12. New drug targets, mechanism of action studies and biochemical pharmacology

504 Antitumor activity of 17-allylaminogeldanamycin (NSC 330507) in melanoma xenografts is associated with decline in Hsp90 protein expression

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17-Allylaminogeldanamycin (17-AAG, NSC 330507) is a semisynthetic derivative of the naturally occurring anticancer agent geldanamycin, which was identified by the NCI *in vitro* screening program. The melanoma panel was among the most sensitive cell types in the 60 tumor cell line screen with GI_{50} concentrations as low as 25 nM. The heat shock protein Hsp90 has been identified as molecular target for geldanamycin and its antitumor effects have been described to result from degradiation of signaling proteins and nuclear hormone receptors by binding their activator Hsp90. In this study, we examined the activity of 17-AAG in 4 human melanoma xenografts *in vitro* and *in vivo* and whether any inhibitory effects would be associated with declining Hsp90 levels. The clonogenic assay (TCA) was used to test 17-AAG in the melanoma xenografts MEXF 276, 989 and 462, and the SRB assay to evaluate 17-AAG in the xenograft derived cell lines MEXF 276L, 514L and 462NL. 17-AAG was most active in both MEXF 276 *in vitro* systems with GI_{50} = 70 nM in the SRB and GI_{50} < 0.02 nM in the TCA, followed by MEXF 989 with GI_{50} = 5 nM in the TCA. MEXF 462 was least sensitive (μM range) *in vitro*. The *in vivo* activity of 17-AAG was evaluated in the s.c. growing human tumor xenografts MEXF 276, 989, 462 and 514 in athymic NCRnu mice. Drug was administered i.p. at 80, 60 and 40 mg/kg/d on days 1–5 and 8–12 with the daily dose split into half given 8 h apart. Partial tumor regression was seen in the MEXF 276 and 989 models with optimal T/Cs at the MTD (80 mg/kg/d) of 6% or 13% respectively, whereas no signficant effects were found in MEXF 462 and 514. Hsp90 protein expression was followed by Western analysis in 17-AAG treated and control melanoma tissues. A marked decline in Hsp90 levels was observed in MEXF 276 tissue treated with 80 mg/kg/d 17-AAG, but no effects were seen in resistant MEXF 462 tumors.

Our data indicate that 17-AAG is markedly active in melanoma xenografts *in vitro* and *in vivo* and that its antitumor efficacy appears to be associated with Hsp90 reduction.

505 Anticancer activity of FGN-1 and its analogs against human tumor specimens taken directly from patients

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FGN-1 (sulindac sulfone) causes regression of and prevents recurrence of colonic adenomas in patients with familial adenomatous polyposis. These results show that apoptosis primarily contributes to growth inhibition by sulindac metabolites. The biochemical pathway does not require COX inhibition or p53 induction and appears to be fundamentally different from the apoptotic response to 5-FU (Piazza et al., Cancer Res 57: 2452, 1997). Apoptosis inducing properties of FGN-1 have been shown against a variety of epithelial cancer cell lines.

To examine the effects of sulindac metabolites against primary tumors, we investigated the activity of FGN-1 and its analogs (CP248, CP132, CP165, and CP265) in a human tumor colony-forming assay against specimens taken directly from patients. FGN-1 and CP248 were tested against 33 specimens representing breast, non-small cell lung, ovarian, prostate, and thyroid cancers. FGN-1 at 144 μM inhibited the growth of 2/2 breast tumors and 5/10 of ovarian cancers. Inhibition is defined as ≤50% survival at the given concentration. With 0.1 μM CP248 inhibition of breast tumor growth was observed in 1/2 tumors and with 1 μM and 5 μM in 2/2 breast tumors, respectively. Inhibition of ovarian tumors was seen in 3/9 tumors with 1 μM CP248 and in 5/10 tumors with 5 μM CP248.

CP132 was tested against 31 primary tumors and demonstrated a concentration-dependent response with activity at the 100 μM concentration in 1/2 colon, 1/2 non-small cell lung and 2/4 ovarian cancer tumors. In the 38 tumors tested, CP165 showed significant inhibition for all tumor types at the 100 μM concentration including 3/4 ovarian and 3/3 prostate tumors. Inhibition of 2/3 colon, 1/2 non-small cell lung, and 3/4 ovarian tumors was seen in the 36 primary tumors tested against CP265 at the same concentration. In conclusion, FGN-1 and its analogs are promising new antineoplastic agents with activity against tumor colony-forming units taken directly from patients.

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506 *In vitro* studies of drug interaction between meglumine gamma linolenic acid epirubicin or mitomycin c: Towards combination chemotherapy in superficial bladder cancer

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Introduction: The use of multi-agent chemotherapy is well established in many oncological specialities. The use of sole agents in intravesical chemotherapy may explain the limited success of this form of treatment in superficial bladder cancer. Tumour cells have a relative deficiency of polyunsaturated fatty acids in their membranes which compromises their permeability and may therefore limit drug uptake. Their membrane composition however, can be altered by incubation with essential polyunsaturated fatty acids such as meglumine gamma linolenic acid (MeGLA). Is the cytotoxic action of epirubicin and mitomycin C enhanced in combination delivery with MeGLA?

Materials and methods: The transitional cancer cell line, MGH-U1 and its multidrug resistant (MDR) variants, were exposed to MeGLA, epirubicin or mitomycin C for 2 hours, in serial doubling dilutions. The MTT assay was used to identify an ineffective dose of MeGLA (5 μg/ml) that was then added to the serial doses of epirubicin and mitomycin C and the experiments repeated.

Results: 5 μg/ml of MeGLA had no effect on the residual viable biomass. The drug concentration required to reduce the viable biomass to less than 50% (IC < 50) was 1.6 μg/ml for epirubicin alone and 0.2 μg/ml for epirubicin in combination with MeGLA. The IC < 50 was 3.2 μ/ml for mitomycin C alone and 0.4 μg/ml for mitomycin C in combination with MeGLA. Strong synergim between MeGLA and both cytotoxics was confirmed by the median effect analytic method of Chou and Talalay.

Conclusions: MeGLA enhances the cytotoxic action of both epirubicin and mitomycin C when used in combination. This results in a reduction in the combination dose to 1/8th of that necessary when either agent is used in isolation. This action is probably mediated via direct alteration of tumour membrane characteristics.

507 Sensitivity of N-Myc amplified and non-amplified human neuroblastoma cell lines to deferoxamine (DFO) and hydroxyurea treatment

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Deferoxamine (DFO) and Hydroxyurea (HU) inhibit cell proliferation through action on ribonucleotide-reductase, an iron-dependent enzyme responsable for the synthesis of deoxyribonucleotide. Recently, it has been reported that the administration of DFO and HU shows a synergistic effect on growth of NB cell lines mainly in those with N-Myc amplified. (Frgala T., 1996). To verify this hypothesis we started a pharmacological study on the sensitivity of two NBcell lines, SKNSH (1 N-Myc copy/genome) and IMR32 (8 N-Myc copies/genome), to treatment with DFO and HU as single agents or in combination. Preliminary results show that there is a dose-response effect in both the cell lines to DFO and HU and the inhibition of cell proliferation (tested by MTT assay) is time-dependent. DFO appears more potent than HU as demonstrated by the ratio between the ICs 50% of the two drugs. At 24 h of exposure the IC50% DFO/IC50% HU is 3,3 in SKNSH line and 10 in IMR32 line while at 120h this ratio arises at about 11 and 64 respectively. The line with amplified N-Myc (IMR32) is more resistant to drugs treatment than SKNSH at all time studied (24–48–120 hours). Interestingly, the co-administration of DFO and low dose of HU (50 μM) shifts the IC50% of DFO treated cells of 20 folds in IMR32 and 10 folds in SKNSH at 48 h.

These results indicate that the line IMR32 is resistant to low doses of DFO and HU given as single agents while the SKNSH line is sensitive; the co-administration of DFO and low dose of HU is more effective in inhibiting cell proliferation in both cell lines but this additive effect is more evident in the N-Myc amplified line.

The data obtained in this experimental model suggest that the pharmacological resistance present in NB cell population with amplified N-Myc could be overcome by the co-administration of low doses of drugs with similar mechanism of action. This effect might be explained by changes in ribonucleotide reductase conformation and function in deoxyribonucleotide synthesis concomitant with genetic selection of amplified N-Myc in NB cells.

*Abstract numbers in italics denote abstracts for publication only

508 Benzoprims: Dual action against DHFR and mutant Ki-*ras*

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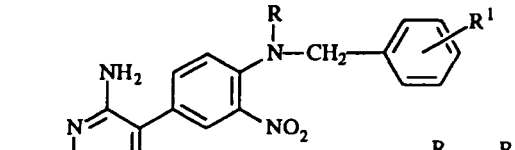
Highly-substituted 2,4-diaminopyrimidines (benzoprims) as exemplified by methylbenzoprim **1** and dichlorobenzoprim **2** were developed as non-classical lipophilic analogues of methotrexate (MTX) and have potent inhibitory activity against mammalian DHFR (Ki = $\sim 10^{-12}$M): the compounds have *in vivo* activity against the mouse M5076 reticulum cell sarcoma, a tumour resistant to MTX by virtue of modification of the folate transporter (Griffin et al., J. Medicin. Chem., 1989, 32, 2468–2476). In certain cell lines (e.g. F28-7 murine breast) the inhibitory activity of **1** and **2** is reversed by hypoxanthine-thymidine or folinic acid, pointing to an antifolate locus of action. In confirmation in an NCI COMPARE analysis (59 cell panel) (Weinstein et al., Science, 1997, 275, 343–349) the highest Pearson Correlation Coefficients (PCC) were to MTX (PCC = 0.86) and trimetrexate (PCC = 0.83).



	R	R^1
1 methylbenzoprim	Me	H
2 dichlorobenzoprim	H	2,4-di-Cl

However there is a strong correlation (PCC = >0.75) with activity of benzoprims against mutant *ras* molecular target expression in a restricted panel of NSCL and colon tumour cell lines (n = 16). The following GI_{50} values (μM) were obtained: in NSCL lines expressing wild-type *ras* – EKVX (1.78), HOP 92 (1.55), NCI-H522 (0.51); and lines expressing mutant Ki-*ras* – A549 (<0.01), NCI H23 (0.016), NCI-H460 (<0.01). A similar selectivity was observed against colon cell lines expressing mutant Ki-*ras* and this activity is **not** related to DHFR inhibition.

We have established a panel of lung, colon and pancreatic cell lines which have been characterised for *ras* expression (wild or mutant) and are seeking to redesign the lead benzoprim structure to identify a pure anti-*ras* molecule.

509 Differentiation-inducing, hybrid polar compounds HMBA and SAHA are inhibitors of histone deacetylase

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Histones are the protein component of eukaryotic chromatin. The reversible acetylation of lysine residues in the N-terminal tails of core histones contributes to the regulation of nuclear processes, as transcription and differentiation. The modification is maintained by histone acetyltransferases and histone deacetylases. Inhibition of histone deacetylase leads to hyperacetylation of histones, thereby affecting growth and differentiation. Inhibition of histone deacetylase leads to terminal differentiation of leukaemic cells, a process that could be explored for the prevention and treatment of cancer. Other inducers of differentiation such as retinoids and vitamin-D-derivatives are already established in cancer therapy and are investigated in clinical trials for cancer chemoprevention. In the course of our studies towards new inhibitors of histone deacetylase we have found promising lead substances. Among them was a hydroxamic acid that led us to SAHA (suberoylanilide hydroxamic acid), a structurally related inducer of differentiation. It belongs to the group of the so called hybrid polar compounds (HPC) whose other prominent representative is HMBA (hexamethylene-bisacetamide). HMBA has shown some success in a clinical study in the treatment of acute myelogenous leukaemia, but as it induces differentiation in millimolar concentrations *in vitro*, large doses were applied and severe side effects occurred. SAHA has an ~1000 fold higher potency and is currently intended for use in clinical studies of leukaemia treatment. The induction of differentiation by the HPC has been studied extensively but a common underlying cellular mechanism has not been identified so far. Given the structure of our inhibitor we investigated deacetylase inhibition by SAHA and HMBA and found that they inhibit purified maize histone deacetylase HD-2 and a preparation from rat liver cytosol with deacetylase activity with concentrations similar to those required for the induction of differentiation *in vitro*. Our results put the application and improvement of HPC on a more rational basis and the value of inhibitors of histone deacetylase in treatment and prevention of malignant disease which has been speculative at most so far is strongly supported and this will stimulate research in that area.

510 Bisimidazoacridones, potent but selective anti-tumor agents which appear to target transcription in sensitive cells

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Previous reports from our laboratory described the synthesis and the potent but highly selective antitumor activity of bisimidazoacridones (BIA) and the closely related bistriazoloacridones. One of the BIA's, 5,5'-[(methylimino)-bis(3,1-propanediylimino)]bis[6H-imidazo{4,51-*de*]acridin-6-one], or WMC26, which had shown potent *in vitro* and *in vivo* activity against colon cancer, was chosen for further mechanistic studies. Spectroscopic studies show that in aqueous solutions WMC26 exists in a folded structure, where the two imidazoacridone ring systems interact by π-stacking. The folded structure becomes relaxed in non-aqueous solvents, in presence of micelles, or when interaction with nucleic acids occurs. Gel shift and ligase assays suggest that the binding of WMC26 to DNA is by mono-intercalation, but binding studies indicate that the binding constants are low, with the molecule rapidly moving from one binding site to another. Confocal microscopy studies showed initial rapid concentration of the drug in the nucleus of exposed cells, especially in nucleoli, but then rapid clearance (minutes) into the cytoplasm, where it became localized in lysosomes. The cell mitochondria were not involved, as evidenced by colocalization studies, and by studies involving yeast. The drug was not a substrate for either the MDR or multidrug resistance associated protein (MRP) pumps. WMC26 induced a sharp and rapid decrease in RNA synthesis, consistent with the nucleolar localization, in sensitive cells but not in resistant cells. Several BIA's were also found to be very potent inhibitors of HIV-1. The target for the anti-viral activity of these compounds was found to be the initiation of transcription of the viral genome, which appears to involve a virus specific component of transcription regulation. The combined data on the activity of WMC26 in sensitive tumor cells suggest that the likely target for the drug is also a component of transcriptional regulation of critical genes in those cells. Our current hypothesis is that WMC26, and compounds related to it, bind to DNA in a reversible manner, which does not produce damage unless the binding is near a locus of transcription. Then the drug highjacks one of the proteins involved in the transcriptional machinery. The resulting ternary complex is very stable, and effectively blocks transcription.

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511 Mechanism of uptake and retention of 'Prognox™', a new agent to assess tumour hypoxia

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Hypoxia in solid tumours has been reported to be an important factor in limiting the success of conventional radiotherapy. Identification of tumour hypoxia could be valuable in predicting tumour resistance and directing patient management. Prognox™ is a novel technetium (Tc-99m) hypoxia marker which has already demonstrated high uptake in a range of human tumours using Gamma Camera Imaging (SPECT). Studies were undertaken to investigate the mechanisms involved in the uptake and retention of Prognox™ in hypoxia. Other hypoxia markers have been shown to enter the cell and undergo reductive metabolism under hypoxic conditions with retention of a protein bound fraction. The uptake, retention and metabolism of Prognox™ were investigated using V79 cells incubated under hypoxic or aerobic conditions. Cell extracts were then analysed by HPLC or subjected to subcellular fractionation or autoradiography. The metabolism of Prognox™ was also studied using S9 and microsome fractions from rat liver and HT29 (human) tumour xenografts. Subcellular localisation experiments performed on hypoxic V79 cells showed accumulation of Prognox™ within the cell nucleus and cytoplasm with little entrapment within the plasma membrane. Autoradiography of V79 cells corroborated these results with activity localised throughout the cell nucleus and cytoplasm. Uptake of Prognox™ into V79 cells under hypoxic conditions was linear. In addition, uptake was inversely correlated with low oxygen tensions. No uptake was seen in V79 cells incubated aerobically. HPLC analysis of the experimental media and V79 cell contents showed that >90% of the Tc-99m complex was unchanged in both the hypoxic and aerobic incubates. Hypoxic incubation of Prognox™ with liver and tumour S9 and liver microsomal fractions indicated no metabolism of the Tc-99m complex whereas metabolism of the hypoxia marker H-3 misonidazole occurred under these conditions. It can therefore be concluded that, rather than accumulating in the extra cellular environment, Prognox™ enters cells and is retained under hypoxic conditions. Retention occurs without any significant change to the Tc-99m complex and is therefore independent of metabolism. This implies that the accumulation of Prognox™ in hypoxic tissues occurs by a process different to that of other hypoxia markers and appears to be dependent upon an alternative mechanism associated with cellular hypoxia.

512 Pharmacological determinants of the antitumour activity of mitomycin C – implications for enzyme directed drug development

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Recent investigations into bioreductive drugs have focused on profiling reductase enzymes and relating their expression to therapeutic activity in an approach referred to as enzyme directed drug development. However, few studies have attempted to validate this approach *in vivo* and even less is known as to how the expression of reductases relates quantitatively and qualitatively to metabolic activation. In the present study, the antitumour activity, pharmacokinetics and metabolism of mitomycin C (MMC) has been determined *in vivo* in two murine adenocarcinomas of the colon, MAC 16 (high DT-diaphorase activity) and MAC 26 (low DT-diaphorase activity) after intra-tumoural injection of drug. Over a broad range of drug concentrations (50–1000 μg), MAC 16 proved to be consistently the more sensitive tumour (e.g. 75 μg MMC, T/C 11% for MAC 16 and 31% for MAC 26). Higher levels of parent drug (peak concentration 103 μg/tumour compared to 58 μg/tumour) were maintained over 45 min in MAC 16 after which time clearance was rapid from both tumours. Four metabolites were detected in both tumours characteristic of different pathways of metabolism. However, by far the major metabolite was 2,7-diaminomitosene (2,7-DM), an accurate indicator of metabolic activation of MMC. Despite higher reductase levels and greater sensitivity to the drug, there was 4-fold less production of 2,7-DM in MAC 16. These results indicate a lack of a simple relationship *in vivo* between reductase expression and metabolic activation and suggest factors other than pharmacological determinants being responsible for the chemosensitivity of the MAC tumours to MMC.

513 Synthesis and evaluation of DT-diaphorase activated naphthoquinones

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DT-diaphorase (DTD, NAD(P)H:Quinone oxidoreductase, EC 1.6.99.2) is considered to be a target for bioreductive drug development because of its ability to activate quinone based prodrugs and elevated levels of DTD have been reported in several tumour types (particularly NSCLC). In the early 1990's, the indoloquinone compound EO9 emerged as a strong candidate for clinical trial based upon the role that DTD plays in its mechanism of action and antitumour activity *in vitro* and *in vivo*. Despite 3 partial remissions in phase I trials, no activity was reported in subsequent phase II trials casting doubt over whether DTD is a target for drug development. Recent studies have, however, suggested that the failure of EO9 in the clinic may not be due to pharmacodynamic factors but may be the result of poor pharmacokinetic properties resulting in inadequate drug delivery to tumours (Phillips et al., Br J Cancer, In press). This result is important in that it keeps the concept of DTD as a target for drug development alive and the aim of our drug development program is to develop new drugs which retain the same properties of EO9 (in terms of bioactivation by DTD) but have better pharmacological properties in terms of drug delivery. Four known naphthoquinone compounds were synthesised by direct substitution of aziridine ring structures to commercially available 1,4 naphthoquinones (menadione, juglone and plumbagin). GS1 (3-aziridinyl-5-hydroxy-2-methyl-1,4 naphthoquinone), GS2 (2-aziridinyl-5-hydroxy-1,4-naphthoquinone), GS3 (2,3-diaziridinyl-5-hydroxy-1,4-naphthoquinone) and GS4 (2-aziridinyl-3-methyl-1,4-naphthoquinone) were initially assessed as substrates for purified human DTD and specific activities compared with the indoloquinone EO9. All four compounds were better substrates for DTD than EO9 with specific activities of 18.95 ± 1.9 (GS3), 18.26± 3.56 (GS2), 15.58 ± 3.71 (GS4), 15.04 ± 1.18 (GS1) and 9.59 ± 2.16 (EO9) μmol cytochrome c reduced /min/mg protein. All four compounds were reduced to species capable of inducing single strand breaks in supercoiled plasmid DNA. Chemosensitivity studies against H460 NSCLC (DTD activity = 1,100 nmol/min/mg) demonstrated that all four compounds were less potent than EO9. Comparison between the response of H460 cells and BE (human colon cells with no detectable DTD activity) demonstrated that both GS3 and GS2 were selectively toxic to DTD rich cells (IC_{50} BE/IC_{50} H460 = > 606 and 187 respectively). The degree of selectivity for GS3 was comparable with EO9 (> 606 and > 531 respectively). Whilst these 4 compounds have been synthesised previously, they have not been evaluated as substrates for DTD. The results of this study have identified 2 compounds, both of which are good substrates for and are bioactivated to DNA damaging species by DTD in cell free systems. GS3 in particular has similar properties to EO9 in terms of selectivity for DTD rich cells and further studies to compare the pharmacological properties of GS3 with EO9 are currently in progress.

514 Inhibition of DT-diaphorase by 5,6 dimethyl xanthenone-4-acetic acid (MeXAA) and flavone acetic acid (FAA): Implications for bioreductive drug development

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DT-diaphorase (DTD, NAD(P)H:Quinone oxidoreductase, EC 1.6.99.2) is a cytosolic flavoprotein which has the paradoxical properties of being able to detoxify or activate quinone based compounds. The ability to activate quinone based prodrugs, in conjunction with reports that some tumours have elevated levels of DTD enzyme activity, has led to DTD being considered as a good target for drug development. In terms of identifying drugs which are activated by DTD, a specific inhibitor of DTD would be a valuable experimental tool. Dicumarol is a strong inhibitor of DTD although it is non-specific in that it also inhibits other one electron reductases (such as cytochrome b_5 reductase). Various flavanoids have been shown to inhibit rodent DTD although no studies have attempted to determine whether they are selective inhibitors of human DTD. The aims of this study were twofold. First, to determine whether or not FAA and the structurally related compound MeXAA can preferentially inhibit human DTD activity with respect to cytochrome b_5 reductase. Second, to determine whether or not the use of FAA as an inhibitor of DTD can modify the cytotoxic activity of menadione (detoxified by DTD) or EO9 (activated by DTD). Both FAA and MeXAA inhibited purified human DTD in a dose dependent manner with ID_{50} (concentration required to inhibit 50% of enzyme activity) values of 103.3 and 62.5 μM respectively. Enzyme inhibition kinetics demonstrates that both compounds are competitive inhibitors of DTD with respect to NADH and non competitive with menadione. Both FAA and MeXAA inhibited DTD activity in a crude homogenate from DLD-1 human colon carcinoma cells with ID_{50} values of 110.9 and 49.6 μM for FAA and MeXAA respectively. At a concentration of 2.5 mM, DTD activity in DLD-1 homogenates was completely inhibited by both compounds. FAA (2.5 mM) had minimal effect on the activity of cytochrome b_5 reductase in DLD-1 cell homogenates (6.51% inhibition) whereas MeXAA (2.5 mM) partially inhibited cytochrome b_5 reductase activity (28.6% inhibition). Combinations of FAA (at the non toxic dose of 2.5 mM) and menadione induced significantly greater toxicity in DLD-1 cells *in vitro* (IC_{50} = 7.5 ± 2.2 μM) compared with menadione alone (IC_{50} = 22.0 ± 1.6 μM). Combinations of FAA (2.5 mM) with EO9 however resulted in significant inhibition of cell kill (IC_{50} = 12.3 ± 5.4 μM) compared with EO9 alone (IC_{50} = 0.32 ± 0.08 μM). These results suggest that FAA is a selective inhibitor of DTD with respect to cytochrome b_5 reductase and could be used in bioreductive drug development to dissect out the role that DTD plays in a compounds mechanisms of action. Further studies are required to determine whether or not other one electron reductases are inhibited by FAA.

515 Involvement of human and murine tumour cytochrome P450 in bioreduction of AQ4N

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The anthraquinone di-N-oxide AQ4N is a prodrug designed to be excluded from cell nuclei until metabolised in hypoxic tumour regions to AQ4 a potent topoisomerase II inhibitor. AQ4N is useful as an adjunct to both radiation and chemotherapy and is scheduled for a clinical trial in 1998. In principle AQ4N undergoes metabolic reduction to yield two products; AQM, a two electron reduced intermediate and AQ4, the four electron reduced cytotoxin. Here we investigate the enzymology of AQ4N reduction by cytochrome P450 (CYP). Using a panel of human liver microsomes phenotyped for various CYP activity, metabolism was correlated with two markers of CYP 3A activity, namely benzoxylresorufin O-debenzylation ($r=0.88$, $P<0.001$, $n=17$) and tamoxifen N-demethylation ($r=0.79$, $P<0.01$, $n=17$). Metabolism under nitrogen was completely inhibited in the presence of ketoconazole and CO used as global inhibitors of CYP and 88% inhibited (n=3) by troleandomycin, a CYP 3A inhibitor. In *ex vivo* murine T50/80, SCCVII and RIF-1 tumours known to be responsive to AQ4N, CYP3A was identified by western blotting. Hplc analysis showed AQ4N was reduced in S9 preparations of these tumours incubated in nitrogen with a half life of 26 min, 42 min and 43 min respectively. Reductive metabolism of AQ4N was increased significantly in S9 fractions from RIF-1 tumour cells transfected with CYP3A4. The results show that CYP3A is involved in AQ4N reduction and is implicated in the bioactivation of this prodrug in hypoxic tumours to generate the active cytotoxin. This has important implications for the potential clinical use of AQ4N since freshly resected human neoplasms have been shown to express CYP3A.

516 The mechanism of irreversible inhibition of human cytochrome $P450_{17\alpha}$ by abiraterone, a potential new drug for the treatment of prostate cancer

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Abiraterone (17-(3-pyridyl)androsta-5,16-dien-3β-ol) is a potent inhibitor of human testicular cytochrome $P450_{17\alpha}$, with an IC_{50} for the hydroxylase step of 4 nM. As its acetate prodrug, it is under development as a potential new treatment for prostatic carcinoma. A potentially important property of abiraterone is its slow, irreversible binding to the target enzyme and the present study aimed to gain insight into the structural feature(s) responsible. Thus maximal inhibition by abiraterone, by the equipotent 4,16-dien-3-one analogue and by the less potent (IC_{50} 13 nM) 3,5,16-triene was slow to occur under the standard conditions of the assay, in which the enzyme was added to the incubation medium containing substrate and inhibitor. However it was instantly achieved by first preincubating abiraterone with the enzyme in the absence of substrate at 37 °C. Inhibition following preincubation with these compounds was not lessened by dialysis for 24 h, indicative of irreversible binding to the enzyme. In contrast the inhibition by structurally related compounds lacking the 16,17-double bond, namely the potent (IC_{50} 27 nM) inhibitor 17α-(4-pyridyl)androst-5-en-3β-ol, and the less potent 17β-3- and 4-pyridyl analogues (respective IC_{50} values 47 and 160 nM), was not enhanced by preincubation of the inhibitor with the enzyme. Furthermore, in contrast to the compounds with the 16,17-double bond, the inhibition of the enzymic reaction by the 17α-4-pyridyl compound was reversed by 24 h dialysis. The results show that the 16,17-double bond is necessary for irreversible binding of these pyridyl steroids to cytochrome $P450_{17\alpha}$.

517 Regulation of sensitivity to cisplatin and platinum-containing drugs by repair and checkpoint control genes in *Schizosaccharomyces pombe*

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The role of genes that affect sensitivity to radiation in determining sensitivity to cisplatin and Pt-containing drug was examined on a panel of strains of *Schizosaccharomyces pombe*. Such strains were hypersensitive to radiation and contained specific mutations in genes involved in DNA damage repair and control of cellular checkpoints. The studied Pt compounds, including JM216, JM335, BBR3464, tetraplatin and oxaliplatin, were chosen since their different structure is likely to induce different lesions in DNA. The cytotoxic effect of the drugs was evaluated using a microtiter growth inhibition assay with a 48-h drug exposure.The mutants fell into 3 groups with respect to sensitivity to cisplatin: 3 mutants (rad1, rad3, and rad18) were extremely hypersensitive (30–58-fold), 15 (rad4–6, 8–10, 12–14, 16–17, 19–21 and 22) were hypersensitive (5–22-fold), and 4 (rad2, 7, 11, 15) demonstrated no change in sensitivity. No mutants were hypersensitive to JM216, whereas a moderate increase in sensitivity to JM335 was observed for most of the mutants and hypersensitivity to BBR3464 was found in rad1 and rad3. Rad mutations did not influence sensitivity to tetraplatin. With the exception of rad 2, 7 and 15, all the mutants were hypersensitive to oxaliplatin. The pattern of cell response was drug-specific, thus providing genetic evidence that different mechanisms are involved in differential cytotoxicity induced by Pt compounds. In particular, the results support that the rad1 and rad3 gene products, which are involved in cell cycle control and DNA repair, are important determinants of sensitivity to cisplatin and other Pt-containing drugs including JM335, BBR3464 and oxaliplatin.

518 The phosphoinositol 3-kinase inhibitor wortmannin sensitises MCF7 cells to ionising radiation and to the topoisomerase I inhibitor topotecan

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The phosphoinositol 3-kinase gene family includes the ataxia telangiectasia gene and DNA dependent protein kinase (DNA-PK). Wortmannin is widely used as a potent inhibitor of phosphoinositol 3-kinase. However, it is also an inhibitor of DNA-PK which is known to mediate repair of DNA double strand breaks. Wortmannin has been shown to sensitise cells to ionising radiation. Sensitisation is thought to be due to inhibition of DNA-PK since it is associated with a reduction in the repair of radiation induced DNA double strand breaks. Since the cytotoxicity of DNA topoisomerase I inhibitors is thought to be due to the formation of DNA double strand breaks we have investigated the effects of wortmannin on the topotecan sensitivity of a human breast tumour cell line MCF7.

Cells were exposed to wortmannin for 24 hours commencing 1 hour before exposure to the cytotoxic drug or to radiation. We have confirmed that wortmannin (4 μM) sensitises MCF7 cells to radiation showing a two fold increase in cell kill at 2 Gy. For topotecan, sensitivity to a 1 hour drug exposure was increased by 10 fold in the presence of a non-toxic concentration of wortmannin (2 μM). The drug combination was confirmed as synergistic by isobologram analysis. Synergy was also observed with bleomycin which is known to cause DNA double strand breaks. In contrast, wortmannin did not sensitise cells to two agents for which double strand breaks are not the major cytotoxic event, cisplatin and taxol. A detailed isobologram analysis revealed additive toxicity with these two drugs.

We have demonstrated a clear synergistic interaction between wortmannin andtopotecan. The basis of the synergy is not known but the pattern of druginteractions observed is consistent with the suggestion that wortmannin acts by inhibition of DNA-PK. Phosphoinositol 3-kinase inhibitors have been proposed as new class of radiosensitisers that inhibit DNA repair. Our results suggest that they may have a role as chemosensitisers as well.

519 Doranidazole, a novel hypoxic cell radiosensitizer: Non-clinical studies

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Doranidazole (PR-350) {○-(2RS,3SR)-3-[(2-nitroimidazole-1-yl)methoxy]butane-1,2,4-triol}, a 2-nitroimidazole nucleoside analog, is a potent hypoxic cell radiosensitizer, which was newly developed in Japan.

It is a racemic compound with two asymmetric centers in acyclic sugar moiety. The value of LD50 for PR-350 was the highest compared with those of other 2-nitroimidazole analogues and the animal toxicities were found much reduced than the others.

Pharmacokinetic studies indicated that PR-350 was excreted rapidly from blood and a good linear regression (r = 0.995) was obtained. The major excretory organ was the kidney. The distribution of this sensitizer in the central nervous system and peripheral nerves was less than that in other organs.

Radiosensitizing effects were observed in two murine tumor cells; mammary-carcinoma (EMT-6) and squamous cell carcinoma (SCCin the *in vitro* and *in vivo–vitro* clonogenic assays. A sensitizer enhancement ratio (SER) of PR-350 was more than 1.3. Tumor growth delay was recognized in SCC tumor *in vivo*. Furthermore, a similar tumor growth inhibition was also observed in a xenograft model which was consisting of human pancreatic cancer cells implanted into the pancreas of nude mice *in vivo*.

Regarding apoptotic morphological changes such as chromatin condensation and DNA fragmentation, radiation-induced apoptotic cells were increased by the addition of PR-350 under hypoxic conditions *in vitro*. One of its radiosensitizing mechanisms is likely to be the induction of apoptosis.

The phase I clinical trial is undergoing in Japan and no severe adverse effects have been reported.

520 The oral platinum analogue JM216 is a radiosensitizer

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Previous studies *in vitro* and *in vivo* have shown that there is a potential interaction between the combination of platinum compounds and radiation (Begg et al., 1986 and Schaake et al., 1992). The nephro- and neurotoxicity of cisplatin is a clinical problem. The oral platinum compound JM216 has been reported to show no nephro- and neurotoxicity (Kelland et al., 1994). The absence of toxicity and the preference for oral administration led us to design a study to compare radiosensitization by JM216 and cisplatin. RIF1 mouse tumour cells were treated with various doses and various exposure times with JM216 and irradiated 15 minutes before the end of the drug exposure. The fraction of cells surviving the treatment was assessed by colony formation. Results were compared with those for equivalent treatments with cisplatin.

JM216 alone showed exponential killing of RIF1 cells, being approximately three times less efficient than cisplatin on a molar basis. For radiosensitization studies, drug doses were used which gave approximately 50% or 90% cell killing. No radiosensitization was seen after two-hour drug exposures, but significant radiosensitization occurred after exposures of 1 h and 0.5 h (shorter times required proportionally higher drug doses, giving equivalent drug kill). The enhancement ratio and time dependence was similar for the two platinum compounds, reaching 1.5 for the magnitude of radiopotentiation at the highest concentrations tested.

Drug-DNA adduct formation was assessed using immunocytochemistry with an NKI-A59 antiserum raised to cisplatin-DNA adducts. After showing that the antiserum recognized JM216-DNA adducts in a dose-dependent manner, maximum nuclear staining was found to be correlated with cell kill for both drugs However, the levels of staining did not correlate with radiosensitization, neither at the time of irradiation nor at the time of maximum adducts, indicating that the numbers of DNA adducts did not determine radiosensitization.

In summary, JM216 was shown to be capable of radiosensitizing a platinum-sensitive tumour line to an extent similar to cisplatin. Radiosensitization was exposure time and concentration-dependent, but not dependent on DNA adduct levels or glutathione depletion. These data suggest that JM216 would be a suitable replacement for cisplatin as oral administrable platinum complex for combined radiotherapy-chemotherapy phase I studies.

521 Radiosensitisation of cultured human glioblastoma cells through inhibition of protein kinase C activity

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Background: The protein kinase C (PKC) system has been recognised to play an important role in the protection of mammalian cells from a variety of apoptotic stimuli, including ionising radiation. PKC activity levels in glioma cells are often higher than in non-transformed glia, which may not only be associated with their faster growth, but also with their inherent radioresistance. Thus, inhibiting PKC activity may restore the apoptotic response to γ-radiation, (re)sensitising malignant gliomas to this treatment modality.

Aim: In this study, we tested the above mentioned hypothesis using a panel of human glioblastoma cell lines.

Methodology: U-373, U-138, and U-87 cells were treated for 4 to 24 hours with the PKC-inhibiting agents tamoxifen, staurosporine, or calphostin C, and then γ-irradiated in the presence of the drugs. Following culturing for 72 additional hours with the drugs, the cell samples were assessed for growth inhibition through sulforhodamine B staining, as well as for PKC activity through histone phosphorylation. Parallel incubations with the PKC-activating agent phorbol 12-myristate 13-acetate (PMA) were used as positive controls.

Results: D_0 values (radiation doses yielding 37% cell survival) in untreated U-373, U-138, and U-87 cells, were about 10, 12, and 12 Gy, respectively. Pre-treatment for 24 hours with tamoxien, staurosporine, or calphostin C at their IC_{50}s, decreased these values about 4-fold. Concomitant measurements of total PKC activities in these cell samples showed a decrease by 80 to 90% when compared to untreated controls (PKC activities of about 5.5, 4.5, and 4.0 nmoles ATP/minute/mg protein in U-373, U-138, and U-87 cells, respectively). On the other hand, pre-treatment with PMA at concentrations as low as 10 nM, stimulated PKC activities up to 3-fold of control levels, and did not significantly affect the D_0 values found for untreated cells.

Conclusion: Our data indicate, that inhibition of PKC activity can sensitise glioma cells to γ-irradiation, suggesting a rationale for pharmacological manipulation of PKC in radiotherapeutic treatment of these tumour types.

522 Olomoucine derived synthetic CDK inhibitors: Selective induction of apoptotic death in tumor cells and *in vivo* anti-tumor activity are mediated via direct inhibition of cdk7

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Studying a new generation of plant hormones – cytokinins – we have identified a group of tri-substituted adenine derivatives with previously reported inhibitory effect on cdc2 family of cyclin dependent kinases (CDKs). The study was performed on olomoucine, roscovitine and a new compound 2-[(3-hydroxypropyl)amino]-6-benzylamino)-9-isopropylpurine, which was named bohemine (BOH). Although these drugs failed to down regulate cdk4 activity *in vitro*, cdk4 has been inhibited under *in vivo* conditions due to direct inhibition of cdk7, and correspondingly, cdk7-mediated activating phosphorylation of cdk4. The olomoucine (OC) derived synthetic cyclin dependent kinase inhibitors (CDKIs) induced apoptosis in many tumor and leukemic cell lines *in vitro* at 1–30 μM concentrations, while the non-malignant cells survived concentrations above 250 μM. Compounds-induced apoptosis was independent of nucleo/proteosynthesis *de novo*, however, it was inhibited by 1) YVAD peptide, a specific inhibitor of ICE proteases, and ocadaic acid, an inhibitor of cellular phosphatases. The activation of ICE proteases during apoptosis was further confirmed by lamin B and RB proteins cleavage. To analyze the mechanism of drug resistance to CDKIs, CEM T-lymphoblasts were treated with gradually increasing subtoxic doses of BOH and drug resistant sub-lines were prepared. These cultures demonstrated unique mechanisms of resistance: 1) decreased proliferation, 2) induction of cellular senescence, 3) inhibition of cdc2 and cdk7 expression, 4) restoration of $p21^{CIP1}$ expression, 5) decrease in DNA-polymerase, and 6) DNA-telomerase activities. Using the mouse model of P388D1 leukemia and B16 melanoma we demonstrated ability of this family of CDKIs to increase medium survival time, or eventually to cure the animals with transplanted tumors. We expect that this new generation of potent anti-cancer drugs with unique mechanism of action could provide significant benefit to cancer patients in the future.

523 O^6-alkylguanines as selective inhibitors of cyclin dependent kinases

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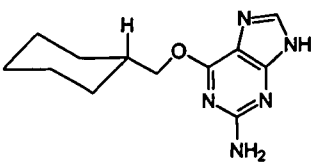

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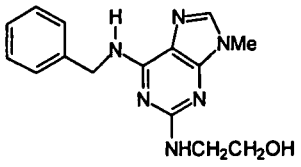

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The cyclin dependent kinases (CDKs) are a family of protein kinases which regulate cell cycle progression in proliferating eukaryotic cells (Doree & Galas, FASEB J 8: 1114, 1994). Cyclin over-expression, loss of endogenous CDK inhibitor function, and CDK substrate alterations, are common genetic changes in human tumours which result in aberrant CDK control and loss of cell cycle checkpoint function. CDKs thus represent potentially important new targets for cancer chemotherapy (Meijer, Trends in Cell Biology 6: 393, 1996). We have identified a series of purine-based CDK inhibitors exemplified by NU2058 (O^6-cyclohexylmethylguanine; 1). This compound exhibits inhibitory activity against CDK1 comparable to the benchmark purine olomoucine 2 (IC_{50} = 7 μM), but is at least 50-fold more active against CDK4 (IC_{50} values 20 μM and > 1 mM, respectively). Crystal structures of selected compounds bound to CDK2 have been determined and observed to bind in a different orientation from that of olomoucine, with the O^6-alkyl substituents overlapping the ribose-binding domain of the substrate ATP. A series of conserved hydrogen bonding contacts between the purine ring and the protein are also evident. This information is being utilised to guide the design of more potent and specific CDK inhibitors.

524 Human recombinant (hr) *polo*-like kinase phosphorylates hr cdc25C which regulates the transition of cells into M phase of the cell cycle

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Cell entry into mitosis is controlled by the cdc2 and cyclin B holoenzyme, which is highly regulated through reversible phosphorylation events. In particular, cdc2 kinase activation is dependent upon the specific dephosphorylation of both the Tyr 14 and Thr 15 residues by the cdc25 phosphatase. The *polo*-like kinases are a family of serine/threonine protein kinases which also play a role in the regulation of mitotic progression. The human *polo* homolog, PLK1, has been cloned but its exact mechanism remains unclear. Recently, Dunphy et al. (Science 273: 1377–1380, 1996) demonstrated that the *Xenopus* homolog, PLX1, positively regulates the Xenopus cdc25C phosphatase via phosphorylation of the amino terminus, leading to increased activation of the cdc2 kinase. To evaluate the role of human PLK-1, human recombinant (hr) PLK and hr cdc25C were expressed with GST tags and purified. Multi-tissue Northern blots showed human PLK1 expression in all cancer cell lines, e.g., HL-60, HelaS3, K-562, MOLT-4, Raji, SW480, A549, and G361 and in adult human testis and fetal liver. Under identical conditions, no expression was noted in adult spleen, thymus, colon, small intestine, ovary, prostate, heart, liver, brain, lung, skeletal muscle, pancreas or kidney. Jurkats and U937 cells were chemically arrested in G_1/S, S or G_2/M. PLK 1 protein appeared low in both G_1/S and S fractions but was highly present and phosphorylated at G_2/M phase. In an *in vitro*, solution-based kinase assay, hr PLK1 efficiently phosphorylated hr cdc25C. A solid-phase flashplate assay was configured for high throughput screening, and initial characterization revealed that the enzyme was inhibited by the cdk inhibitor, olomoucine (–50% at 20 μM), as well the p38 inhibitor, SB203580 (–50% at 1 μM), but not by either the PKA inhibitor, HT-89 (<100 μM), or the tyrosine kinase inhibitor, lavendustin A (<100 μM). Together, these data demonstrate that human PLK 1 phosphorylates cdc25C and as such, its inhibition should interfere with cell cycle progression, providing an intriguing target for identifying novel anti-cancer therapeutic agents.

525 Characterization of covalent binding to DNA of antitumor imidazoacridinone C-1311, after metabolic activation

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Imidazoacridinones are new antitumor compounds developed in our laboratory (Cholody et al., J. Med. Chem., 1992, 35, 378). They undergo intensive preclinical studies and one of them, C-1311, is in the first phase of clinical trials. We previously demonstrated that C-1311 induced formation of DNA interstrand crosslinks in tumor cells, in a dose dependent manner (Dziegielewski & Konopa, 87th AACR Meeting, 1996, abst. 2800). To extend our knowledge on imidazoacridinones interactions with DNA, we studied in more detail irreversible binding of C-1311 to DNA in noncellular and cellular systems.

Calf thymus DNA was incubated with C-1311 for 3 hours in the presence of horseradish peroxidase (Mazerska et al., Exp. Toxic. Pathol., 1996, 48, 374). Drug-treated DNA was purified by proteinase K digestion, phenol-chloroform-ethyl ether extraction and ethanol precipitation – under these conditions only irreversibly complexed compound remains attached to DNA. To quantify the amount of covalently bound C-1311 we employed fluorescence measurements of samples at the wavelength specific for the imidazoacridinone fluorochrome. The level of fluorescence intensity of modified DNA depended on C-1311 concentration, incubation time and reaction cofactors. Under the optimal conditions one C-1311 molecule binds per 30 base pairs. DNA incubated with the drug and without activation system displayed negligible fluorescence. Irreversible, covalent binding of C-1311 to DNA is thermally unstable.

In tumor cells treated with C-1311, at optimal conditions, one drug molecule binds per 300 DNA base pairs, as estimated by the same procedure. No binding to DNA was observed in cell lysates with inactivated enzymes. In conclusion, metabolically activated C-1311 binds irreversibly, presumably covalently, to DNA in non-cellular and cellular systems.

526 Inefficient cellular repair of DNA adducts induced by *cis*-bis-neodecanoato-trans-R,R-1,2-diamminocyclohexane platinum (II)

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NDDP [*cis*-bis-neodecanoato-trans-R,R-1,2-diamminocyclohexane-platinum (II)] is a lipophilic cisplatin derivative of the 1,2-diammino-cyclohexane family currently in phase II clinical trials in a liposomal formulation (L-NDDP). L-NDDP is non-nephrotoxic and non-cross-resistant with cisplatin (CDDP) in several mouse and human tumor models. The mechanism for the lack of cross resistance remains unclear. Previous studies have shown that at equimolar concentrations, L-NDDP induces the formation of more DNA adducts than CDDP in whole cell systems. (Han, Cancer Res. 53: 4913–4919, 1993). This may explain the lack of cross-resistance to L-NDDP. Liposomes are known to alter the cellular uptake and subcellular distribution of drugs and DNA adduct formation is inversely correlated with cellular repair activity. Therefore, it is important to determine whether the enhanced cellular susceptibility to the formation of NDDP-induced adducts is attributable to liposome entrapment or cellular inefficiency in repairing the adducts. The purpose of the present work was to determine how efficiently cells repair NDDP-induced adducts versus CDDP-induced adducts. An *in vitro* repair assay employing whole-cell extracts from repair-competent SKOV3 cells and NDDP-damaged pBS plasmid was used to measure the repair of NDDP-induced adducts. The repair activity was reflected by the specific incorporation of [α-^{32}P]dAMP into damaged pBS during DNA re-synthesis. To prepare the damaged plasmid substrate, pBS was incubated with either NDDP or CDDP (control) at room temperature overnight using a molar ratio of drug to nucleotide ranging from 0.0025 to 0.02. The total platinum content in the damaged pBS was then determined by flameless atomic absorption spectrophotometry. The results of the *in vitro* assay showed that NDDP-induced adducts were repaired with a significantly lower efficiency ($26 \pm 2 \times 10^{-26}$ mol dAMP/DNA molecule), than CDDP-induced adducts ($181 \pm 15 \times 10^{-26}$ mol/ DNA molecule). These data suggest that the lower efficiency of cells in repairing NDDP-induced DNA adducts may be an important factor in the antitumor effectiveness of NDDP against CDDP-resistant cells.

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527 Site- and region-specificity of tallimustine lesions in cellular DNA

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Tallimustine (FCE 24517) is a benzoic acid mustard (BAM) derivative of distamycin, currently in clinical trials. Tallimustine retains distamycin's AT-specific minor groove binding to DNA, while the BAM moiety can alkylate the N3 of purine residues with a consensus sequence of 5′-TTTTPuA-3′ established in naked DNA. Similar sites were suggested in intact cells, although tallimustine binding to cellular DNA has been less extensively investigated. In this study, tallimustine action on intracellular DNA was examined and compared to an unrelated AT-specific drug, bizelesin, which prefers 5′-(A/T)$_5$A-3′ motifs. Sites of drug-induced lesions were analyzed by the repetitive primer extension in several regions of simian virus 40 (SV40) DNA isolated from drug-treated, virus- infected BSC-1 cells. Of the four strongest drug-adduct sites identified, three conformed to the consensus sequence of 5′-TTTTGPu-3′. One such site (TTTTTGC) was also an atypical bizelesin site. Non-classical yet relatively strong sites were seen at ATTTGPu and AGTTGPu. Among other moderate to weak sites such as CTTTGG, ATTTGT, and AATTGT, the minimum shared motif seemed to be TTG. Another moderate site (TTTTTAC) was shared with bizelesin. Additional non-consensus sites included a multi-site seen at CCCCAAA, as well as a weaker site at GACCAA. Both these sites were opposite strong consensus sites on the complementary DNA strand, suggestive of interstrand DNA crosslinks by the BAM moiety. However, no evidence of crosslinks was seen in genomic DNA of nuclei treated with up to 20 μM tallimustine. The same treatment conditions produced a strong evidence of drug lesions in nuclear DNA monitored as thermally-inducible strand breaks. Further studies by quantitative PCR stop assay examined region-specific lesions induced by tallimustine in drug-treated COLO320DM cells. Tallimustine lesions were detected in such regions as c-myc MAR, c-myc ORI, and an AT island in GenBank sequence Z79699 (0.85, 0.66, and 0.94 lesions/kbp/μM, respectively). In comparisons with equimolar bizelesin, however, tallimustine induced 3.4, 3.0 and 4.6-fold fewer lesions, respectively. Thus, the relative vulnerability of the tested regions to both drugs was similar. However, tallimustine was at least 1000-fold less cytotoxic than bizelesin, with an IC_{50} in the MTT assay of 13 μM, compared to an IC_{50} of 7 nM for bizelesin. Our results suggest that either tallimustine lesions are less cytotoxic than bizelesin lesions and/or the sites typically modified by tallimustine are less critical for cell growth than bizelesin binding motifs.

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528 Targeting of AT-rich islands in genomic DNA by bizelesin, an AT-specific DNA-reactive antitumor drug

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A non-random distribution of DNA lesions may be important for the antiproliferative action of sequence-specific DNA-targeting agents such as bizelesin, a cyclopropylpyrroleindole (CPI) AT-specific drug, currently in clinical trials. Our previous studies demonstrated bizelesin preference for $T(A/T)_4A$ sites in cellular DNA. In this investigation, we combined the following approaches. First, a long-range search for the distribution of bizelesin binding motifs in human sequences was performed. Next, actual region-specific lesions in cellular DNA were selectively determined by QPCR stop assay and compared to lesions bulk DNA in CEM and Colo320DM cells. Analysis of >24 Mbp of human genome sequences for the distribution of $T(A/T)_4A$ motifs showed, on average, 11 hits/kbp. However, ~130 loci were identified which contained clusters of potential bizelesin binding sites, with local motif density from 120 up to 400 hits/kbp. These loci typically span 200–600 bp and consist of $(A/T)_n$, where $n \approx 20$. Experimental data on lesion frequencies in CEM cells, obtained for selected loci, generally corresponded to predictions. Apo B MAR, a locus shown to be most vulnerable to bizelesin (with ~4.4 lesions/kbp/μM), also scored high in the search with a local density of ~280 hits/kbp. The highest-scoring locus in the search (GenBank Z79699, positions ~37–38 k, ~400 hits/kbp) was confirmed to be highly affected by bizelesin with ~3.3 actual lesions/kbp/μM. Another bizelesin-sensitive region, *c-myc* MAR (~1.7 lesions/kbp/μM), was identified by the search but with relatively modest ~50 hits/kbp. Several loci not identified by search showed only low levels of DNA lesions (e.g., <0.4 lesions/kbp/μM in a β-globin locus). Likewise, the overall lesion frequency in bulk cellular DNA (~0.8 lesions/kbp/μM) was lower than in the AT-rich loci. The identified AT islands may represent a subset of MARs, i.e., sites which presumably define the borders of DNA loops and organize their attachment to the nuclear matrix. The distribution of potential bizelesin binding sites was matched closely by loci identified as likely MAR candidates using the MAR-Finder algorithm (Kramer et al., Genomics, 33, 302, 1997). The identified AT-rich repetitive sequences could be an important target for bizelesin. Thus, our findings demonstrate for the first time that if drug binding motif clusters in repetitive sequences, small molecules of a *non-oligonucleotide* type are capable of region-specific DNA lesions.

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529 PNU 151807 (α-bromoacryloyl distamycin A): A new non-covalent minor groove DNA binder with antineoplastic activity

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PNU 151807 is a new synthetic α-bromoacryloyl derivative of distamycin A. We have investigated the DNA interaction and the mechanism of action of this compound in parallel with the distamycin alkylating derivative tallimustine. PNU 151807 possesses a good cytotoxic activity in *in vitro* growing cancer cells, even superior to that found for tallimustine. By footprinting experiments we found that PNU 151807 and tallimustine interact non covalently with the same AT rich-DNA regions. However differently from tallimustine, PNU 151807 failed to produce any DNA alkylation as assessed by Taq stop assays and N3 or N7-adenine alkylation assays in different DNA sequences.

PNU 151807, like tallimustine, is able to induce an activation of p53, and consequently of p21 and BAX in a human ovarian cancer cell line (A2780) expressing wt p53. However, in A2780/E6 cells, derived from the parental A2780 by transfection with a plasmid encoding for the HPV16-E6 (which causes disruption of p53 function), the lack of wt p53 does not significantly modify the cytotoxic activity of the compound. Flow cytometric analysis of cells treated with equitoxic concentrations of PNU 151807 and tallimustine showed a similar induction of accumulation of cells in the G2 phase of the cell cycle but with a different time course. When tested against recombinant proteins, only the compound PNU 151807 (and not tallimustine or distamycin A) is able to abolish the *in vitro* kinase activity of CDK2-cyclin A, CDK2-cyclin E and cdc2-cyclin B complexes. The results obtained showed that the compound seems to have a mechanism of action completely different from that of its parent compund tallimustine and clearly indicate PNU 151807 as a new promising non-covalent minor groove binder with cytotoxic activity against cancer cells.

530 An Ecteinascidin-DNA hydrogen bond network plays a major role in determining DNA sequence specificity of Et 736 and Et 743 for their favored three base target sequences

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The marine natural product, Ecteinascidin 736 (Et 736), differs from Et 743 by the substitution of a tetrahydro-b-carboline C-subunit for a tetrahydroisoquinoline C-subunit, a substitution that results in reduced biological activity. The more active Et 743 is currently in phase I clinical trials involving different solid tumor types. We undertook parallel structural and modeling studies of Et 736 and Et 743 adducts of the same DNA sequence in order to ascertain the structural basis for the activity differences. Aside from the C-subunit-based differences, there were similarities including parallel drug-DNA hydrogen bonding patterns. We propose that this parallel hydrogen bonding network linking the common Et 736 and Et 743 A- and B-subunits to a three base-pair region is the major factor governing sequence recognition and reactivity. In order to test this sequence recognition proposal our structural results were compared to the findings of an earlier sequence selectivity analysis (Yves Pommier and co-workers). The possibility that a unique hydrogen bonding network directs the course of sequence recognition was examined by first using DNA adduct NMR properties to characterize the hydrogen bonding network. Using these experimental findings, DNA 12-mer models containing either of the favored sequences, 5'-AGC-3' or 5'-CGG-3', associated with Et 736 (binding or covalent form) were examined by molecular dynamics (MD) in order to test the stability of the resulting conformations and the individual network hydrogen bonds. In conclusion, the modeling results confirm that parallel hydrogen bonding specific to the 5'-AGC and 5'-CGG sequences provides a rationale for the rules that govern sequence specificity. These rules are as follows: For the target sequence, 5'-XGY-3', the favored base to the 3'-side, Y, is either G or C. When Y is G, then a pyrimidine base (T or C) is favored for X. When Y is C, a purine (A or G) is favored for X. MD hydrogen bonding analysis demonstrates that these favored sequences provide the optimal donor/acceptor positions for maximizing the number of ecteinascidin drug-DNA hydrogen bonds prior to covalent attachment.

531 Protein-linked DNA breaks induced by Ecteinascidin 743 in human colon carcinoma cells

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Ecteinascidin 743 (Et743) is a potent antitumor agent presently in phase I clinical trials. Et743 has been shown recently to alkylate selectively guanine N_2 from the DNA minor groove. This alkylation can only take place in duplex DNA and is reversed by DNA denaturation (Biochemistry, 1996, 35: 13303–9). This differentiates Et743 from other DNA alkylating agents presently in the clinic. We characterized cellular DNA damage, cell cycle response and cytotoxicity of Et743 in human colon carcinoma cell lines. Et743 exhibited an IC50 in the low nanomolar range indicative of its cytotoxic potency and produced cell cycle arrest in G2. Alkaline elution experiments demonstrated that exposure of human colon carcinoma HCT-116 cells to micromolar concentrations of Et743 produced DNA protein-crosslinks and DNA single-strand breaks. These lesions were temperature-sensitive and were not formed at 0 °C. Furthermore, the DNA single-strand breaks were protein-concealed as they were not detectable under non-deproteinizing conditions. Experiments using radiolabeled oligonucleotides indicated that a single protein specie was specifically crosslinked to DNA in the presence of Et743. The identity of the crosslinked protein is yet unknown. It does not appear to be topoisomerase I or II. These data indicate that minor groove DNA alkylation by Et743 induces the formation of protein-linked DNA breaks, G2 arrest and apoptosis in human colon carcinoma cells.

532 Mechanism for the catalytic activation of Ecteinascidin 743 and its subsequent alkylation of guanine N2

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Ecteinascidin 743 (Et 743) is the first of a novel class of marine alkaloids to be introduced into phase 1 clinical trials against a variety of solid tumor types. These alkaloids are believed to exert *in vivo* activity, at least in part, via interactions with duplex DNA. We have previously reported an NMR-based model of Et 743 bound to a 12-mer oligonucleotide; now the protonation state of the drug and DNA in the covalent adduct is reported. Reaction of Et 743 with an isotopically labeled (13C, 15N) and a natural abundance 12-mer oligonucleotide containing an AGC alkylation site yielded a stable GN2-alkylated Et 743-DNA adduct. Two-dimensional NMR spectroscopy (NOESY, COSY, TOCSY) of the natural abundance Et 743-DNA adduct in H2O-D2O (9 : 1) demonstrated that N12 of Et 743 is the only site of protonation of the drug. The labeled Et 743-DNA adduct was studied using HMBC and HMQC NMR spectroscopy, and the results indicated that the DNA bases were not protonated in the covalent adduct. These data, in combination with earlier results, were employed to propose a specific mechanism for the catalytic activation of the carbinolamine and subsequent alkylation of GN2 by the iminium intermediate. Our mechanism utilizes the proton on 12N of the free Et 743 to activate the carbinolamine for formation of an iminium intermediate. This intermediate alkylates DNA, which is followed by a water mediated proton transfer of one of the GN2H protons back to 12N of Et 743. These data provide information regarding the chemical and structural features of Et 743 that is required for *in vivo* activity. In general, these results suggest a general mechanism for the catalytic activation of other carbinolamine-containing antibiotics.

533 *In vitro* cytotoxicity of Ecteinascidin 743 (ET-743) against human hemopoietic progenitors and cancer cells

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ET-743 is a marine compound with promising *in vitro* and *in vivo* antitumour activity and phase I evaluation of different schedules of administration is ongoing. The aim of the present study was to compare the cytotoxicity of different schedules of exposure of ET-743 on human hemopoietic progenitors and tumour cell lines.

Hemopoietic progenitors from human cord blood were incubated with ET-743 for 1 hour (h), 24 h or 1 h daily × 5. Additional experiments were performed to assess if incubation for 24 h or 5 days could change the biology or sensitivity of cells or the activity of ET-743. Nine solid tumour cell lines were also incubated for 1 h or 24 h, then assayed on clonogenic assays to obtain by comparison an '*in vitro* therapeutic index' (TI).

We observed that the 24 h and the daily × 5 schedules were more toxic (GM-CFC ID70) as compared to 1 h exposure (3.1 and 3.2 ng/ml versus 9.2 ng/ml). More differentiated cells (colonies scored at 7 days incubation) were more sensitive than immature progenitors (scored after 14 days). ET-743 lost 30% of its inhibiting capacity when left for 24 h under culture conditions. Incubated cells were more resistant to the drug than fresh cells, but cells incubated after treatment were much more sensitive. When applying these observations to the schedule-dependency data, it can be extrapolated that ET-743 myelotoxicity is AUC dependent.

On tumour cell lines the prolonged exposure showed an increased activity, with more favorable TI, as compared to the 1 h exposure; this result was even more striking for the ovarian cancer cell lines OVCAR-3 and IGROV-1 (TI: 6.3 and 8.3 at 24 h versus 0.9 and 1.7 at 1 h).

We conclude that prolonged exposure might be the most promising schedule of ET-743 for clinical evaluations and that ovarian cancer might be a good candidate for early clinical studies.

534 Effect of Ecteinascidin-743 (ET-743) on the interaction between transcription factors and DNA

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ET-743 is a tetrahydroisoquinoline alkaloid isolated from *Ecteinascidia turbinata*, a tunicate growing in mangrove roots in the Caribbean Sea. It has been shown that it binds in the minor groove of DNA forming covalent adducts by reaction of the N2 of guanine with the carbinolamine moiety. We investigated ET-743 ability to inhibit the binding of different transcription factors to their consensus sequences by using gel shift assays. We have selected 3 types of factors such as I) oncogene products such as Myc, c-Myb and Maf, II) transcriptional activators regulated during the cell cycle as E2F and SRF and III) general transcription factors as TBP, Sp1 and NF-Y. We observed no inhibition of the binding of some factors such as Sp1, Maf, Myb and Myc. Inhibition of DNA binding was observed for TBP, E2F, SRF at ET-743 concentrations ranging from 50 to 300 μM. The inhibition of binding of NF-Y occurs even at lower concentration (i.e. 10–30 μM) when the recombinant subunits of NF-Y were preincubated with the drug, indicating that the inhibition of NF-Y binding does not require previous ET-743 DNA binding. Since NF-Y is a trimer containing two sub-units with high resemblance to histones H2B and H2A we have investigated the effect of ET-743 on nucleosome reconstitution. ET-743 caused a decrease of the nucleosomal band already at 100 nM with the complete disappearance of the band at 3–10 μM. Although the precise mechanism of inhibition of ET-743 requires further studies it seems attractive to speculate that the mode of action of this novel anticancer drug is related to its ability to modify the interaction between some DNA binding proteins and DNA.

535 Mode of action of Ecteinascidin 743, a natural marine compound with antitumoral activity

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Ecteinascidin 743 (ET743) is a natural marine compound isolated from the Caribbean tunicate *Ecteinascidia turbinata*. ET743 was found to bind to minor groove of DNA forming adducts at N2 position of guanine (Pommier, Bioch. 1996; Hurley, J. Am. Chem. 1997). The precise mechanism of cytotoxicity of ET743 is unknown. ET743 does not inhibit DNA topoisomerase I and II and does not cause DNA breakage or DNA intrastrand cross links as assessed by alkaline elution methods. The clonogenic potential inhibitory effect of ET743 was investigated on IGROV-1, OVCAR-3, SW626 ovarian cancer, LoVo, SW620 colon cancer, A549 small cell lung cancer. A375M melanoma and MCF7 breast cancer cell lines. IC 50 ranged from 1 to 144 nM for 1 h exposure and from 0.06 to 27.4 nM for 24 h exposure. By biparametric BrdU/DNA flow cytometric analysis we detected cell cycle phase perturbation induced by 1 h ET743 exposure at equitoxic concentration on LoVo and SW620 colon cancer cell lines. ET743 caused an important accumulation of cells in S phase and a dramatic block in the G2M phase of the cell cycle. In addition, in various cancer cell lines ET743 induced apoptosis evaluated by morphological and biochemical assays. ET743 was much less active against MDR cancer cells expressing Pgp. Prolonged exposure of IGROV-1 and LoVo cancer cells to ET743 induced resistance to ET743 associated with overexpression of Pgp. Studies are in progress to evaluate the mechanism of cell cycle perturbations and cell death induced by ET743 in cancer cell lines and in tumor xenografts.

536 *In vitro* telomerase inhibitors in human cancer models using a biotinylated-primer extension assay

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We characterized the telomerase activity *in vitro* and *in vivo* in human cancer cells using a non-amplified primer extension assay which allows direct measurement of telomerase through its incorporation of a labeled nucleotide without PCR amplification. A ^{32}P-dGTP labeled telomerase-extended 5'-biotinylated $(TTAGGG)_3$ primer was separated using streptavidin-coated magnetic beads. This procedure improved the characterization and the quantification of the banding pattern resulting from telomerase extension by reducing the radioactive background. Telomerase activities (31.8 ± 24.9% of that of HeLa cells, range: 0–100) and telomere lengths (6.24 ± 6.16, range: 3.84–33.5 Kbp) vary markedly in a panel of 39 human cancer cell lines but no correlation between the level of telomerase and telomere length (Sau 3A1 digestion in southern blots with hybridization to a TTAGGG telomeric probe) was observed suggesting that a high processivity is not required to maintain telomeres. From this study, we selected breast (MCF7, MX1, BT20), lymphoma (Raji), and prostate (DU145, PC3, TSUPr1) cancer models that showed reproducible telomerase activity and a relatively limited telomere length for the testing of potential telomere-telomerase interacting agents. Using cisplatin and porphirin compounds, we showed that our model was able to detect a slight down regulation of the telomerase activity in human cancer cells in culture and/or in xenografts. Based on these results, human cancer models for evaluating telomerase and telomere interactive agents are proposed.

537 Comparative effects of DNA damaging drugs on telomerase activity in a testicular cell line

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Telomerase is a ribonucleoprotein reverse transcriptase which adds telomeric repeat units of 5-(TTTAGGG)-3' onto the ends of human chromosomes. In non-transformed cells, telomeres, the specialised region at the end of the chromosome, shorten after successive replications. Extreme attrition of the telomeric region appears to cause initiation of cellular senescence. Activation of telomerase is thought to be one of the precursors for the immortalisation of cells.

It was previously reported that cisplatin inhibits telomerase while other DNA damaging agents, such as melphalan and transplatin, had no effect (Burger et al , Eur. J. Cancer, 26, 638, 1997). However, in another report, decreased telomerase activity was associated with non-specific tumour cell killing 'in vitro' (Faranoi et al., Clin. Cancer Res., 3, 579, 1997). The objectives of the present study were to confirm the specific inhibition of telomerase by cisplatin and investigate the underlying mechanism.

Using the testicular cell line Susa CP, telomerase activity was measured using the Telomeric Repeat Amplification Protocol (TRAP assay).

Following a 4 hour exposure of Susa CP cells to 100 μM cisplatin or melphalan and growth in drug free medium for a further 20 or 44 hours, telomerase activity was measured. After 24 hours incubation, telomerase activity was found to be reduced by cisplatin but in contrast, there was no evidence of such a decrease following melphalan, in agreement with previous studies. However, after 44 hours in drug free medium, telomerase activity was reduced after treatment with cisplatin or melphalan. This suggests the rate of loss of telomerase activity may be drug dependent.

Growth inhibition of Susa CP cells using the sulphurhodamine B assay (SRB) showed the IC_{50} of cisplatin and melphalan to be 0.73 μM and 0.80 μM respectively. Thus although the drug doses used to study telomerase inhibition were high, each was used at equivalent toxicities (100× its IC_{50}). Furthermore, at the drug exposures used, the time courses for apoptosis induction were found to be similar for both drugs. Thus the differential effect on telomerase inhibition may not simply be due to different overall rates of cell death.

538 Correlation between telomerase activity, clonogenicity and chemo-sensitivity in a panel of human tumor xenografts

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Telomerase is a ribonucleoprotein enzyme which has been associated with cell immortalization and cancer. Telomerase has emerged as an attractive target for cancer chemotherapy because it is found in more than 85% of human tumors, but is absent in most normal tissues and high telomerase activity appears to correlate with disease progression and poor prognosis. We have determined telomerase activity in 58 s.c. growing, well characterized human tumor xenografts and xenograft derived, permanent cell lines with the view to identify feasible *in vivo* models for the evaluation of anti-telomerase strategies. Carcinomas of the prostate (4), kidney (9), lung (6), ovary (5), breast (7), pancreas (2), head and neck (3), bladder (3), colon (6), uterus (1), testis (1), stomach (3), melanomas (4), sarcomas (2), a lymphoma and a hepatoma were examined. Telomerase activity was determined using a semiquantitative PCR Elisa method together with the conventional telomeric repeat amplification protocol (TRAP assay) to allow comparison of specimens. Xenograft tissues collected for telomerase measurements were tested in parallel for colony formation in the clonogenic assay.Telomerase activity was present in all human tumor xenografts and cell lines evaluated at levels ranging from very low to high. Activity was high in the lung and breast cancer panel, particularly those tumors responding well to chemotherapy such as DNA-interactive standard agents. In contrast, enzyme levels were low in the head and neck cancers and soft tissue sarcomas, which are less chemosensitive. Variable levels of activity were found among other tumor types. However, tumors with marked telomerase activity grew well in soft agar, indicative for the presence of a large number of tumor stem cells. Moreover, xenografts from which permanent cell lines could be established were strongly telomerase positive. Tumors with very low telomerase activity e.g. prostate carcinoma PRXF 1369 and renal cell cancer ACHN LX had no colony forming ability, which is accompanied by slow *in vivo* growth. Our data suggest that clonogenicity of tumors is associated with marked telomerase activity. Thus, conventional *in vivo* xenograft testing in combination with clonogenic assays of treated tumors, might prove a way to evaluate telomerase inhibitors.

539 Potential of peptide nucleic acids as inhibitors of human telomerase catalytic activity

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Telomerase, an RNA-dependent DNA polymerase which contributes to maintain the stability of telomere by preventing their progressive shortening, may represent a promising target for innovative anticancer strategies owing to its differential expression pattern in normal and tumor cells. In fact, telomerase is activated in most tumors that concurrently have short telomeres and is generally not expressed in normal differentiated cells. Moreover, human germ cells and stem cells, which also express telomerase activity, have long telomeres and, therefore, would be affected by telomerase inhibitors later than cancer cells. Different classes of possible telomerase inhibitors, designed according to the continuous improvement in the knowledge of enzyme properties, have been recently proposed. We pursued the possibility to inhibit the catalytic activity of the ribonucleoprotein by the use of chemically modified oligonucleotides, known as peptide nucleic acids (PNAs) and directed against the RNA component of the enzyme. For comparative purposes, phosphorothioate oligonucleotides (PS) of analogous sequence were also used. PNAs contain a nonionic backbone in which the deoxyribose linkages have been replaced by N-(2-aminoethyl) glycine units. The uncharged nature of the internucleotide linkage increases the melting temperatures of associated strands and affords greater resistance to protease and nuclease degradation. Extracts from human cutaneous melanoma cell lines and primary cultures were exposed for 30 min at 37 °C to scalar concentrations of PNAs and PS. Telomerase activity was measured by using the telomeric repeat amplification protocol (TRAP). Telomerase products were visualized by gel electrophoresis, and the extent of inhibition was quantified by phosphorimager analysis. PNAs inhibited the enzyme activity more efficiently than PS, with IC50 values in the nanomolar range. When applied to mildly permeabilized melanoma cells, PNAs continued to inhibit telomerase activity but with IC50 values higher than those required for cell extracts. The present data suggest that PNAs could be useful lead compounds for the development of specific and stable inhibitors of telomerase, although the problem of cell membrane permeability to the agents needs to be resolved.

540 Inhibition of human telomerase and growth of human ovarian carcinoma cell lines by 2,6-disubstituted amido-anthracene-9,10 diones

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In recent years, telomerase, the enzyme responsible for maintaining telomeres on the ends of chromosomes, has emerged as a potential highly selective drug target for anticancer chemotherapy. Activation of telomerase has recently been directly shown to be involved in bypassing cellular senescence, around 85% of tumours exhibit telomerase expression and moreover, high telomerase activity has been shown to correlate with poor prognosis in some tumour types. This study aims to develop telomerase inhibitors through the design of small molecules capable of stabilising G-quadruplexes, specialised nucleic acid structures that may be associated with human telomeres and telomerase. Initial biological evaluation of putative inhibitors is aimed at examining selectivity of polymerase versus telomerase inhibition by testing the ability of compounds to inhibit *Taq* polymerase in a cell-free assay. Whereas, the anthraquinone-based anticancer drugs doxorubicin and mitoxantrone were potent inhibitors of *Taq* polymerase at 10 μM, some compounds within a series of 2–6 disubstituted amido-anthracene-9,10-dione derivatives exhibited no inhibition of *Taq* polymerase at concentrations of either 10 or 50 μM but were potent inhibitors of human telomerase (50% inhibition at concentrations ranging from 4.5 for BSU1021 to >50 μM for BSU1032) in a PCR-based cell free assay utilising telomerase extracted from a human ovarian cancer cell line. Companion cytotoxicity studies conducted with human ovarian carcinoma cell lines (96 h drug exposure, sulforhodamine B assay) have shown the 2–6 disubstituted compounds to be substantially less cytotoxic than doxorubicin (mean IC_{50} of approx 10 nM) or mitoxantrone (mean IC_{50} of approx 0.5 nM) with IC_{50} values typically in the 0.5 to 5 μM range. Long-term culture of the human ovarian cancer cell line, A2780, at sub-cytotoxic concentrations of BSU9048, resulted in detectable telomerase inhibition from 18 days onwards with a maximum inhibition of 40% at 30 days concomitant with a marked decrease in cell growth. These small molecule inhibitors represent promising leads to the design of selective telomerase inhibitors for further *in vitro* and *in vivo* antitumour studies.

541 Mutated DNA polymerases γ and δ in tumor cells as targets for antineoplastic drugs

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Hypothesis: Tumor development is often characterized by accumulating mutations. Such mutations when arising in DNA polymerase genes at early tumor stages would confer mutator properties to the DNA replication machinery ('mutator hypothesis' of carcinogenesis).

Aims: To prove or to disprove the mutator hypothesis we compared DNA polymerases γ and δ from cancer cells with those from normal cells. As a model, we used the highly malignant Novikoff hepatoma cells and normal rat liver as a control.

Materials and methods: DNA polymerases γ and δ were purified and compared with regard to i) their sensitivity towards nucleotide analogues and antineoplastic drugs, ii) copying fidelity, and iii) full-length cDNA sequences which were amplified by polymerase chain reaction.

Results: i) DNA polymerase γ and δ from Novikoff cells showed lower K_{50} values for nucleotide analogues (e.g. butylphenyl-dGTP) and antineoplastic drugs (e.g. doxorubicin, topotecan, netropsin). ii) DNA polymerase δ from malignant cells exhibited decreased copying fidelity using primer-templates which contained O^6-methylguanine. iii) In the full length cDNA (3325 bp) of the polymerase δ gene from Novikoff cells was sequenced and a single heterozygous transition mutation (CGG ⇒ CAG) was detected in codon 648, resulting in an exchange of Arg to Gln. Position 648 lies close to domain no. VI which is involved in nucleotide binding.

Conclusions: Enhanced sensitivity of DNA polymerases from malignant cells towards nucleotide analogues and antineoplastic drugs and reduced fidelity are suggestive of mutator properties. The point mutation detected in the DNA polymerase δ gene from Novikoff cells seems to cause the observed changes in enzyme characteristics. If mutations in DNA polymerase genes do not arise at random but occur in hot spots (resulting in characteristically altered but still functional enzymes) then these enzymes can be made targets for the development of improved, i. e., more selective drugs. Human colon carcinomata are presently used to verify the findings obtained with Novikoff tumor cells.

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542 Thiocoraline, a novel DNA polymerase inhibitor with a higher cytotoxic potency against cancer cells with mutated or inactivated p53

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Thiocoraline is a natural compound isolated from the actinomycete *Micromonospora marina*. It was found to be cytotoxic against several cancer cell lines *in vitro* and to *possess in vivo* antitumor activity. Thiocoraline mode of action is still not fully elucidated yet. It is not a DNA topoisomerase I or II inhibitor, nor it causes detectable DNA damage. *In vitro* it inhibits DNA polymerase α, a finding which explains why cells exposed to the drug are accumulated in G_1 phase of the cell cycle. The observation that thiocoraline was more potent against human colon SW620 cancer cell line which expresses wt p53 prompted us to investigate the influence of p53 status on thiocoraline cytotoxicity using isogenic systems. HCT116 (WT p53), HCT116/E6 (with inactivated p53) and A2780 (WT p53), A2780/E6 (with inactivated p53) were exposed to thiocoraline. In both cell lines inactivation of p53 increased the sensitivity to thiocoraline more than 3 times in a reproductible manner. Studies are in progress to elucidate the mechanism by which p53 exerts a protective effect in the cytotoxicity caused by thiocoraline.

543 Retinoids in head and neck cancer: Antiproliferative effects, metabolism and mRNA expression of nuclear receptors and binding proteins

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Retinoids can reverse potentially premalignant lesions in the oral cavity and prevent second primary tumors following head and neck squamous cell carcinoma (HNSCC). All-*trans*-retinoic acid (ATRA) and its metabolites (e.g., 13- and 9-*cis*-RA) may regulate growth and differentiation via two types of nuclear receptors (RARs and RXRs), which act as transcription factors. The aim of this study was to identify the criteria that define the response to retinoids. Three HNSCC cell lines were selected on the basis of a large variation in the all-*trans*-RA induced growth inhibition. After an exposure of 1 ±M ATRA medium and cells were analyzed for retinoid metabolites during three days. The amount of growth inhibition was proportional to the extent at which all-*trans*-, 13-*cis*, and 9-*cis*-RA disappeared from the medium as from the cells. This turnover process coincided with the formation of relatively polar metabolites in the medium in which the cells had been cultured. With the exception of the major metabolites ATRA, 9-, 13-*cis*-RA, and retinol, no retinoid metabolites were detected in the cell-associated fraction. When the three cell lines were exposed to 9-, 13-*cis*-RA or retinol the growth inhibiti on and the rate of metabolism were similar as with ATRA. The types of metabolites were however different. The mRNA expression levels of retinoid receptors (RAR-α, -β, -γ, and RXR-α, -β, -γ) and retinoid binding proteins (CRABP-I and -II) were measured before and after exposure to ATRA and did not seem to be correlated with the level of growth inhibition. Eight more HNSCC cell lines were tested on growth inhibition and metabolism when exposed to ATRA. No direct correlation was found in these cell lines between growth inhibition and the rate at which ATRA disappeared from the medium. At present we measure transcription levels of retinoid specific genes in these cell lines. In conclusion, there is not a straightforward relation between growth inhibition and metabolism, indicating that other factors involved in the retinoid signal transduction pathways (e.g., the retinoid receptors) are involved.

544 Cytotoxic effects of two gamma linoleic salts (Gla) on human glioblastoma cell lines: Lithium gammolinolenate (LiGla) or meglumine gammalinolenate (MegIa) alone or associated with a nitrosourea, fotemustine (Fote)

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GLA salts may exert a direct antiproliferative activity on tumoral cells. The cytotoxicity is linked to the generation of conjugated dienes, peroxyl radicals and superoxide radicals. LiGla and MeGla have been recently developed for enhancing water solubility of these compounds. MeGla or LiGla (10^{-5}–10^{-4} moles/l) and Fote ($2\,10^{-6}$–$2\,10^{-4}$ moles/l) were applied, alone or in combination, during up to 9 days to two human glioblastoma cell lines A172 and U373MG. Fote was applied first and only once whereas exposure to LiGla and MeGla was renewed daily. Cytotoxicity was evaluated by the MTT test and the effects of drug combinations were analyzed by the isobolographic representation according to Chou and Talalay method (combination indexes, CI). For both Gla salts cytotoxicity was manifested after 4 days of cell exposure and with very sharp doseresponse curves. Comparison of IC50 values indicated that MeGla was more active than LiGla. There was a constant reduction in IC50 values following an increase in exposure time for A172 cells: between 4 and 9 days of cell exposure, IC50 (10–5 M) changed from 7.3 to 4 6 for LiGla and from 4.9 to 3.1 for MeGla ($P < 0.05$). With U373MG cells, there was no influence of exposure duration on IC50 values. CI values indicated that association between Fote and Gla salts globally resulted in slightly antagonist effects. These informations may be useful for futher development of Gla salts at the clinical level.

545 Binary catalytic systems as new antitumor drugs

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For the first time binary catalytic systems generating free radicals have been proposed as new antitumor drugs. These systems consist of complexes of transitional metals with macrocyclic and chelating ligands and low weight reducing agents. Cytotoxic effect was estimated for 60 catalytic systems *in vitro* experiments on human tumor cells of A-549 lung adenocarcinoma, ovary adenocarcinoma, HEP 2 laryngopharynx adenocarcinoma, and Raji B-cell lymphoma. 2 binary systems: a derivative of cobalt phthalocyanin + ascorbic acid and a cobalt derivative of corrin + ascorbic acid with the best antitumor properties have been selected during screening investigations. Their pronounced antitumor activity has been confirmed and their chemo-and radiomodifying properties have been detected in *in vivo* experiments on animals bearing transplanted P388, L1210, Ehrlich, LLC, B16, hepatoma, Ca755 and Sa37 tumors. Preclinical trials of these binary systems have already been fulfilled by the present time. Experimental specifications and particular results are presented in other reports of the authors.

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546 Calixarene derivatives as novel anticancer agents

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Background: A quarter of the population of the U.S.A. and Western Europe will develop cancer, one fifth of these people will die from their disease. Faced with these statistics there is a need to investigate novel therapies for the treatment and cure of cancer.

Synthetic calixarenes are a set of compounds that we are investigating as potential anticancer agents. Calixarene is the collective name given to the cyclic aromatic compounds formed by the condensation of phenol and formaldehyde. A multitude of derivatives can be made by further modifying these calixarenes.

Results: Using the MTT assay, we have shown an *in vitro* cytotoxic effect for one calix[4]arene derivative against the human breast adenocarcinoma line MCF7. Cell viability was significantly reduced, compared to solvent (DMSO) treated cells, when the calixarene was administered over a 0.005–50 μM concentration range (analysed by Students t-test, $P = 0.025$, $IC_{50} = 5$ μM). We are currently characterising this calixarene induced cell death.

It was suggested that the predicted amphipathic structure of the calixarene may enable it to interact with a membrane bilayer. We wanted to know if this could happen and whether the subsequent membrane disruption caused by the calixarene was the trigger for MCF7 cell death. Biophysical studies have tested this by looking at the calixarene's effect on artificial membrane bilayers and single shelled liposomes. Electron microscopy was used to visualise calixarene-treated soybean liposomes, and the Schindler technique for soybean bilayers preparation was employed to measure any channel-like properties of the calixarene.

We have shown that the calixarene has the ability to modify a liposome membrane resulting in altered bilayer morphology. Also, when the calixarene was added to ion-impermeable bilayers there was a measurable increase in membrane conductance with time.

Conclusions: We suggest that membrane disruption could be one explanation for the calixarenes observed toxicity *in vitro*.

547 Interaction between n-3 and n-6 essential fatty acids and therapeutic radiation in apoptosis and reactive oxygen intermediate formation in the C6 glioma cell line and in human glioma cells

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Human gliomas are characteristically radioresistant. *In vivo* and *in vitro* studies indicate that radiation therapy and treatment of tumours with essential fatty acids (EFA) are associated with increased production of transiently formed reactive oxygen intermediates (roi) and enhanced apoptosis. Human glioma cells, treated *in vitro* with n-6 EFA showed higher sensitivity than cells from normal brain tissue, in terms of roi and apoptosis responses (Williams et al., Prostaglandins, Leucotrienes and Essential Fatty Acids & Eur. J. Clin. Invest., in press 1997).

The aim of this study was to determine the interaction between potential therapeutic n-3 and n-6 EFA and radiation in glioma cells on roi and apoptosis. Glioma cells were incubated with n-3 EFA (eicosapentaenoic acid, Lithium salt) or n-6 EFA (arachidonic acid, Na salt; gamma linolenic acid, Li salt) and roi formation was analysed using using flow cytometry of 2',7', dichlorofluorescin oxidation. Apoptosis was analysed using flowTUNEL. Glioma cells were irradiated with 2 Gy gamma radiation in the presence or absence of EFA (20 μM) and roi, apoptosis and cell membrane integrity were analysed at 0.25–24 h (1 h intervals) after radiation by flow cytometry. The characteristic EFA and radiation induced kinetics of apoptosis and roi formation (Williams et al. Biochem. Soc. Trans., 1997) were observed when the two stimuli were administered together (EFA 30 min before irradiation). But the amplitude of these changes was upregulated by up to 10 fold. These findings suggest that exogenous n-3 and n-6 EFA increased the radioresponsiveness of glioma cells *in vitro*.

548 Prevention of chemical carcinogenesis in mice immunized with tularemia vaccine

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Tularemia live vaccine (TLV) is widely used in the Former USSR and about 12 million people are annually immunized with TLV. Recently we have shown that immunization of rats with TLV decreases significantly carcinogenic and clastogenic effects of 7,12-dimethylbenz(a)anthracene (DMBA) and benzo(A)pyrene (BP).The aim of the present work was to study carcinogenic and clastogenic effects of DMBA, BP and urethane in mice immunized with TLV. Experiments were performed with male Swiss albino mice immunized with TLV 15 days before carcinogens administration. Control groups of mice received carcinogens at the same doses. Some mice from every group were killed 24 h after carcinogen administration and micronuclei (MN) were evaluated in bone marrow cells. The rest mice were killed 6 months after the start of the experiment and their organs were studied histologically.Subcutaneous tumors induced by DMBA and BP were observed in 100% and 71.4% of control mice, respectively. In immunized mice the incidence of tumors was significantly lower than in corresponding control groups (65.2% and 41.4%, respectively). Mean latency period of tumors development increased and mean tumor weight decreased significantly in immunized mice. Lung tumors induced by urethane were observed in 83.3% of control vs 47.4% in immunized mice. The level of MN induced by all carcinogens in bone marrow cells of immunized mice was significantly lower than in corresponding controls. To study the possible mechanisms of anticlastogenic/anticarcinogenic effects of TLV we investigated DNA repair activity in somatic cells of mice using the comet assay and activity of cytochrome P-450 system using indirect method (duration of mice sleep induced by barbiturates). We have shown that immunization of mice with TLV increases significantly DNA repair activity and, at the same time, decreases cytochrome P-450 activity. Hence, anticarcinogenic and anticlastogenic effects of TLV are due to decrease of carcinogens metabolic rates in liver of rodents and also to increase of resistance of DNA to genotoxic action of carcinogens. It would be of interest to carry out further investigations of anticarcinogenic action of TLV, widely used in the Former USSR, if only because the tularemia vaccination process in rats is quite similar to that in humans.

549 Molecular targets for antiproliferative and antimetastatic agents in human osteosarcoma cells

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Aims: To improve the efficacy of drug therapy in childhood osteosarcoma it has been decided to elucidate the cellular and molecular alterations induced by cytostatic agents or potential antimetastatic agents. To this end damage of DNA and inhibition of proteoglycan biosynthesis were investigated in experimental model systems.

Methods: Osteosarcoma surgically removed from a 17 year old boy was xenotransplanted in immunosuppressed mice and cultured *in vitro* (OSCORT). Cytostatic and cytotoxic action of the test compounds were measured by applying the sulphorhodamine test and determining the apoptotic index resp. DNA damage in the individual cells were detected in comet-assay, drug actions were also monitored by flow cytometry. The intracellular concentrations of p53, ras, Rb, bcl-2 oncoproteins were measured by using Western technique. To measure proteoglycan production, cells were labelled with ^{3}H-glucosamine, radioactivity was measured in proteoglycans isolated from both the medium and the cells by using DEAE column chromatography and polyacrylamide-gel electrophoresis.

Results: In the OSCORT culture the cytostatic drugs showed the following IC50 values: adriamycin: 2,9 μM, 5-fluorouracil 19,9 μM, methotrexate 213 μM, vincristine 15,6 μM and cisplatin 25 μM. Similarly these drugs showed a very moderate activity in the xenograft model and also in the comet assay. It is noteworthy that cisplatin which was not very active as antiproliferative agent, increased the bcl-2 concentration. Clodronate and 5-hexyl-2'deoxyuridine with antimetastatic efficacy showed only modest antiproliferative activity but inhibited the production of proteoglycans implicated in the complex mechanism of the metastatic cascade.

Conclusion: The OSCORT cell line showed very modest response to cytostatic agents. However it may be worthwhile to consider agents acting on proteoglycan production in the adjuvant treatment protocol for osteosarcoma.

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