494 POSTER

Preliminary results from a phase I clinical trial of the bioreductive drug, RH1

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RH1 (2,5-diaziridinyl-3-[hydroxymethyl]-6-methyl-1,4-benzoquinone) is a novel bioreductively activated drug which is an excellent substrate for the two electron reductase, DT-diaphorase (EC. 1.6.99.2). DT-diaphorase has been shown to be over expressed in many tumours relative to the normal tissue, especially lung, breast and colorectal tumours. RH1 will be activated in such tumours, allowing targeted drug delivery to cancer cells with minimal normal tissue toxicity. RH1 is presently being assessed in a Cancer Research UK phase I trial (PH1/089).

Pharmacokinetic analysis was performed using Waters Alliance HT Separation Module coupled to a Waters/Micromass Quattro Mass Spectrometer. The primary pharmacodynamic endpoint used was DNA interstrand cross-linking as measured using the comet-X assay. For the comet-X assay, peripheral blood lymphocytes were isolated at pre-infusion and post-infusion on both days 1 and day 5 of treatment and irradiated with gamma radiation. It was expected that interstrand cross-links produced by RH1 would retard the migration of DNA during electrophoresis resulting in less DNA in the tail of these comets compared to the irradiation only controls. RFLP analysis on peripheral blood DNA was also performed to detect a polymorphism in the NQO1 gene responsible for inactivation of DT-diaphorase.

From September 2003 to May 2004, 7 patients with solid tumours refractory to conventional chemotherapy have been enrolled into this study. Pharmacokinetic analysis of plasma samples taken on day 1 and day 5 of cycle 1 has demonstrated detectable levels of drug with a half life of approximately 6 minutes for clearance from the blood. The peak levels range from 17 to 113 nM with escalating dose. These dose levels are consistent with those causing significant biological activity *in vitro*. For the comet-X assay, the pooled data for all patients on day 1 shows 70–80% DNA in the tail similar in distribution to the irradiated control, however by day 5 the population showed peaks at 60–70% DNA in the tail consistent with low level DNA cross-linking.

495 POSTER

The synthesis of novel 3- and 5-piperidinyl substituted indolequinone bioreductive prodrugs: mechanism of hypoxic/reductive activity

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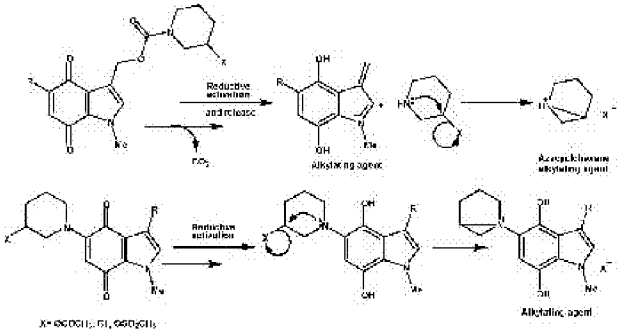
Introduction There is continued interest in the design and synthesis of novel indolequinone bioreductive prodrugs as hypoxia selective agents and in a GDEPT approach to increase their therapeutic effects specifically to solid tumours. We have previously synthesised and evaluated 5-aziridinyl substituted indolequinone prodrugs that are activated preferentially under hypoxic conditions or in tumour cell lines that were genetically manipulated to over-express reductive enzymes (e.g. NQO1). Thus maintaining selectivity to hypoxic solid tumours.



Aims and Objectives In this study the synthesis of novel indolequinone-substituted piperidinyl conjugates is described. The cytotoxic moieties were attached either at the C-3 and C-5 postions of the indole ring in order to establish a structure activity relationship. The mechanism of activation is proposed in which the substituted piperidinyl compounds can either be activated (C-5) or released (C-3) to ultimately afford the azabicyclohexane DNA alkylating agent. This mechanism is inhibited by molecular oxygen.

Chemistry The synthesis of 3-substituted indolequinones was achieved in several steps starting from 3-hydroxymethyl-5-methoxy-1-methylindole-4,7-dione, which was reacted with nitrophenylchloroformate, to afford the 5-methoxy-1-methyl-3-methyl-(4-nitrophenyl-carbonate)indole-4,7-dione intermediate. Nucleophilic substitution of the substituted piperidinyl (3-chloro and 3-methanesulfonate) gave the desired target carbamate prodrugs. The 5-susbtituted indolequinones were similarly prepared from 3-hydroxymethyl-5-methoxy-1-methylindole-4,7-dione. Nucleophilic substitution at the C3-position of the substituted piperidinyls gave the desired compounds. The lead compounds are currently being evaluated for their cytotoxic properties in a range of breast and colon tumour cell lines.

496 POSTER

Water-soluble dinitrobenzamide mustard phosphate pre-prodrugs as hypoxic cytotoxins

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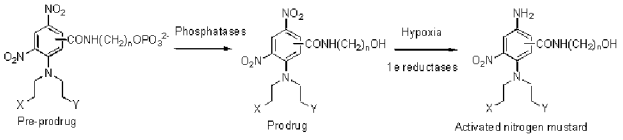
The activity of tirapazamine (TPZ) in phase **II/III** clinical trials validates hypoxia as a target in the treatment of solid tumours, but TPZ causes substantial normal tissue toxicity. The dinitrobenzamide mustards (DNBMs) offer several theoretical advantages as hypoxia-activated prodrugs, including activation only under severe (pathological) hypoxia, improved extravascular transport, and bystander effects due to formation of relatively stable cytotoxic metabolites. However, the first generation DNBMs (e.g. SN 23862) had poor aqueous solubility and limited hypoxic selectivity prompting the development of DNBM phosphate esters. These have excellent solubility and formulation characteristics, and act as "pre-prodrugs"; systemic phosphatase activity generates the corresponding alcohols (prodrugs) which are subsequently activated by nitroreductases, including one-electron reductases under hypoxia and *E. coli* nitroreductase (NTR) in the context of GDEPT. In the present study we apply this strategy to optimization of DNBMs as hypoxic cytotoxins.



A set of 40 DNBM alcohols with activity as NTR prodrugs were screened for hypoxia-selective cytotoxicity in proliferation (IC_{50}) assays against A549 cells, providing hypoxia cytotoxicity ratios (HCR) in the range 1.4–29. HCR values in an A549 line that overexpresses human P450 reductase (9-fold) were greater (6.6–160), indicating substantial one-electron activation. HCRs were highest for mixed halogen/mesylate mustards, and hypoxic potency was greatest for the 2-mustard 3,5-dinitrobenzamide regioisomers (IC_{50}s 3–5 μM). A preferred subset of 16 structurally diverse compounds showed a broadly similar SAR across a panel of 5 human tumour cell lines (HCRs 1.5–82). Phosphate pre-prodrugs of the alcohols, evaluated in athymic CD-1 mice, generally showed low host toxicity with maximum tolerated doses (MTD) in the range 0.56–3.2 mmol/kg. The DNBM phosphates lacked the retinal toxicity characteristic of TPZ at its MTD (0.32 mmol/kg), and displayed markedly greater activity than TPZ against hypoxic cells in SiHa tumour xenografts at 75% of the MTD as determined by clonogenic cell killing following administration of the pre-prodrugs after 15Gy irradiation (IR). The logarithmic kill *in addition* to IR (1.6 logs alone) was substantially greater (2.1 to >3.5 logs) than for the pre-prodrugs alone (0.9–2.6 logs), showing clear selectivity for hypoxic cells. In contrast, TPZ displayed modest hypoxic activity post-IR (0.66 logs) and no single-agent activity. The tumour activity and host toxicity profile of the DNBM phosphates is clearly different from TPZ, and appears to offer a superior therapeutic ratio *in vivo*. Selection of an optimized lead for clinical development is in progress.

497 POSTER

A bioreductive prodrug of combretastatin A4

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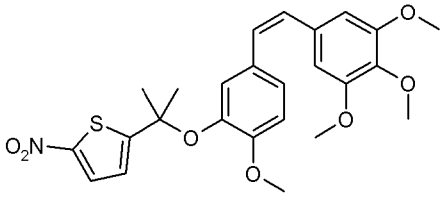
Combretastatin A4 (CA4) is a tubulin depolymerising agent with *in vivo* vascular targeting activity and *in vitro* antimitotic properties. Its phosphate ester prodrug is currently in Phase **II** clinical trials for a number of solid tumour indications. Although this agent has shown encouraging

antivascular activity in the clinic, derivatives of CA4 that have a more selective antitumour activity would be advantageous.
We have designed ANG704 as a bioreductive prodrug of CA4 using the trigger-effector principle, wherein a moiety (the trigger) attached to the active drug renders the prodrug inactive until release of the drug by fragmentation, which occurs selectively under the hypoxic conditions found in solid tumours *via* an oxygen-sensitive free radical intermediate. Unlike CA4, ANG704 was itself inactive as an inhibitor of bovine brain tubulin polymerisation (CA4: IC50 2.8 μM; ANG704: no inhibition at 20 μM) and inhibition of A549 cell growth (CA4: IC50 0.25 μM; ANG704: no activity at 2 μM). Under anoxic conditions ANG704 efficiently released CA4 in the presence of supersomal cytochrome P450 reductase or homogenates of CaNT (syngeneic mouse mammary adenocarcinoma) or FaDu (human squamous cell carcinoma xenograft) tumours. The release of CA4 was inhibited in air under similar conditions. Oxygen-inhibited release of CA4 was also catalysed by lysate from A549 cells and by whole A549 cells in culture. ANG704 was stable when incubated under aerobic conditions with homogenate prepared from mouse liver, with rates of both prodrug loss and CA4 production of less than 0.01 nmol/min/mg protein at 5micromolar prodrug concentration.
ANG704 has promising *in vitro* activity as a metabolically-stable bioreductive prodrug of combretastatin A4.



ANG704

498 POSTER

Selective potentiation of the hypoxic cytotoxicity of the bioreductive drug tirapazamine

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Of the 3 mono-N-oxides examined so far by clonogenic assay of hypoxic cell suspensions, SN 29051 provided similar dose dependent potentiation of TPZ hypoxic cytotoxicity as SR 4317, while SN 29254 and SN 29047 provided greater potentiation (an extra 4 logs of killing relative to TPZ alone at a non-toxic potentiator concentration of 0.3 mM).

SN	R	X	Hypoxic TPR	Aerobic TPR	Hypoxic Cytotoxicity Ratio*
TPZ					66±10
SR 4317	H	H	2.6±0.2	0.8±0.3	240±35
29051	NH(CH2)2 N(CH3)2	H	21±3	1.3±0.3	1090±220
29047	OCH3	H	7.3±4.0	0.9±0.1	520±200
29254	NH(CH2)2 pyrrolidone	H	41±5	1.1±0.1	3145±215
29334	NH(CH2)2 N(CH3)2	6iPr	5.6±0.3	0.9±0.1	415±50
29112	NH(CH2)2 N(CH3)2	6CF3	13±6	0.9±0.2	680±190
29059	NH(CH2)2 N(CH3)2	8CH3	17±8	0.9±0.1	805±85

*Aerobic IC50/hypoxic IC50 of TPZ combined with the maximum non-toxic concentration of potentiator.

The plasma pharmacokinetics of TPZ (0.13 mmol/kg), determined from HPLC analysis of tail vein bleeds of CD-1 nu/nu mice, were not altered by co-administration of SN 29051 (0.42 mmol/kg). This combination also provided no toxicity against the retina – a hypoxic normal tissue that is damaged by TPZ at its MTD in mice. The results of HT29 xenograft excision assays, currently under progress will be reported.
Conclusions: Benzotriazine mono-*N*-oxides can selectively potentiate hypoxic cell killing by TPZ, and may improve the therapeutic utility of TPZ as a hypoxic cytotoxin in cancer treatment.

499 POSTER

N(O)-(Substituted)-b-(2),4,5-tetramethyl-3,6-dioxo-1,4-cyclohexadinen-1-propa(noate) propanoamide: bioreductive delivery systems for selective delivery of therapeutic agents into solid tumours

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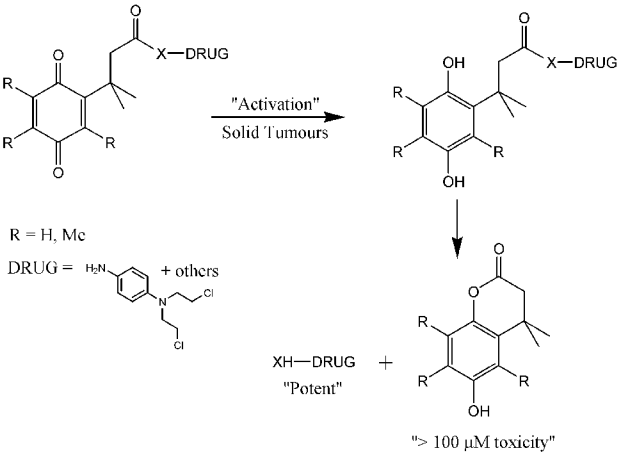
1,4-Cyclohexadiene (benzoquinone) agents have been previously developed to target the aerobic and hypoxic function of solid tumours. They are prodrugs that can either be activated under hypoxic or reductive conditions e.g. NQO1 to afford a therapeutic effect. The 1,4-cyclohexadienes synthesised here can be utilised to deliver a therapeutic entity (toxic or non-toxic) under solid tumour conditions. Upon hypoxic or reductive activation, the 1,4-cyclohexadiene product is converted to the 1,4-cyclohexadiol which spontaneously cyclises to afford the active drug (see figure).



Here we report on the synthesis of a series of 1,4-cyclohexadinenes drug conjugates (in which the drug is attached via an ester or amide link). Starting from methyl substituted 1,4-cyclohexadiols and methyl-3,3-dimethylacrylate to produce the desired lactone 6-Hydroxy-4,4,7,8-tetramethyl-1-benzopyran-2-one. Oxidation of the lactone affords the proponic acid precursor. Addition of a therapeutic agent via and ester or amide link affords the desired prodrugs. The lead cyctotoxic compound of this series has been evaluated for it toxicity in the A549 breast carcinoma cell line. The prodrug was at least 3-fold less potent (inactivated) under aerobic conditions when compared to the free drug (*N,N-bis* (2-chloroethyl)-benzene-1,4-diamine) (IC_{50} (air) prodrug: 14.04 μM IC_{50} (air) free drug 4.52 μM) Under hypoxic conditions similar toxicity was observed for the two compounds (IC_{50} (hypoxia) prodrug 6.70 μM, IC_{50} (hypoxia) free drug 4.52 μM). These results suggest that the 1,4-cyclohexadiene drug conjugates can be utilised to selectively deliver potent agents specifically and selectively into solid tumours.

500 POSTER

Bioreductive activation and in vitro cytotoxicity of MUP03/704: a novel bioreductive cytotoxic drug conjugate for solid tumours

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MUP 03/704 is a novel quinone based cytotoxic prodrug (see structure), initially designed to target NQO1 (NAD(P)H: quinone oxidoreductase 1), a cytosolic flavoprotein catalysing two-electron reductions, for its bioactivation. When reduced, the compound spontaneously breaks down to afford a potentially non-toxic lactone and the activated alkylating agent (N,N-*bis*-2-[chloroethyl]-benzene-1,4-diamine). In the absence of reduction, the prodrug is potentially non-toxic. The purpose of this study is to establish the proof of principle of MUP 03/704 cytotoxicity in cancer cell lines.
The affinity of NQO1 for MUP 03/704 as a substrate was measured using spectrophotometry. The MTT assay was used to evaluate the cytotoxicity of the prodrug in H460 lung cancer cells (high NQO1 activity) and BE colon cancer cells (no NQO1 activity). MUP 03/704 interstrand crosslinks (ICLs) induction and repair were evaluated in the same cell lines using the comet