC), HT 1376 (grade 3 TCC), and T24 (grade 3 TCC) were treated with MCC (0.1-100 μg/ml) *in vitro*. Determination of cell cycle arrest (flow cytometry), cell division (MTT reduction), intracellular signaling changes (protein phosphorylation), intracellular free radical levels (dichlorofluorescein acetate probe), caspase-3 levels (flow cytometry), PARP degradation (flow cytometry) and apoptotic nuclei (Hoechst 33258 staining) was carried out at 24-72 hours post treatment. MCC caused cell cycle arrest and inhibited the division of all the bladder cancer cell lines tested. Cell lines derived from high grade TCC appeared to be the most susceptible. There was no correlation between cell cycle arrest/inhibition of division and the presence of mutated or absent cell cycle/apoptosis regulators (p16, p21, p53, pRb). Cell cycle arrest occurred at 24-48 hours post-treatment, predominantly at the G0/G1/S phase of the cell cycle. Significant changes in intracellular protein hypophosphorylation and elevated free radical levels occurred at 48-72 hours post-treatment. Apoptosis was present at 72 hours post-treatment, as demonstrated by activated caspase-3, PARP degradation product and apoptotic nuclei. Treatment of MCC with DNase-I significantly reduced its ability to induce apoptosis in the bladder cancer cell lines, demonstrating that the complexed DNA in MCC is responsible for its anticancer activity. The ability of MCC to cause cell cycle arrest/inhibition of division and induce apoptosis in the bladder cancer cell lines tested appears to be independent of defects in cell cycle/apoptosis regulators. MCC is most effective against bladder cancer cell lines derived from high grade TCC, and may therefore have application in the treatment of high grade bladder cancers that are known to be associated with mutations in cell cycle/apoptosis regulators.

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Anticancer activity of a 6-base length phosphodiester oligonucleotide, Oligomodulator™BT 99-25, against lymphoma

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Oligomodulator™BT 99-25, a synthetic 6-base length phosphodiester oligonucleotide with the sequence 5'-G3TG2-3', has apoptosis-inducing activity towards a number of leukemia cell lines. In this study, the activity of BT 99-25 towards lymphoma cells has been determined. BT 99-25 inhibited the division of murine EL-4 T lymphoma cells, human HH cutaneous T cell lymphoma cells, human HB B cell lymphoma cells and human U 937 histiocytic lymphoma cells in a concentration-dependent manner (0.5 to 50 μM). Inhibition of cellular division was associated with cell cycle arrest in the G0/G1/S phase of the cell cycle. BT 99-25 treatment induced apoptosis of EL-4 and U937 cells as measured by phosphatidylserine plasma membrane translocation. In addition, BT 99-25 triggered the release of soluble nuclear mitotic apparatus protein (NuMA), a marker of apoptosis, in a concentration-dependent manner. The murine syngenic EL-4 lymphoma model was used to assess the *in vivo* anticancer activity of BT 99 25. A single intratumoral injection of BT 99-25 (0.4 mg/kg body weight) inhibited subcutaneous EL-4 tumor growth by 53% and cured 37.5% of treated mice (N=8). Anticancer activity was also observed at a lower dose of BT 99-25 (0.04 mg/kg body weight, 34% inhibition of tumor growth, 12.5% mice

cured). BT 99-25 was stable in biological milieu with a half-life of approximately 72 hours. These results demonstrate that a phosphodiester 6-base length oligonucleotide, Oligomodulator™ BT 99-25, may have potential for the treatment of lymphoma.

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Rhythms in BCL-2, cell cycle distribution and circadian clock gene expression in normal tissues and in tumor for improving novel targeted cancer therapy

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Objectives: To establish prerequisites for circadian timing of novel therapies targeted at cell cycle or apoptosis.
Methods: Two studies involved mice from 2 different strains (B6D2F1 and C3H/HeN, with or without mammary carcinoma) in order to assess 24-hour changes in cell cycle phase distribution (flow cytometry), clock gene mRNA (Per2, Bmal-1, Clock, Tim with RNAse protection) and BCL-2 protein (western blot) in bone marrow. The human oral mucosa was used to study (8 subjects), cell cycle proteins (immunohistochemistry), clock genes (hPer1, hBmal1, hClock, hTim, hCry1) and hBcl-2 (RT-PCR). 24-h changes were validated with ANOVA and cosinor. In the experimental models, a circadian rhythm was found for the proportion of G1 phase and S phase cells ($p < 0.05$) in the total bone marrow of both strains. BCL-2 expression varied 3 to 5-fold along the 24 h ($p < 0.02$), with a peak near the middle of the rest-phase of the rest-activity cycle. The transcriptional activity of mPer2 and mBmal1 varied rhythmically in mouse bone marrow and could control the BCL-2 rhythm. In mammary carcinoma-bearing C3H/HeN mice, the bone marrow cell cycle phase and BCL-2 rhythms were near normal. Circadian changes characterized G1 and G2/M phase in tumor cells ($p<0.01$), with peaks respectively occurring 2 and 7 h earlier than in bone marrow. No BCL-2 rhythm was found. The circadian regulation of cell cycle and apoptotic pathways was severely altered in this tumor known to display a marked circadian dependency of docetaxel efficacy (Granda et al. Cancer Res 2001). In humans, a 2- to 3-fold change along the 24-h time scale was documented for p53, and cyclins-E, -A and -B1, with peaks occurring at 11:00, 15:00, 16:00 and 21:00 respectively (Bjarnason et al. Am J Pathol 1999). These rhythms could result from the rhythmic expression of oral mucosa clock genes (Bjarnason et al. Am J 2001). Mucosal hBcl2 mRNA expression also varied by 50%, with a peak near 01:00 at night. Cell cycle and Bcl-2 pathways are under circadian clock regulation both in mouse bone marrow and in human oral mucosa, with a fixed relation with the rest-activity circadian cycle. In both species, BCL-2-mediated protection of normal cells against apoptotic processes may be increased during the rest span. These data support the incorporation of circadian concepts in the development of novel therapeutic agents targeting cell cycle and apoptosis. Supported in part by ARTBC, Villejuif and Aventis, Vitry, France

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Cyclic AMP inhibits caspase-8-mediated, pH-dependent, apoptosis by attenuating cellular acidification

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The G protein-coupled receptor agonist somatostatin (SST) regulates multiple second messenger systems to inhibit cellular secretion and proliferation. Inhibition of secretion by SST is due, largely, to its ability to inhibit the activation of adenylylcyclase thereby decreasing cyclic AMP (cAMP) production. By contrast, the inhibition of cell proliferation by SST results from its positive regulation of the protein tyrosine phosphatase SHP-1. We have previously shown that activation of the cysteine aspartate-specific protease caspase-8 by SST promotes cytoplasmic acidification thereby triggering apoptosis in MCF-7 human breast cancer cells. In some models caspase-8-mediated apoptotic signalling occurs in the absence of any change in pHi. cAMP increasing agents have been shown to modulate apoptosis both positively and negatively. For instance cAMP can promote apoptosis either directly or by potentiating the action of a variety of apoptotic inducers in thymocytes and lymphocytes. By contrast it inhibits apoptosis in T lymphocytes, T cell hybridomas and in promonocytic leukemia cells. Likewise activation of adenylylcyclase directly by forskolin (Fsk) or by agonists of receptors that are positively coupled to this enzyme has been reported to either facilitate or suppress apoptosis. Whether these findings reflect differing effects of cAMP on pH-dependent and -independent apoptosis is not known. These considerations prompted us to investigate the effect of increased cAMP on SST- and TNF-a-induced apoptosis in MCF-7 cells. We report here that SST-, but not TNF-a-, induced apoptosis is acidification-dependent and is inhibited by elevated cAMP levels. The protective action of cAMP against SST-induced apoptosis is due both to an elevation of resting pHi and attenuation of SST-induced acidification distal to SHP-1-mediated caspase-8 activation. Interestingly, cAMP is not able to rescue the cells from SST-induced apoptosis once the pHi has fallen below 7.0. The cAMP-dependent protein kinase (PKA) inhibitor H-89 partially reversed the pH lowering effect of SST suggesting that both PKA-dependent and independent mechanisms mediate the protective action of cAMP on acidification. These findings reveal for the first time that cAMP can protect against SST-induced, acidification-dependent, apoptosis by attenuating the reduction in pHi.

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Apoptosis signaling by 2-methoxyestradiol in DS-sarcoma cells

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Introduction: The anti-cancer potential of the natural estrogen metabolite 2-methoxyestradiol (2-ME) is independent of estrogen-receptor binding. Its selective growth inhibition of cancer cells and tumours is associated with microtubuli interaction, anti-angiogenetic effects and inhibition of superoxide dismutase. Recently, Huang et al. showed that 2-ME induces apoptosis in leukeamia cells by blocking the activity of superoxide dismutase which results in excessive production of superoxide anions. Addition of antioxidants or overexpression of superoxide dismutase prevents apoptotic cell death of these cells. In order to clarify whether this mechanism is generally induced by 2-ME in any cancer cells, the present study investigated apoptosis signaling of 2-ME in DS-sarcoma cells.
Results: Translocation of the pro-apoptotic protein Bax to the mitochondria was identified as initial apoptotic event, followed by a decrease in mitochondrial transmembrane potential and the release of AIF out of the mitochondria. In addition, upregulation of FasL and TNFa by 2-ME, two death receptor ligands, was observed. Although, 2-ME administration resulted in activation of caspases, pan caspase inhibitor Z-VAD-FMK could not block 2-ME induced apoptotic cell death pointing to a caspase-independent mechanism. Furthermore, an increase in formation of reactive oxygen species was observed after 2-ME treatment. However, supplementation with different antioxidants could not decrease the toxic effect of 2-ME.
Conclusion: These findings may indicate, that reactive oxygen species are not involved in apoptosis induction in DS-sarcoma cells, rather they are a consequence of mitochondrial damage. Thus we could not validate the results of Huang et al., who investigated the effect of 2-ME in leukaemia cells. Hence, the mechanism of apoptosis induction by 2-ME seems to be cell line dependent.

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Modulation of STAT activation by DNA damaging anti-cancer drugs

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Members of the STAT (Signal Transducers and Activators of Transcription) family of transcription factors in particular STAT1, 3 and 5, have been shown to both promote and inhibit apoptosis. Normally, active STAT1 plays a role in mediating growth arrest and apoptosis in response to stimuli such as IFNg. It follows that its absence *in vivo* can lead to an increase in tumour formation. Furthermore, a number of tumour cell types show a defective response to STAT1 activation, accounting for a lack of growth arrest or apoptotic induction. Previously, we have shown that DNA-damaging anti-cancer drugs can activate NFkB, a transcription factor known to modulate apoptosis (1). We now show that the same family of drugs, which include the topoisomerase II-targeted drugs, doxorubucin and mitoxantrone, can also effect the duration and intensity of STAT1 activation. Specifically, we observed that treatment of the breast cancer cell line, MDA435, with IFNg results in activation of STAT1 as measured by increased phosphorylation of tyrosine 701. This activation was potentiated by both mitoxantrone and doxorubucin, drugs that give rise to DNA double stranded breaks. This potentiation was accompanied by enhanced nuclear localisation of STAT1 and modulation of downstream STAT1 targets. In addition, we observed a potentiation of