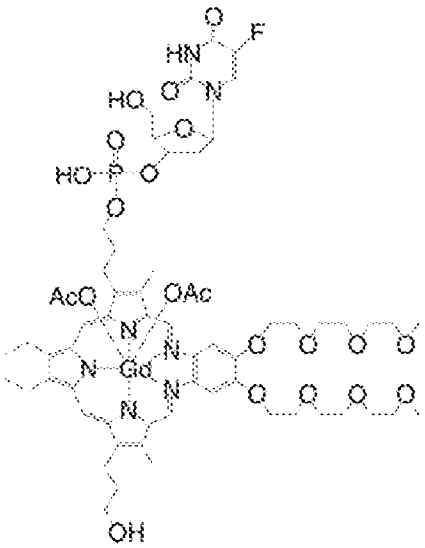

Structure of MGd-FdUrd conjugate.

Methods: Compound stability was assessed by reversed phase HPLC on an HP1050 system. Flow cytometry was performed using a BD FACSCalibur instrument. Cell proliferation was evaluated in 96-well format using a formazan reduction (MTT) assay.
Results: Uptake of MGd and MGd-FdUrd conjugate into the A549 human lung carcinoma cell line was quantified by flow cytometry. Based on the median fluorescence >650 nm, there was approximately 40% uptake of the conjugate relative to MGd. Intracellular enzymatic cleavage of the phosphodiester linker joining the nucleoside and MGd moieties was demonstrated by HPLC analysis of cell pellets and extracellular treatment medium. Moreover, an anti-nucleoside antibody-based flow cytometric assay determined that the nucleoside portion of a similar conjugate containing 5-bromo-2′-deoxyuridine (BrdU) was incorporated into DNA. MGd-FdUrd conjugate cleavage and stability in human serum was addressed. The majority of the compound was uncleaved after 24hr incubation in serum and displayed comparable stability to that of MGd. In addition, preliminary data using MTT indicate that MGd-FdUrd and FdUrd inhibit cell proliferation to a similar degree.
Conclusions: The phosphodiester linkage between MGd and FdUrd is stable in human serum. The MGd-FdUrd conjugate is taken up by A549 cells, and is released in active form under intracellular conditions. This approach may allow the targeted delivery of nucleoside analogues to tumors.

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Two photoaffinity analogs of HTI-286, a synthetic analog of hemiasterlin, interact with alpha-tubulin

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HTI-286 is a synthetic analog of hemiasterlin, a naturally occurring tripeptide derived from marine sponges. The molecule depolymerizes microtubules, is a poor substrate for MDR1, and overcomes paclitaxel-resistance in human tumor xenograft models. Phase I trials with HTI-286 are in progress. Two tritium-labeled benzophenone analogs of HTI-286 were synthesized and their interaction with purified tubulin was investigated. Each analog had a benzophenone-reactive group in a distinctly different region of the molecule. It was found that both analogs specifically and solely photolabeled alpha-tubulin. Photolabeling was inhibited by unlabeled photoaffinity analog, HTI-286, vinblastine, and another peptide-like anti-microtubule agent, dolastatin-10. However, similar concentrations of paclitaxel and colchicine were found to either enhance binding of the photoprobe to tubulin or have no effect; the result depended on the temperature of the reaction. To identify the binding site(s), alpha-tubulin bound to photoprobe was subjected to sequential formic acid and LysC digestions. A 16-kDa formic acid fragment was found to contain the radiolabel and is predicted to be the C-terminal fragment of alpha-tubulin. Following both formic acid and LysC digestions, a 3-kDa peptide was obtained. The identification of this peptide and the amino acid site of interaction are in progress. Our studies support the previous proposal that HTI-286 and other peptide-like anti-microtubule agents have similar binding domains and these regions overlap with the Vinca-binding site previously speculated to be in beta-tubulin. However, this data is the first to suggest that a tubulin-binding peptide may interact with alpha-tubulin and is consistent with mutations in alpha-tubulin that have been already reported in HTI-286-resistant cells.

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Molecular beacon based photosensitizers for imaging guided cancer therapy

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Near-infrared (NIR) dyes are presently attracting considerable interest as fluorescence probes for detection of cancer and as photosensitizers for cancer treatment by photodynamic therapy (PDT). Since tissue is relatively transparent to NIR light, NIR active fluorescence imaging (NIRF) and PDT are capable of detecting and treating, respectively, subsurface tumors, including breast cancer. Stable bacteriochlorophyll (BChl) analogs derived from R. Sphaeroides are excellent NIR dyes for NIRF and PDT because of because of their favorable photophysical properties (1O2 yield: 45%) and long activation and fluorescence emission wavelengths (labs 825nm; lem 840nm). A current limitation of both NIRF and PDT modalities is their lack of sufficient tumor-to-tissue contrast due to the nonspecific nature of delivering the dye to the tumor, which has led to false negatives for NIRF and a limited therapeutic window for PDT. Hence, agents targeting "cancer signatures," i.e. molecules that accumulate selectively in cancer cells, are particularly attractive. We are currently focused on two of such signatures: the tumor-specific mRNAs and the LDL receptor (LDLr) overexpressed in certain tumors. Our first approach is to develop BChl based molecular beacons (hairpin antisense oligonucleotides) so that the dye would be activated only in cancer cells when the beacon hairpin hybridizes to the target mRNA. This will unfold the hairpin and these agents will light up (by emitting fluorescence) and destroy (by producing reactive oxygen species) the cancer cells, while leaving normal cells undetectable and unharmed. In the second approach, BChl cholesteryl oleate conjugates are synthesized and reconstituted into the LDL lipid core. Imaging studies showed that such LDL beacons were selectively internalized by LDLr overexpressing tumors both *in vitro* and *in vivo*. By targeting to these cancer signatures, our goal is to significantly improve the tumor-to-normal tissue ratio of NIRF guided PDT for subsurface cancers.

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Indolequinone carbamate prodrugs of mustards as hypoxia-selective cytotoxins

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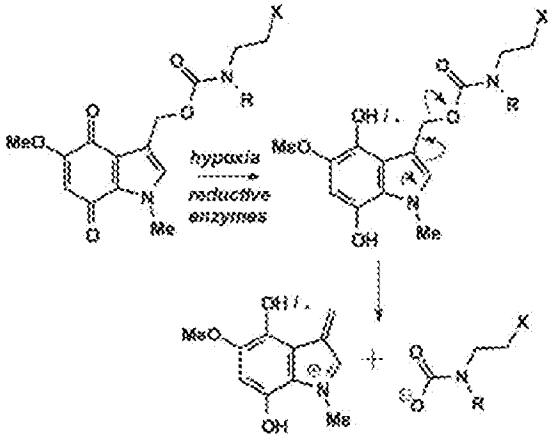
Introduction: The indolequinone class of bioreductive drugs have been developed whereby the p-quinaniod prodrug is reduced under hypoxic condi-

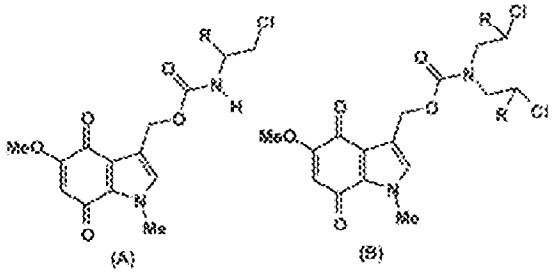


tions to afford the hydroquinone/semiquinone 'activated' species. We have previously shown that under chemical and radiolytic reductive conditions, indolequinone-drug conjugates (attached at the [3-indolyl]methyl position) are bioreductively-activated and subsequently release the drug.

Here, we describe the synthesis and biological activity of indolequinone-mustard conjugates and shows that the mustard moiety is released preferentially under hypoxic conditions leading to a greater hypoxic cell kill when compared with the prodrug under normoxic conditions.

Aims and Objectives: In this study, the T47D human breast tumour cell line was chosen as it has low levels of endogenous reductive enzymes and A549 cell lines which has high expression of reductive enzymes. The synthetic indolequinone analogues were evaluated for their cytotoxicity against the cell lines under both aerobic (air) and hypoxic (N_2) conditions. The compounds were compared against MMC.

Results: Overall, the novel analogues were more potent than MMC under aerobic condition and even more so under hypoxic conditions. All the compounds show greater hypoxic selectivity (HCR between 2.6-16.7) when compared to MMC (HCR 1.4-3.0) in the cell lines (see Table).



(A) (B)

Drug	R	A549 (μM)		HCR
A1	H	0.024 ± 0.004	0.005 ± 0.002	4.8
A2	Me	0.993 ± 0.036	0.128 ± 0.013	7.8
B1	H	0.194 ± 0.028	0.034 ± 0.0077	5.7
B2	Me	0.0415 ± 0.112	0.0160 ± 0.0049	2.6

Drug	R	T47D (μM)		HCR
		IC50 (air)	IC50 (N_2)	
MMC		2.3 ± 2.0	0.75 ± 0.22	3.0
A1	H	1.285 ± 0.352	0.44 ± 0.166	2.3
A2	Me	21.31 ± 0.335	6.293 ± 2.196	4.0
B1	H	7.34 ± 1.36	0.44 ± 0.20	16.7
B2	Me	7.46 ± 0.75	0.46 ± 0.14	16.2

The lead compounds (B1 and B2) show greater selectivity in the T47D especially under hypoxic conditions, which may be due to reduced enzymatic expression.

Conclusion: The results indicated that the prodrug could efficiently release the mustard preferentially under hypoxic conditions especially in cell lines where there is reduced expression of reductive enzymes. The mechanism of delivery seems to suggest that the indolequinone class of bioreductive prodrugs may retain the mustard (to some extent) in its inactive form under aerobic conditions (via electron withdrawing effects). However, under hypoxic conditions, the quinone is reduced to the hydroquinone (or semiquinone radical) resulting in the efficient release of the mustard moiety and causing enhanced cell kill.

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Transmembrane inhibitors of ABC transporters

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Drug resistance mediated by ABC transporters such as P-glycoprotein (P-gp), is a major problem for cancer chemotherapy. We showed previously that the function of polytopic membrane proteins such as G-protein coupled receptors could be inhibited by synthetic peptide analogs of transmembrane domains (TMs) of the protein. We developed a panel of highly specific peptide inhibitors of P-gp and of the recently discovered transporter ABCG2. The inhibitors, structural analogs of the TMs of the transporters, disrupt the assembly of the target proteins. Initially, the design of peptide inhibitors was based solely on the primary structures of the target protein. It allowed us to generate compounds that selectively inhibited the transporters with IC_{50} in 5-10 mM range. Optimization of the structures resulted in more than 10-fold increase in the potency. The major gain was due to more precise localization of the TMs that were initially predicted inconsistently by several available computer programs. A novel 96-well plate assay was developed based on the efflux of fluorescent ABCG2 substrate, BODIPY-prazosin, and was used for structure-functional characterization of transporter inhibitors. The most potent inhibitors of ABCG2, which is a 6 TM protein, were derived from TMs 1, 2 and 4 (IC_{50} 0.1-1 mM). Analogs of domains 5, 6 and 11 of P-gp were found to be the most active in inhibiting efflux of fluorescent substrate of this 12-TM pump. The minimal length for an active peptide was 15 amino acids, including the negatively charged residues added for membrane orientation and solubility, which is significantly shorter than the thickness of the plasma membrane. Thus, a truncated peptide derived from TM5 of P-gp, YASYALAFWYGTTDD, was as potent as cyclosporin, the standard for in-vitro P-gp inhibition, the full length 23-residues long peptide was 5-fold more potent. Circular dichroism and fluorescence studies of TM peptides in lipid micelles indicated significant differences between the domains in terms of degree of helicity, ability to form regular structures and entry into lipid bilayers. SAR studies of P-glycoprotein and ABCG2 inhibitors strongly suggest that potent and selective inhibitors of ABC transporters can now be developed based solely on the primary structures of the target proteins. Retro-inverso peptides synthesized from D-amino acids and peptido-mimetics of the a-helices are now being investigated as improved rationally designed ABC transporter inhibitors.

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The synthesis of hypoxia-selective nitroaromatic compounds as bioreductive drug delivery agents

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Hypoxic (Ischaemic) cells exist due to the poor and disorganised vasculature that is present in most solid tumours. These cells are generally resistant to conventional radiotherapy and some chemotherapy. Bioreductive prodrugs, for example tirapazamine and Mitomycin C, were designed to target hypoxic cells of solid tumours. Under hypoxic conditions, these drugs are activated by reductive enzymes such as nitroreductase (NTR), DT-diaphorase (DTD) and cytochrome P450 reductase (cP450R) to afford the active species. The reaction involves a series of one-electron reduction processes first to the hydroxylamino (4 electron reduction) and then to an amino (6 electron reduction) group which is the active trigger. This concept has recently been extended to develop these agents as hypoxia – selective drug delivery systems. We have designed and synthesised novel bioreductive prodrugs, which comprise of a nitroaromatic trigger joined to the therapeutic drug by a propenyl, carbamate or urea linker. The trigger domain controls the tumour cell selectivity of the prodrug as it undergoes reduction only under hypoxic / reductive conditions. The linker deactivates the cytotoxic moiety. Upon bioreduction, the activation signal is transmitted from the trigger through the linker to cause the release of the active drug. A series of N-(1-(2,4-dinitrophenyl)ethyl urea derivatives bearing either an aromatic or an aliphatic nitrogen mustards have been synthesised (see Figure 1 for an example).

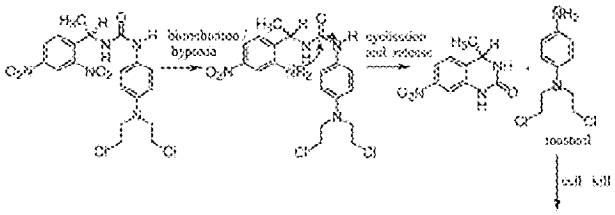

Figure 1.

It is anticipated that under appropriate reductive conditions, the *ortho* nitro moiety of the trigger component of the prodrug will be reduced to the corresponding reactive amino derivative. Cyclisation of the trigger onto the linker will cause selective release of the cytotoxic mustards in hypoxic cells. These toxic mustards would then diffuse and kill neighbouring non-hypoxic tumour cells (by-stander effect). We have shown that under biomimetic chemical reduction (NaBH4 in presence of 10% Pd/C), the *ortho*-nitro group is reduced to the amino moiety causing a "through space" cyclisation to con-currently release the therapeutic drug. This technology, therefore, can be used to deliver cytotoxic drugs, enzyme inhibitors or diagnostic imaging agents to hypoxic tumours.