Design of Anticancer Prodrugs for Reductive Activation

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Abstract: Anticancer prodrugs designed to target specifically tumor cells should increase therapeutic effectiveness and decrease systemic side effects in the treatment of cancer. Over the last 20 years, significant advances have been made in the development of anticancer prodrugs through the incorporation of triggers for reductive activation. Reductively activated prodrugs have been designed to target hypoxic tumor tissues, which are known to overexpress several endogenous reductive enzymes. In addition, exogenous reductive enzymes can be delivered to tumor cells through fusion with tumor-specific antibodies or overexpressed in tumor cells through gene delivery approaches. Many anticancer prodrugs have been designed to use both the endogenous and exogenous reductive enzymes for target-specific activation and these prodrugs often contain functional groups such as quinones, nitroaromatics, N-oxides, and metal complexes. Although no new agents have been approved for clinical use, several reductively activated prodrugs are in various stages of clinical trial. This review mainly focuses on the medicinal chemistry aspects of various classes of reductively activated prodrugs including design principles, structure-activity relationships, and mechanisms of activation and release of active drug molecules. Med Res Rev, 29, No. 1, 29–64, 2009

Key words: bioreductive activation; gene-directed enzyme prodrug therapy (GDEPT); hypoxia; metal complex; nitroaromatic; nitroreduction; N-oxide; prodrug; quinone; target-specific activation

1. INTRODUCTION

Cancer is an important health issue that caused 7.6 million deaths in 2007 according to American Cancer Society. Localized cancer can be removed by surgery, but cancer after metastasis has to be treated systematically by chemotherapy in combination with radiotherapy and surgery. However, most chemotherapeutic agents employed are cytotoxic agents and suffer several limitations including

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lack of selectivity toward tumor cells, emergence of drug resistance, and low therapeutic index. The lack of selectivity is responsible for the dose-limiting side effects and toxicities associated with cytotoxic agents. One approach to improve the therapeutic effectiveness and decrease systemic side effects of current anticancer agents is through the design of targeted anticancer prodrugs for tumor site-specific activation.[1] These targeted prodrugs will be stable and inactive over a wide range of physiological conditions and will be activated preferentially by a specific enzyme or metabolic pathway only present or predominantly present in the targeted cancer cells. This type of anticancer prodrugs, by utilizing the special features associated with cancers, can selectively act on those tumor cells and improve the therapeutic index over traditional chemotherapy. A number of prodrug designs have been proposed to meet these requirements and are currently under investigation.[2–4] Enzymes targeted by these prodrugs can be either endogenous enzymes such as DT-diaphorase, β-glucuronidase, prostate-specific antigen (PSA) and cytochrome P450 enzymes, or exogenous enzymes such as carboxypeptidase and nitroreductase that are delivered to cancer cells *via* antibody, gene and virus directed technologies. A number of reviews appeared over the last few years on targeted prodrugs[2,5–19] covering various aspects of the approach from enzyme biology[16] and design strategies[3,4,20] to clinical studies;[18] however, this current review will focus on prodrugs activated by reductive enzymes with special emphasis on the prodrug design principles and the structure-activation relationship (SAR) to help medicinal chemists in their design of targeted anticancer prodrugs for reductive activation. After a brief discussion of the strategies and enzymes employed in reductive activation, prodrugs with varying core structures or key functional groups that can be used to trigger reductive activation will be discussed in the order of quinones, nitroaromatics, N-oxides, and metal complexes.

2. STRATEGIES FOR REDUCTIVE ACTIVATION OF ANTICANCER PRODRUGS

A. Tumor-Specific Activation Under Hypoxic Conditions

Hypoxia is a common characteristic of most solid tumors resulting from inefficient microvascular systems associated with rapid tumor growth.[21] Hypoxic tumor cells confer resistance to radiotherapy and chemotherapy and present a tremendous challenge to cancer therapy for the following reasons: (i) hypoxic cells are distant from blood vessels and anticancer drugs usually cannot reach these hypoxic tumor cells;[22] (ii) any treatment that kills better-oxygenated cells will allow hypoxic cells to be reoxygenated and start to grow, making a significant contribution to repopulation of the tumors; (iii) tumor cells that survive in the hypoxic environment are more hypoxia-tolerant and might promote the growth of more malignant tumor cells;[23,24] (iv) tumor cells adapted to hypoxia also upregulate genes involved in drug resistance including genes encoding p-glycoprotein.[25] On the other hand, hypoxia also distinguishes tumor cells, especially those solid tumor cells, from normal cells, thus presenting new opportunities for selective cancer treatment. Hypoxia-selective prodrugs have obvious advantages over traditional anticancer cytotoxic agents in that the action of the prodrugs after activation is limited to hypoxic tumor regions thus minimizing the systemic side effects. The rationale employed in the design and development of hypoxia-selective agents targeting tumor cells is that hypoxia-selective prodrugs are able to release the active cytotoxic agents upon reduction under hypoxic conditions. Although reducing enzymes that can activate these hypoxia-selective prodrugs may also be present in aerobic cells, the reduced prodrug forms can often be rapidly oxidized back to the parental prodrugs by molecular oxygen. This redox cycling process ensures that the prodrugs are stable in normal cells and activated only in the hypoxic area of tumor tissues to deliver a selective treatment. Many enzymes including NADPH-cytochrome P450 reductase, DT-diaphorase, xanthine oxidase/xanthine dehydrogenase, and cytochrome b_5 reductase are involved in the reductive activation either alone or in combination with each other.

B. Tumor-Specific Activation by Exogenous Reductive Enzymes Delivered to Tumor Cells

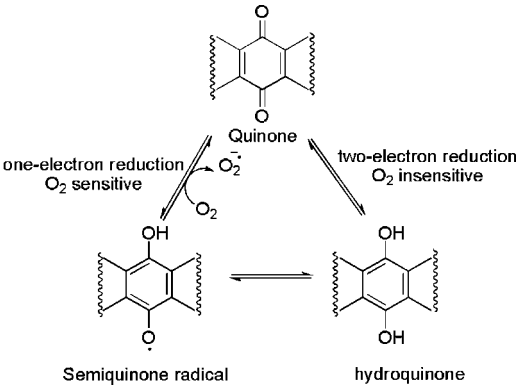
In addition to targeting endogenous reductive enzymes present in hypoxic cells, alternative strategies such as gene directed enzyme prodrug therapy (GDEPT) and virus directed enzyme prodrug therapy (VDEPT) have also been developed to express or deliver exogenous reductive enzymes into targeted tumor cells for prodrug activation. A number of enzyme/prodrug systems for bioreductive activation *via* GDEPT/VDEPT were explored and some have been tested in clinical trials recently.[14,18,23,26] Reductive enzymes employed in these strategies include cytochrome P450s, *E. coli* nitroreductase, and DT-diaphorase. Cytochrome P450s (CYP450s) represent a large family of heme-containing enzymes that metabolize both exogenous and endogenous compounds. Although the most common reaction catalyzed by CYP450s is monooxygenase reaction, CYP450s are also required for the activation of some bioreductive anticancer prodrugs. Cytochrome P450 reductase (EC 1.6.2.4, CYP450R) is a flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) containing enzyme and functions as an electron donor for CYP450. This NAPDH-ferrohemoprotein oxidoreductase catalyzes one electron-reduction reducing aldehydes and quinones to alcohols and semiquinones, respectively. Tirapazamine and AQ4N are two examples of prodrugs activated by CYPs/CYP450Rs. Nitroreductase, specifically *nfsB* gene product, from *E. coli* is another flavoenzyme with a bound FMN cofactor, which catalyzes the reduction of aromatic nitro groups to hydroxylamino groups. This reduction of nitro to hydroxylamino group represents a very large electron density change and can thus be used as an efficient ''electronic switch'' to release or generate potent cytotoxic agents as exemplified by the combination of CB1954 with virally delivered *E. coli* nitroreductase for the treatment of cancer.[26,27] DT-diaphorase (EC 1.6.5.2, NAD(P)H quinone dehydrogenase, NADPH-quinone oxidoreductase-1, NQO1) is an oxygen-independent flavoenzyme that catalyzes two-electron reduction using NAD(P)H and has a preference for short-chain acceptor quinones, such as ubiquinone, benzoquinone, juglone, and duroquinone. DT-diaphorase is a flavoprotein containing FMN as a cofactor and reduces quinones to the corresponding hydroquinones (diols).

3. PRODRUGS DESIGNED FOR BIOREDUCTIVE ACTIVATION

Four major classes of anticancer prodrugs have been investigated specifically for reductive activation, each with a distinct core structure. They are quinones, nitroaromatics, N-oxides, and metal complexes.

A. Quinones

Natural and synthetic compounds containing a quinone core structure are an important class of biologically active agents from coenzyme Q, vitamin K to anticancer antibiotics doxorubicin. Due to the strong tendency to form a fully aromatic system, quinones can be easily reduced by various enzymes to hydroquinones. The bioreduction of quinones can be through a one-electron pathway to produce semiquinone radicals by reductive enzymes like CYP450 reductase, cytochrome b_5 reductase and ubiquinone oxidoreductase or through a two-electron pathway to hydroquinones by reductive enzymes like DT-diaphorase. The one-electron reduction is a reversible and oxygen-dependent process which can be inhibited by molecular oxygen under normoxic conditions. Under hypoxic conditions, however, the semiquinone radicals will continue to be reduced to hydroquinones (Scheme 1). The hypoxia selectivity of quinone-containing prodrugs arises from this one-electron reduction pathway. DT-diaphorase is the major enzyme responsible for the two electron reduction of quinones and it is expressed at high levels in many human solid tumors, such as thyroid, adrenal, breast, ovarian, colon, and non-small-cell lung cancer. Therefore, DT-diaphorase became an attractive target for enzyme-directed anticancer prodrugs designed for reductive activation. However, the distribution of DT-diaphorase in normal tissues also presents potential adverse effect related to this type of prodrugs.[28]

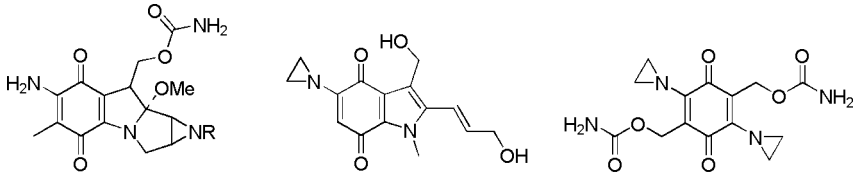


Scheme 1. Quinone reduction pathway by CYP450s and DT-diaphorase.[29]

Quinone-containing prodrugs of cytotoxic agents are designed to be selectively activated either by the hypoxic environment of tumor cells or by the two-electron reducing enzyme DT-diaphorase. Prodrugs that are good substrates of DT-diaphorase will be more cytotoxic under normoxic conditions but with lower hypoxia selectivity while prodrugs that are good substrates of a one-electron reductase but poor substrates of DT-diaphorase usually possess good hypoxia selectivity. It has also been shown that hypoxia selectivity of quinone-containing prodrugs is very sensitive to structure modification; slight changes on quinone core structure could result in substantial reduction in hypoxia selectivity.

1. Quinones as Part of Active Drugs

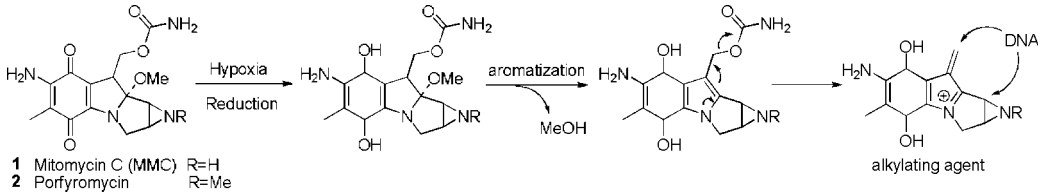
Mitomycin C (MMC, **1**) is the earliest clinically used quinone-containing drug recognized as a bioreductive and hypoxia-selective alkylating agent.[30] As the prototype agent in this group, the mechanism of action of mitomycin C has been extensively investigated and is believed to involve bioreductive activation as shown in Scheme 2. MMC and its analogues produce their cytotoxicity through reductive metabolism followed by well-defined fragmentation to bifunctional alkylating species that crosslink DNA *via* guanine–guanine in the major groove.[31] However this first hypoxia-targeting prodrug only shows marginal hypoxia selectivity while its N-methyl analogue porfiromycin (**2**) shows higher selectivity and has been clinically evaluated as a hypoxia-selective prodrug.[32] In addition to reduction by CYP450 reductases, MMC and its analogues are also substrates of DT-diaphorase[33,34] which is widely overexpressed in many types of tumor cells. EO9 (**3**) and AZQ (**4**) are another two principal aziridinylquinones, analogues of MMC developed as hypoxia selective agents. They are simplified analogues with aziridine attached directly to benzoquinone or indolequinone ring. These two types of quinone compounds were shown to be potent alkylating agents upon reduction to the corresponding aziridinyl hydroquinone, which effectively increases the pK_a of the aziridine nitrogen for protonation and activation toward nucleophilic attack at physiological pH values.[35]



1 Mitomycin C (MMC) R=H
2 Porfiromycin R=Me

3 EO9

4 AZQ



Scheme 2. Bioreductive activation of mitomycin C and porfiromycin leading to the formation of bifunctional alkylating agents.

EO9 (**3**), an indolequinone-based bioreductive prodrug, was identified as a good substrate of both human and rodent DT-diaphorases and can be activated to form DNA-damaging species under both normoxic and hypoxic conditions.[36–39] Under normoxic conditions, its cytotoxicity correlated well with DT-diaphorase activity, while the cytotoxicity under hypoxic conditions also correlated with but could not be attributed completely to the low levels of DT-diaphorase activity in tumor cells. These results indicate that EO9 has the potential to kill either the aerobic fraction of DT-diaphorase-rich tumors or the hypoxic fraction of DT-diaphorase-deficient tumors.[40] EO9 exhibited excellent activity in solid tumor animal models and showed no significant bone marrow toxicity in animal toxicology studies.[41] EO9 was tested in a phase II clinical study using a marker lesion strategy to evaluate its activity for the intravesical treatment of bladder cancer. Complete response, defined as complete disappearance of the marker lesion, was achieved in 67% and local toxicity was similar to that seen with other intravesical chemotherapeutic agents. Larger clinical trials will be needed to evaluate the potential clinical use of this drug.[42] However, clinical trials of EO9 failed to show significant therapeutic benefits in humans. The main reason for this failure is believed to be due to its poor pharmacokinetic properties such as the very short half-life and poor tissue penetration.[43] Since the discovery of EO9, indolequinone-based bioreductive prodrugs have drawn considerable interest. The indolequinone core has been modified in efforts to develop analogues that would retain the hypoxia-selectivity and low toxicity while exhibit improved pharmacokinetic properties. SAR studies mainly focused on the 2, 3, or 5 position on the indolequinone core as shown in Figure 1.

5-position: An aziridinyl group at 5-position is essential for potency and selectivity under both normoxic and hypoxic conditions. Addition of a methyl group onto the carbon of aziridinyl ring introduces steric hindrance that would reduce DT-diaphorase-dependent metabolism leading to increased hypoxia selectivity.[44–46]

3-position: Elimination from the indol-3-yl methyl position was found to be important in clearly differentiating the hypoxic cells from the normoxic DT-diaphorase-rich cells. Several model compounds were synthesized and shown to undergo elimination of the leaving groups at 3-yl position as shown in Scheme 3 under chemical or radiolytic conditions exhibiting significant hypoxia selectivity in the range of 10–200.[47] However, very few changes at the indol-3-yl methyl position can be made without adversely affecting substrate specificity. Introduction of bulky leaving groups like carbamoyloxy methyl at the 3-position usually gave poor substrates of DT-diaphorase.[44] On the other hand, when a small good leaving group like chlorine was attached at 3-position, inactivation of the reductive enzyme was observed. That is believed to be due to alkylation of the enzyme active site.[45,46] This finding was utilized later to design mechanism-based inhibitors of DT-diaphorase with a series of phenolic leaving groups at the 3-position.[48] Replacement of the hydroxymethyl group with an

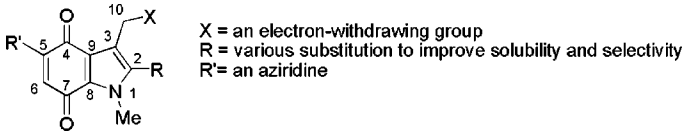
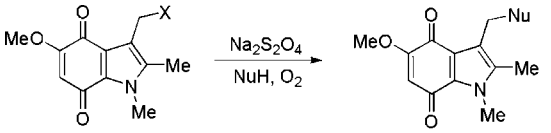


Figure 1. SAR of indolequinone analogues.



X = a leaving group such as carbamate, carboxylate, phenol, thiol

Scheme 3. Elimination of the leaving group at the 3-position of indolequinones.

aldehyde completely lost the substrate activity of DT-diaphorase under both normoxic and hypoxic conditions.[49] It is, thus, possible to preferentially target the hypoxic fractions of tumors with such modifications at the 3-position that gave poor substrates of DT-diaphorase but with high hypoxia selectivity. When desired, reintroduction of the hydroxymethyl group at the 3-position would render it active as a substrate of DT-diaphorase, making it possible to target the normoxic DT-diaphorase-rich fractions of tumors.

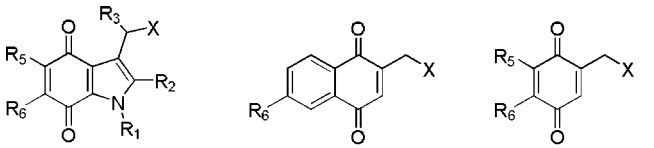
2-position: The effect of substitution at indol-2-yl position on cytotoxicity and selectivity under hypoxic conditions has been investigated by Stratford and co-workers.[47] It was demonstrated that the bulkiness of the alkyl group at 2-position dramatically affected the *in vitro* cytotoxicity and hypoxia selectivity upon reductive activation under chemical or radiolytic conditions. Analogues without any substitution at C-2 were up to 300 times more cytotoxic but less hypoxia-selective than analogues with an alkyl substituent at C-2.[47] However, substrate specificity for DT-diaphorase was not adversely affected by such modifications and was substantially enhanced in some cases. For example, 2-piperidin-1-ylethyl aminocarbonyl, 2-morpholinylethyl aminocarbonyl, or cyclopropyl substitution at C-2 position was well tolerated resulting in good substrate activity for DT-diaphorase that is comparable to EO9.[45] 2-Phenyl-5-methoxy indolequinone derivatives were shown to be six times better as substrates of DT-diaphorase.[45,50] Recently, a series of indolequinones bearing p-nitrophenoxymethyl group at indol-2-yl position were reported to be poor substrates of DT-diaphorase.[51]

Benzoquinone-based bioreductive prodrugs like AZQ (**4**)[52–55] and prodrugs based on benzimidazolequinone, and naphthoquinone[56–61] were also widely investigated as hypoxia-selective bioreductive anticancer agents. Such SAR studies are not only essential for optimization of quinone-based small molecules as active drugs but also facilitate the design and development of new quinone-based molecules to deliver cytotoxic agents to hypoxic tumor tissues or tumor tissues transfected to express reductive enzymes.

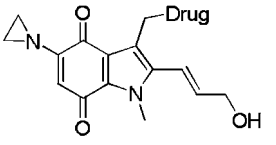
2. Quinone as a Promoiety for the Targeted Delivery of Cytotoxic Agents

Indolequinones (**5**) have been extensively used as promoieties to effectively release the parent drugs attached at 3-position upon bioreduction or radiolytic reduction.[46,47,62] Reductive activation of indolequinone prodrugs is accompanied by the concomitant formation of the electrophilic iminium cations, which may also contribute to the cytotoxicity to tumor cells. Other similar promoieties include naphthoquinones (**6**) and benzoquinone (**7**). A recent example of indolequinone prodrugs is an FUDR prodrug **8a** shown in Scheme 4. **8a** exhibited only minimal cytotoxicity against EMT6/KU cells under aerobic conditions, while it showed significantly enhanced cytotoxicity under hypoxic conditions with an IC_{50} as low as 150 nM. This is approximately 50-fold lower than the IC_{50} of 8.1 μM for the parent drug FUDR. This enhanced cytotoxicity was attributed to the strong cytotoxic effect of the electrophilic iminium cation formed in addition to the release of the parent drug FUDR.[63] Camptothecin is a potent inhibitor of DNA topoisomerase leading to irreversible and lethal strand breaks of DNA during its replication. However, unfavorable physicochemical properties of camptothecin such as poor water solubility, lactone ring instability prevented its clinical use in the treatment of cancer. Various approaches such as camptothecin analogues, camptothecin prodrugs, or

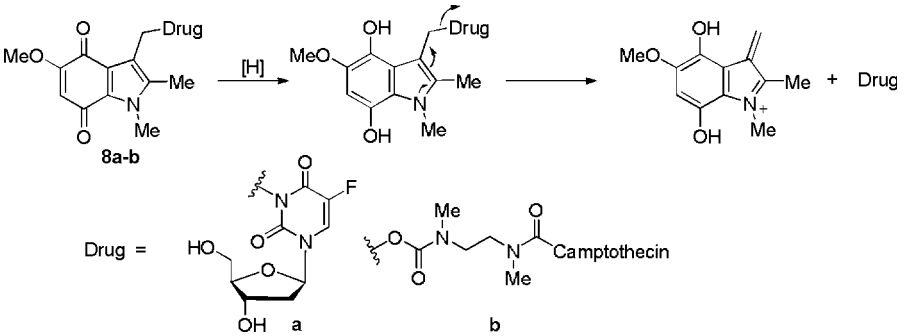
new drug delivery systems have been developed to overcome these unfavorable physicochemical issues.[64–70] One of these approaches is to develop water soluble camptothecin prodrugs with selectivity toward tumor cells by targeting tumor hypoxia. Camptothecin was attached to the 3-position of indolequinone as in **8b** through N, N′-dimethyl-2-aminoethylcarbamate linker as a new class of water soluble prodrugs that can be activated quickly by DT-diaphorase. The reduction was followed by cyclization releasing the parent active drug camptothecin. This camptothecin prodrug showed at least one order of magnitude lower cytotoxicity and higher hypoxia selectivity than the parent drug camptothecin.[71]



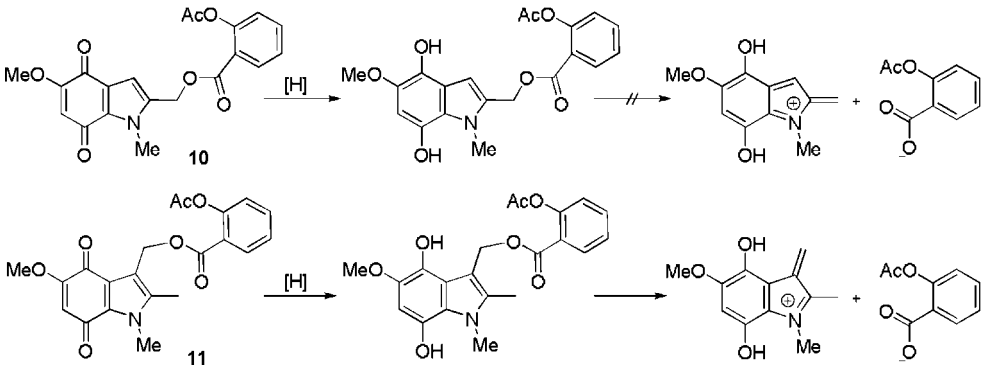
Anticancer agent EO9 has also been proposed as a promoiety to attach to an anticancer drug at its indol-3-yl methyl position as a leaving group to form a dual acting prodrug (**9**).[72] Upon reduction, both the anticancer drug itself and the active metabolite of EO9 can be released *via* spontaneous elimination.



In addition to indol-3-yl position, SAR study has demonstrated that modification at 2-position might be superior to the 3-position in terms of generating better substrates of DT-diaphorase. Molecular modeling of the enzyme-substrate complex also indicated that the active site of DT-diaphorase can accommodate a broad range of substituents at 2-position since this position is located at the entrance to the binding site.[45,73] In light of these findings, it is believed that a prodrug strategy utilizing bioreductive delivery of cytotoxic agents from the 2-position of the indolequinone would provide compounds having a high correlation between DT-diaphorase activity and cytotoxicity. Aspirin was chosen as a model drug to be attached to indolequinone at 2- or 3-position to provide potential prodrugs **10** and **11** for bioreductive activation.[74] As shown in Scheme 5, it was found that only 3-substituted regioisomer **11** can generate aspirin under radiolytic conditions, suggesting that modification of C-2 position might not be suitable for drug delivery through bioreductive activation.



Scheme 4. Release of the active drugs from prodrugs in the form of indolequinone-drug conjugates.

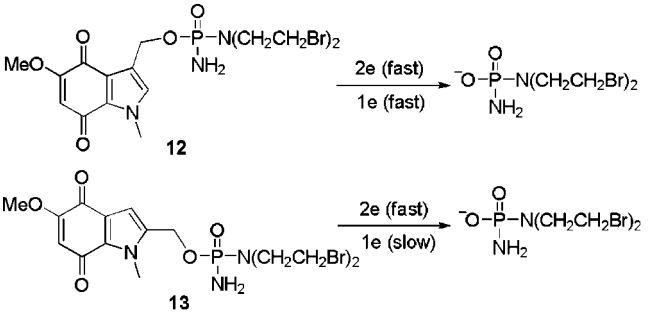


Scheme 5. Drug release of model prodrugs **10** and **11** of aspirin upon radiolytic reduction.

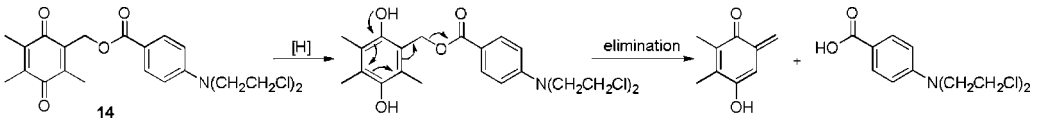
This conclusion was reexamined by Borch and co-workers when they extended this indolequinone prodrug strategy to deliver phosphoramide mustards to prepare phosphoramidate prodrugs for activation by DT-diaphorase.[62,75] It was found that both 2- and 3-substituted prodrugs undergo rapid activation *via* two electron reduction. Furthermore, the 3-substituted prodrug **12** also underwent rapid activation following one electron reduction while 2-substituted prodrug **13** was activated very slowly by one electron reduction (Scheme 6). Although both prodrugs were shown to be nanomolar inhibitors of cell proliferation *in vitro*, there was no correlation between cytotoxicity and DT-diaphorase activity for the 3-substituted regioisomer.[62] Further investigation under similar conditions found that the 3-substituted regioisomer could also be activated by glutathione rapidly while the activation of 2-substituted regioeisomer was considerably slower by glutathione. These results suggested that drug delivery *via* two-electron reduction from the 2-position was a more selective prodrug strategy.[75]

Anticancer drugs can also be released from benzoquinone conjugates. One example of a benzoquinone prodrug is the conjugate **14** of a nitrogen mustard. Under hypoxic conditions, prodrug **14** can be activated to produce 4-[bis(2-chloroethyl)amino]benzoic acid as shown in Scheme 7. Under physiological pH, the anionic nature of the carboxylate increases the electron density of aromatic ring that favors the formation of the aziridinium species capable of alkylating DNA.[76]

The ''trimethyl lock'' type of conformational restriction was also employed to facilitate cyclization activation of benzoquinone-derived prodrugs.[77] Upon reduction to hydroquinone, the ring cyclization process was facilitated by three strategically placed methyl groups—one the quinone ring *ortho* to the side chain and two on the side chain β to the carbonyl but α to the quinone ring as shown Scheme 8; such arrangement has been shown to restrict the rotation of the side chain and bring the carbonyl group closer to the attacking nucleophilic OH, an effect referred to as the ''trimethyl lock.''



Scheme 6. Activation of indolequinone prodrugs of phosphoramide mustard.



Scheme 7. Reductive activation of a benzoquinone prodrug of 4-[bis(2-chloroethyl)amino]benzoic acid.

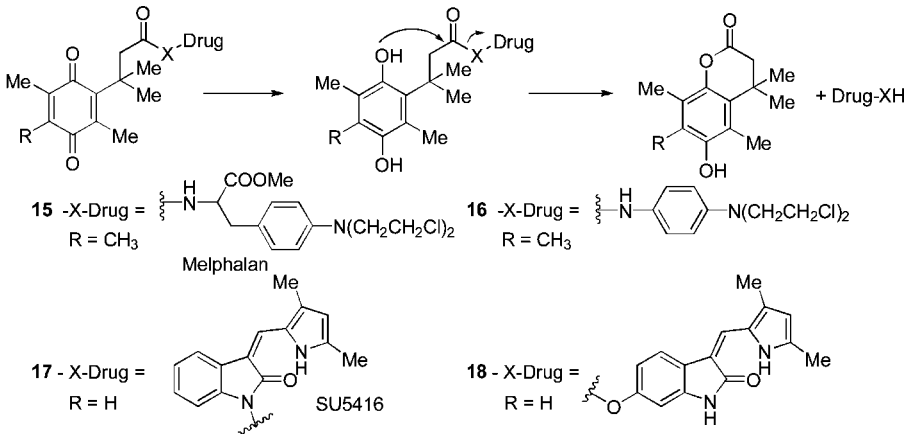
Trimethyl lock was first applied to prodrug design by Carpino et al.[78] and then explored by others in the design of prodrugs (Scheme 8).[20,79,80] Alkylating agent melphalan was shown to be released *via* lactonization following reduction of compound **15**. Recently, an antiangiogenic agent SU5416, a potent inhibitor of the VEGF receptor tyrosine kinase Flk-1/KDR, and its 6-hydroxy derivatives were used as the parent drugs in this prodrug system and the prodrugs **17** and **18** were shown to release parent drug molecules under reductive conditions, although hydrolysis of the amide or ester bond between the parent drug and benzoquinone promoiety could not be ruled out.[81]

Another example of a prodrug using the "trimethyl lock" concept is a benzoquinone conjugate **16** of 4-aminophenyl nitrogen mustard.[82] Prodrug **16** was shown to be a good substrate of DT-diaphorase with a *K*m of 2.70 ± 1.14 μmol/L, release the 4-aminophenyl nitrogen mustard upon reduction by DT-diaphorase under cell free conditions, and exhibit selectivity toward both the DT-diaphorase-overexpressing cells under normoxic conditions and T47D cells that overexpress cytochrome P450 enzymes with a hypoxia selectivity of 15.8.

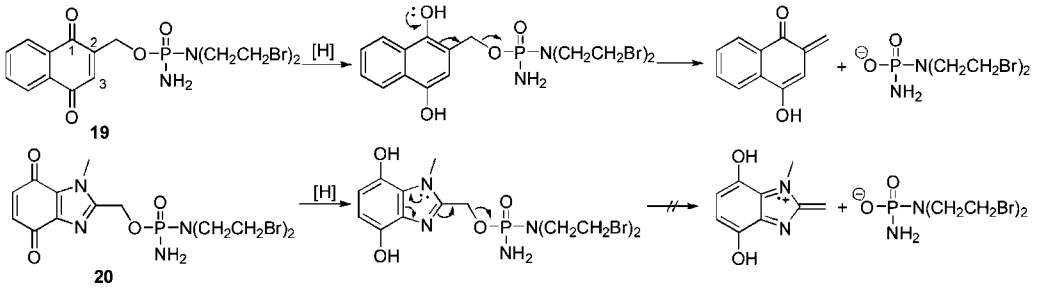
Naphthoquinone and benzoimidazolequinone have been tested as prodrug promoieties to release phosphoramide mustard.[83] The naphthoquinone prodrug **19** was a good substrate of DT-diaphorase and underwent facile activation and rapid expulsion of the cytotoxic phosphorodiamidate as shown in Scheme 9; however, its cytotoxicity was not correlated to DT-diaphorase activity suggesting the presence of other activation mechanism. Michael addition of sulfur nucleophile GSH to 3-position of naphthoquinone followed by elimination is believed to be the alternative activation mechanism of this prodrug. To solve this problem, benzoimidazolequinone analogue **20** was designed and synthesized. Prodrug **20** was stable to the nucleophilic attack; however, the reduced product was also stable and failed to expel the phosphorodiamidate anion.[83]

B. Nitroaromatics

Nitroaromatic compounds can be reduced in cells by a number of flavoprotein enzymes *via* stepwise addition of up to six electrons as shown in Scheme 10; however, the major enzymatic metabolite is



Scheme 8. Benzoquinone prodrugs designed using the "trimethyl lock" concept.

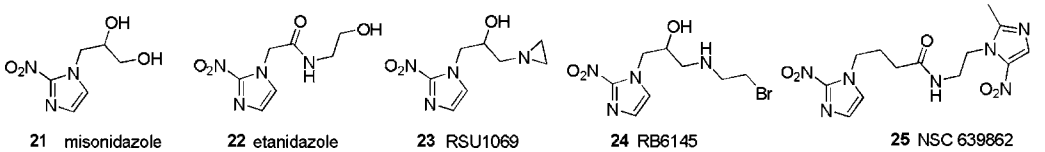


Scheme 9. Reductive activation of naphthoquinone and benzoimidazolequinone prodrugs of phosphoramide mustard **19** and **20**.

usually the four-electron product, hydroxylamine. The first electron adduct of nitro group is a nitro radical anion which can be efficiently oxidized back by O_2 to the nitro group, thus limiting prodrug activation to hypoxic environments. This leads to the hypoxia selectivity of nitroaromatic compounds.

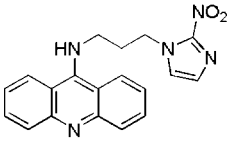
1. Nitroaromatics as Prodrugs

Hypoxia-targeted prodrugs using nitroaromatics were initially developed from the early nitroimidazole-based radiosensitizers such as misonidazole **21** and etanidzole **22** as mimetics of oxygen. These radiosensitizers are selectively metabolized under hypoxic conditions but with only weak cytotoxicity in tissue culture and moderate hypoxia selectivity.[8] Since it was found that misonidazole could enhance radiotherapeutic outcome *in vivo*, nitroimidazole-based derivatives were extensively exploited to increase its cytotoxicity and hypoxia selectivity. For example, alkylating side chains were introduced to nitroimidazole analogues. The first agent showing a remarkable enhancement in hypoxia selectivity was RSU1069 (**23**). This 2-nitroimidazole contains an aziridine moiety in the N1 side chain enabling RSU1069 to function as a bifunctional alkylating agent upon reduction.[84,85] RB6145 (**24**), the mustard analogue of RSU1069, was shown to be 2.5 times less toxic but only slightly less active than RSU1069 partially due to the improved pharmacokinetic profile with RB6145.[86] Both R- and S-enantiomers of RB6145 were potent hypoxia-selective agents;[87] however, due to the retinal toxicity related to its R enantiomer (also called CI-1010), RB6145 was not further developed.[88] Bis bioreductive compounds were also explored as an approach to increase potency and selectivity of prodrugs.[89–91] The bis-nitroimidazole **25** is one such example; Compound **25** joins a 2-nitroimidazole and 5-nitroimidazole moiety *via* a carboxamide linker, was shown to be more selective than the mononitroimidazoles for hypoxic AA8 Chinese hamster cells.[91] The bis bioredutive mechanism could be responsible for the high hypoxia selectivity; but, DNA cross-linking was unlikely to be involved as originally conceived.[90,91]

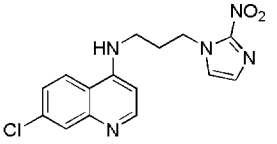


Scheme 10. Reduction of nitroaromatics and back-oxidation of nitro radical anion by oxygen.

DNA intercalating moieties have also been tethered to nitroimidazoles in efforts to increase their potency.[92–98] However, NLA-1 (**26**) with a nitroimidazole tethered to an acridine, which is a tight DNA binding moiety, exhibited slow dissociation kinetics and limited extravascular diffusion to hypoxic regions of tumors and was, thus, ineffective *in vivo*. Weak DNA intercalating moiety with DNA affinity high enough to produce cytotoxicity yet low enough to allow efficient diffusion and penetration were more desirable.[99,100] NLCQ-1 (**27**), a quinoline analogue of NLA-1, met these requirements and showed significant hypoxia selectivity in several rodent and human tumor cell lines. NLCQ-1 substantially enhanced the antitumor effect of alkylating agents, as well as anti-metabolite 5-FU and anti-mitotic paclitaxel without increasing bone marrow or hypoxia-dependent retinal toxicity.[100]

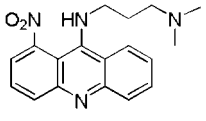


26 NLA-1
Tight DNA intercalator

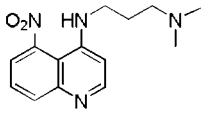


27 NLCQ-1
Weak DNA intercalator

Compared to nitroimidazole derivatives **26** and **27**, 1-nitroacridine (nitracrine, **28**) showed more potency and similar hypoxia selectivity toward AA8 cells in culture.[101] However, nitroacrine and its derivatives showed rapid metabolism and tight DNA binding with limited extravascular diffusion thus no activity against hypoxic cells in solid tumor *in vivo*.[93,102,103] Nitroquinoline derivatives like **29** were considered better candidates due to their lower DNA binding affinity than nitroacridine but with comparable selectivity in AA8 cell cultures. Various substituted nitroquinolines were prepared to improve their extravascular diffusion which was thought to be the key factor limiting the performance of such prodrugs *in vivo*. However, none of nitroquinolines synthesized showed activity toward hypoxic cells *in vivo*.[104]

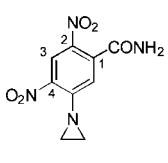


28 1-nitroacridine

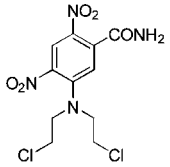


29 5-nitraquine

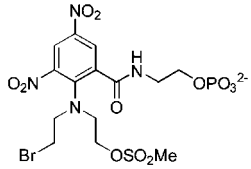
Nitrobenzene directly attached with alkylating agents formed an important group of bioreductively activated prodrugs. The nitro group is a strong electron-withdrawing group (Hammett σ_p electronic parameter = 0.78) and is converted to an electron-donating hydroxylamino group ($\sigma_p = -0.34$) upon NTR-reduction.[27] Enzymatic reduction of an aromatic nitro group to a hydroxylamine results in a huge difference in electronic effect ($\Delta\sigma_p = 1.12$) on the aromatic ring which can activate the latent alkylating species directly attached on it.



30 CB1954



31 SN23862



32 PR-104

Among this group of prodrugs, 5-aziridinyl-2, 4-dinitrobenzamide (CB1954, **30**)[105] was the most widely studied for GDEPT with *E. coli* nitroreductase and for reductive activation under hypoxic conditions.[106] CB1954 was initially found to have potent anti-tumor activity against the Walker rat carcinoma associated with its reduction at 4-position by rat DT-diaphorase; however,

human DT-diaphorase is much less efficient than the rat enzyme isoform in activating the prodrug. CB1954 was found later to be a good substrate of *E. coli* nitroreductase with reduction at either 2- and 4-position and was evaluated as a prodrug with virally delivered *E. coli* nitroreductase in clinical trials.[107] Although reduction of CB1954 with *E. coli* nitroreductase in cell free systems gives an equimolar mixture of 2- and 4-hydroxylamine metabolites, 4-hydroxylamine metabolite was shown to be more cytotoxic than the 2-hydroxylamine metabolite.[108] A recent study suggests that the 2-amino instead of the 4-hydroxylamino was the major metabolite responsible for the bystander effect of CB1954 when it was activated by *E. coli* nitroreductase in tumors.[109] SN23862 (**31**), the mustard analogue of CB1954, was not a substrate of DT-diaphorase; unlike CB1954, SN23862 is activated by *E. coli* nitroreductase only at the 2-position providing a single metabolite that has superior cytotoxicity and diffusion property as compared to CB1954 metabolites. In addition to efficiently killing cancer cells through bystander effects, it also showed potential to target tumor cells with severe hypoxia.[110]

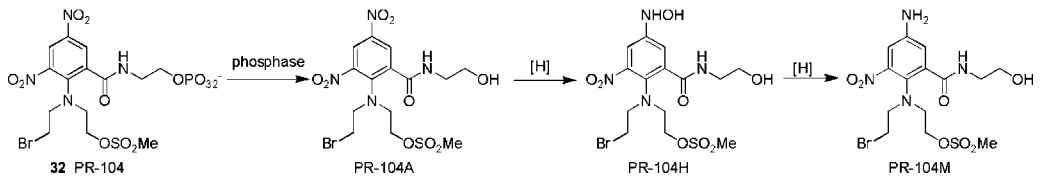
To develop hypoxia-selective cytotoxic agents, extensive SAR studies of SN23862 were conducted by Denny and collaborators mainly focusing on modification of the carboxamide moiety, the mustard leaving groups, and various regioisomers to improve aqueous solubility and at the same time retain or enhance its hypoxia selectivity.[111,112] The optimization efforts led to the development of analogue PR-104 (**32**) in clinical trials as a hypoxia-activated prodrug.[113] PR-104 is a phosphate ester pre-prodrug[114] which is released *in vivo* through the action of phosphatases, followed by cellular metabolic reduction to its 5-hydroxylamino and 5-amino metabolites where the mustard moiety is activated (Scheme 11).[113] DT-diaphorase was initially thought to be the enzyme responsible for PR-104 reduction.. It was demonstrated recently that NADPH:cytochrome P450 oxidoreductase along with other unknown flavoproteins were the enzymes responsible for the activation of PR-104.[115]

To develop prodrugs useful in gene therapy (GDEPT) in combination with *E. coli* nitroreductase, SAR studies are still ongoing focusing more on improving the bystander effects through balancing the lipophilicity for adequate membrane penetration and hydrophilicity for aqueous solubility. Optimal bystander effects in GDEPT will be critical to the success of the cancer therapy.[114,116,117]

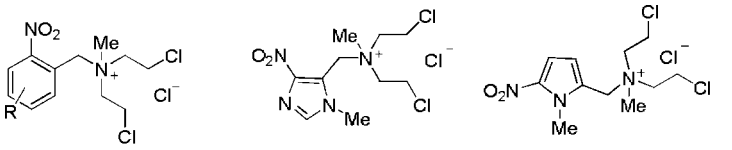
2. Nitroaromatics as Promoieties in Prodrug Design

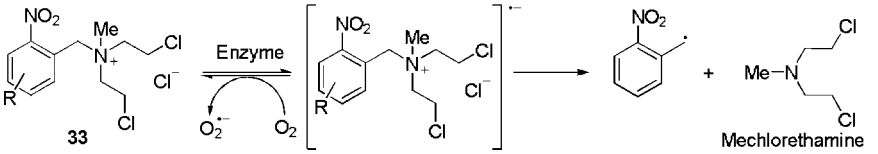
Due to their selectivity to tumor hypoxia or reductive enzymes used in GDEPT mentioned above, nitroaromatics have been used as promoieties to release various anticancer cytotoxic agents upon bioreduction. Various linking functional groups have been employed to attach the anticancer cytotoxic agents through amines, hydroxyls, thiols, and carboxylic acids.

a. Drug Release Through Radical Cleavage. Nitrobenzyl mustard quaternary ammonium salts were first reported as a new class of hypoxia selective cytotoxic agents exemplified by **33**.[118] This design has obvious advantages such as highly deactivated mustard moiety by positive charge and excellent water solubility. The prodrugs (**33**) were originally thought to undergo activation through fragmentation upon one electron reduction followed by release of the cytotoxic nitrogen mustard mechlorethamine and benzyl radical as depicted in Scheme 12. This nitrobenzyl quaternary ammonium salt was found to exhibit high hypoxia selectivity *in vitro*; however, only marginal activity



Scheme 11. Hydrolytic and reductive activation of PR-104.[115]

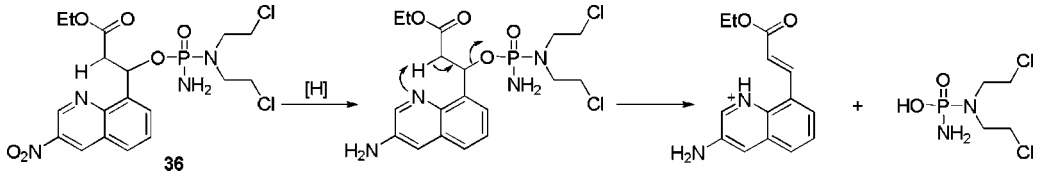




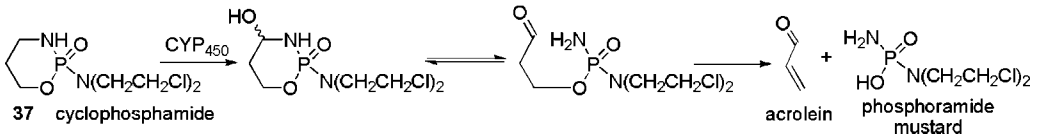
Scheme 12. Proposed mechanism of activation and drug release from nitrobenzyl mustard quaternary ammonium salts upon bioreduction.

against hypoxic cells was observed *in vivo*. Analogues with various substituents on benzene ring were investigated to alter the reduction potential of the nitro group. Although the reduction potential was altered and the observed hypoxia selectivity varied over a wide range from 1 to 3,000-fold against hypoxic AA8 cells, there were no correlations found between reduction potential and hypoxia selectivity.[119] Detailed mechanistic studies by steady-state and pulse radiolysis suggested that multi-electron reduction of **33** was required before fragmentation could occur and that the fragmentation was accompanied by the formation of many aromatic byproducts. On the other hand, nitroimidazole and nitropyrrole analogues **34** and **35** showed high yield of mechlorethamine formation with clean fragmentation upon reduction. However, they too failed to produce predictable cytotoxicity *in vivo* and were not subjected to further development. These mustard quaternary ammonium salts were found to be unstable leading to nonspecific release of mechlorethamine, which could be the major reason responsible for the observed variable cytotoxicity.[120]

b. Drug Release and Activation Through Elimination. Nitroquinoline **36** was first developed in 1991 as a targeting moiety for bioreductive activated prodrug to achieve selective cytotoxicity towards hypoxic cells.[121] As depicted in Scheme 13, phosphoramide mustard attached to β-position was released following nitro reduction under hypoxic conditions and the subsequent β-elimination was facilitated by the nearby basic quinoline nitrogen. Cytotoxicity assays showed the prodrug **36** was 11-fold more toxic toward HT-29 human colon tumor cells under hypoxic conditions than under normoxic conditions. DNA interstrand cross-links confirmed the release of phosphoramide mustard. Phosphoramide mustard is the active metabolite of cyclophosphamide **37**, one of the most successful anticancer agents developed over the past few decades. Because of its activity against both cycling and noncycling cells, cyclophosphamide is one of the few anticancer agents effective in the treatment of slow growing solid tumors. Cyclophosphamide has to be activated by cytochrome P-450 enzyme in the liver. As shown in Scheme 14, hepatic cytochrome P-450 oxidation coverts



Scheme 13. Release of phosphoramide mustard from prodrug **36** following bioreduction and intramolecular catalytic β-elimination.

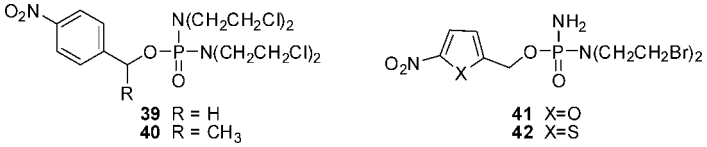


Scheme 14. Mechanism of activation of cyclophosphamide by liver CYP450.

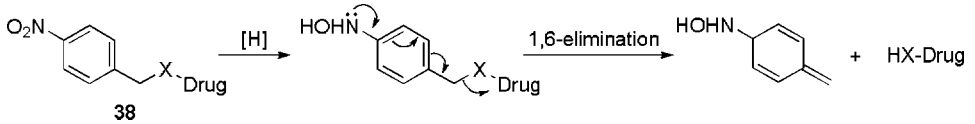
cyclophosphamide to 4-hydroxycyclophosphamide, followed by general base catalyzed β-elimination. Acrolein is a byproduct that is responsible for hemorrhagic cystitis, a life-threatening side effect associated with cyclophosphamide. Thus, targeted delivery of phosphoramide mustard through the design of prodrugs could avoid the systemic release of acrolein and the problems associated with it.

The interest in developing more selective alkylating anticancer agents led to the development of a variety of phosphoramidate prodrugs. 4-Nitrobenzyl derivatives in the form of **38** are among the most widely investigated promoiety for delivering cytotoxic agents selectively to hypoxic tumor cells or in gene therapy in combination with *E. coli* nitroreductase. Bioreduction of the nitro group in **38** gives the corresponding hydroxylamine intermediate, which then undergoes a spontaneous 1,6-elimination to release the cytotoxic drug molecule as shown in Scheme 15. Among them, prodrugs that can generate an alkylating agent upon activation are the most widely exploited.

4-Nitroaryl phosphorodiamidates were first explored by Borch's group as a potential hypoxia-selective alkylating agent.[122] The analogue without the nitro group was not cytotoxic confirming that the presence of the nitro group is essential for activity. Both **39** and **40** showed selective cytotoxicity to HT-29 cells under hypoxic conditions resulting in a greater number of DNA interstrand cross-links. Compound **39** without the methyl at benzylic position showed a selective cytotoxicity ratio of only 2.0 while the corresponding analog **40** with a methyl substitution at the benzylic position showed an improved selectivity ratio of >90. The basis for these differences is not yet clear. However, toxicity to bone marrow progenitor cells was also observed for almost all 4-nitroaryl phosphorodiamidates tested.[122]



Nitrofuryl and nitrothienyl derivatives bearing phosphoramidate (**41** and **42**) were also studied as hypoxia-selective alkylating agent. SAR study indicated that electron-withdrawing substituents on the furan and thiophene ring increased cytotoxicity under aerobic conditions and thereby decreased hypoxia selectivity while electron-donating substituents markedly decreased both aerobic and hypoxic cytotoxicity but enhanced hypoxia selectivity. These nitroheterocyclic analogues were remarkably of low toxicity as compared to other alkylating agents, exhibiting only moderate toxicity to bone marrow progenitors at the maximum tolerated dose.[123] Highly potent but moderately selective analogues were selected for preclinical evaluation. Pharmacokinetic studies showed that both were rapidly metabolized, but the nitrofuryl compound had a somewhat longer plasma half-life. It was concluded that the nitrofuryl phosphoramidate **41** was the most promising as a single agent



Scheme 15. Activation and release of active drug from 4-nitrobenzyl drug conjugates upon bioreduction followed by 1,6-elimination.

because it was highly potent and moderately selective to hypoxia; it could kill both aerobic and hypoxic cells, exhibiting excellent antitumor activity and minimal toxicity at therapeutically effective doses in mice. However, enzymes responsible for the activation of this prodrug has not identified yet.[124]

Efforts from our group have focused on the design of cyclophosphamide analogues through the incorporation of a trigger-activation mechanism by *E. coli* nitroreductase for gene therapy. The design of our nitroaromatics is based on the activation mechanism of cyclophosphamide shown in Scheme 14, in the hope of moving the site of activation from the liver to nitroreductase-expressing tumor cells. As shown in Scheme 14, hepatic cytochrome P-450 oxidation converts cyclophosphamide **37** to 4-hydroxycyclophosphamide, followed by a general base-catalyzed β-elimination. Our first analogue **43** was a nitrobenzene-fused cyclophosphamide (Fig. 2).[125] The strong electron-withdrawing effect reduces the oxidation potential of the phosphorinane ring system toward hepatic cytochrome P-450 oxidation thus avoiding liver metabolism. The strong electron density change upon reduction to hydroxylamine by *E. coli* nitroreductase facilitates the cleavage of benzylic C-O bond and activation of highly cytotoxic phosphoramide mustard portion as shown in Scheme 16. This nitrobenzene fused cyclophosphamide exhibited good substrate activity for *E. coli* nitroreductase with a half-life of 13 min and a modest >33-fold enhanced cytotoxicity toward *E. coli* nitroreductase-expressing cells. Though not sufficiently potent, **43** represented a new structure type for reductive activation and a new lead for further modification in the development of better analogues with much improved selective toxicity to be used in gene-directed enzyme prodrug therapy.[125]

Our interest then turned to related exocyclic cognate, 4-nitrophenyl-substituted cyclophosphamide **44**.[126] Structure-activity relationship studies demonstrated that cytotoxicity and selectivity were dependent on not only good substrate activity toward *E. coli* nitroreductase but also the presence of a benzylic oxygen *para* to the nitro group. Compared to the nitrobenzene-fused cyclophosphoramide **43**, the 4-nitrophenylcyclophosphoramide (**44**) showed an over 100-fold increase in cytotoxicity and nearly 1,000-fold increase in selectivity toward *E. coli* nitroreductase-expressing cells. The *trans*-isomer was shown to be a better substrate of *E. coli* nitroreductase than the corresponding *cis* isomer as indicated by its relatively shorter half life in the presence of the enzyme, suggesting that configuration might affect the substrate binding to and/or the catalytic activity of *E. coli* nitroreductase. Surprisingly, the acyclic analogue 4-nitrobenzyl phosphoramide mustard **45** (LH7), which was originally synthesized as a control compound to explore the mechanism of activation of cyclic phosphoramide analogues, turned out to be the most active compound in all our assays. Prodrug **45** showed 170,000-fold selective cytotoxicity toward *E. coli* nitroreductase-expressing V79 cells and an IC_{50} as low as 0.4 nM. When compared to CB1954, an excellent substrate of *E. coli* nitroreductase currently in phase II clinical trials, 4-nitrobenzyl phosphoramide mustard **45** is 100-fold more active and 27-fold more selective, along with excellent bystander effects with a TE_{50} of 3.3% as compared to 4.5% for CB1954.[127] All nitroaryl phosphoramide mustard analogues were stable in phosphate buffer at pH 7.4 and 37°C. They are all good substrates of *E. coli* nitroreductase with half lives between 2.3 and 13 min. They have low cytotoxicity before reduction and are converted to phosphoramide mustard or like reactive species upon reduction. The excellent activity of compound **45** in nitroreductase-expressing cells was unexpected considering the fact that only a twofold increase in cytotoxicity was observed for 4-nitrobenzyl *N*,*N*,*N*,*N*-tetrakis (2-chloroethyl)phosphorodiamidate (**39**) toward cancer cells under hypoxic conditions as reported

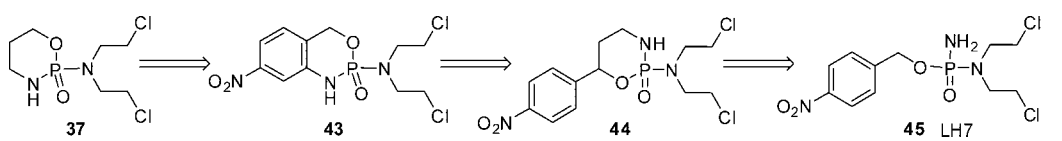
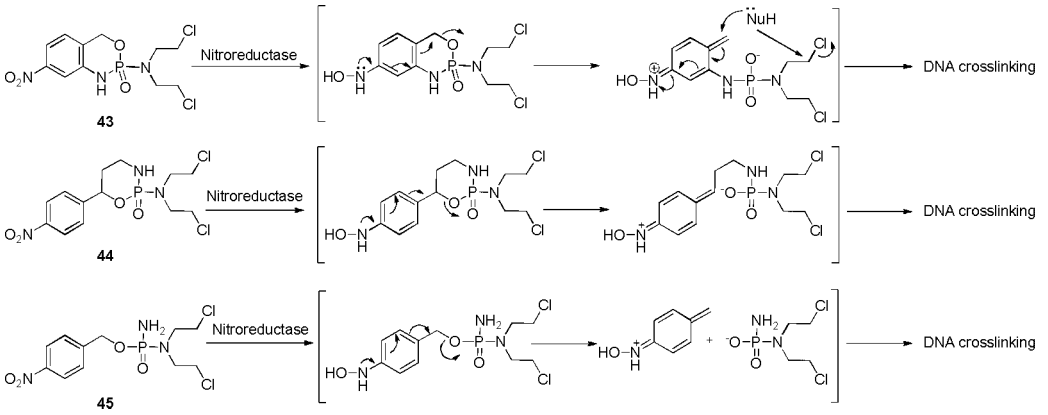


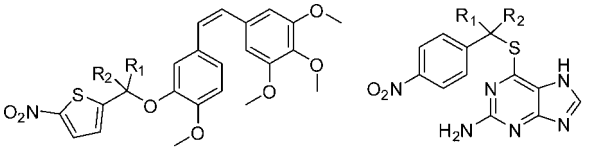
Figure 2. Design of cyclic and acyclic nitroaryl phosphoramide mustards for reductive activation by *E. coli* nitroreductase.



Scheme 16. Reductive activation of cyclic and acyclic nitroaryl phosphoramide mustard prodrugs.

by Borch's group.[122] This suggests that either these compounds were poor substrates of the human reductase(s) present or the expression of these reductase(s) was limited under the hypoxic assay conditions used. The low IC_{50} and the high selectivity of these types of prodrugs in *E. coli* nitroreductase-expressing cells suggest their potential of becoming a drug candidate in enzyme-prodrug therapy.

Nitrothienyl heterocycle has been used to deliver combretastatin A-4 *via* bioreductive activation.[128] SAR studies showed that only the dimethyl-substituted analogue **46b** is metabolically stable for 16 hr in the presence of liver homogenates under aerobic conditions whereas the monomethyl analog or the unsubstituted analog **46a** were not stable under the same conditions. Apparently, the dimethyl substitution successfully inhibited the undesired aerobic metabolism, thus, increasing the selectivity under hypoxic conditions. When this strategy was applied to the 4-nitrobenzyl system in delivering 6-mercaptopurine, it was shown that *S*-(α,α-dimethyl-4-nitrobenzyl)-6-mercaptopurine (**47b**) effectively released the parent drug 6-mercaptopurine under hypoxic conditions while the unsubstituted *S*-(4-nitrobenzyl)-6-mercaptopurine (**47a**) failed to release the parent drug.[129]

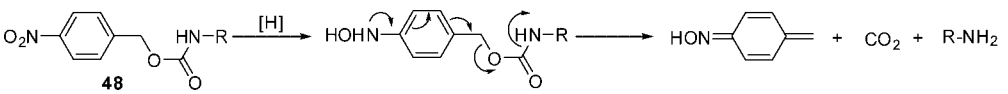


46a R_1 = H R_2 = H
46b R_1 = Me R_2 = Me

47a R_1 = H R_2 = H
47b R_1 = Me R_2 = Me

Nitroarylmethyl carbamate is one type of prodrugs that have been explored in GDEPT.[12] Nitrobenzyl carbamates (**48**) undergoes enzymatic reduction to its hydroxylamine, followed by 1,6-elimination to release the amine-containing parent drug as shown Scheme 17.[130]

Since the reduction potential of these compounds are probably too low to be activated efficiently by human cellular reductases, application of these prodrugs have turned from targeting hypoxia to GDEPT using *E. coli* nitroreductase. Crystal structure of *E. coli* nitroreductase revealed that there are



Scheme 17. Reductive activation of 4-nitrobenzyl carbamates prodrugs *via* 1,6-elimination.

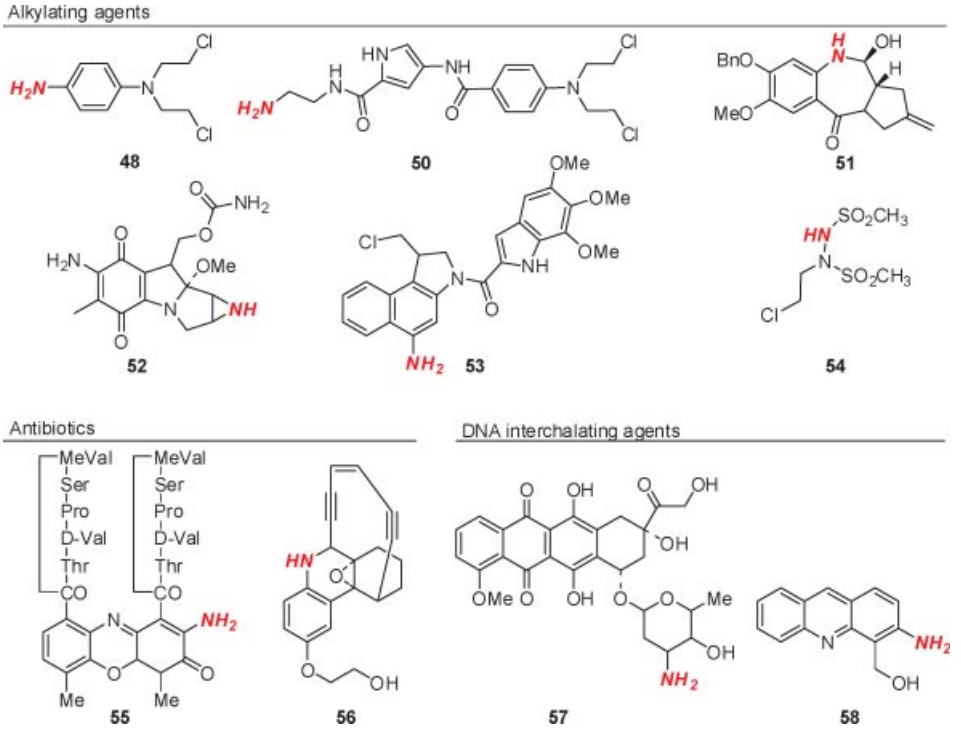
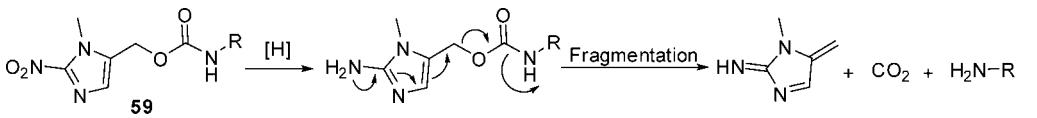


Figure 3. Structures of representative anticancer drugs that have been used in the 4-nitrobenzyl carbamate prodrug system. The nitrogen in each drug used to form the carbamate is shown in red. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

only a few specific contacts between the enzyme active site and the ligand, which might explain the broad substrate specificity observed for the enzyme. As shown in Figure 3, alkylating agents **49**–**54**, antibiotics **55** and **56**, and DNA intercalating agents **57** and **58** have been used as the parent drug in the 4-nitrobenzyl carbamate prodrug system.[90,131–137] However, efficacy studies have yet to identify the conjugates for further evaluation in GDEPT.

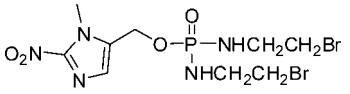
1-Methyl-2-nitroimidazol-5-ylmethyl carbamates in the form of **59** were also tested as potential prodrugs that share a similar fragmentation mechanism (Scheme 18) as the 4-nitrobenzyl carbamates.[81,138,139] Minor groove alkylating agent 5-aminobenz[e]indoline, phenyleneamine mustard and other cytotoxins analogues have been tested for nitroreductase mediated gene therapy.[140] Despite exhibiting potent and selective activity in culture in several cases, they failed to show activity against NTR+ cells in tumors. Further optimization of pharmacokinetic parameter will be required.

Similar to the design of 4-nitrobenzyl phosphoramide mustard **45** for reductive activation, 1-methyl-2-nitroimidazol-5-ylmethyl was attached to phosphoramide as in prodrug **60** (TH-302).[141] Compound **60** is achiral and releases the corresponding bromo analog of isophosphoramide mustard upon reduction. It was shown to be activated under hypoxic conditions with a 400-fold enhanced cytotoxicity toward H460 human non-small cell lung cancer cells in culture under hypoxic versus



Scheme 18. Reductive activation of 1-methyl-2-nitroimidazol-5-ylmethyl carbamate prodrugs.

aerobic conditions. *In vivo* studies using an orthotopic xenograft model of pancreatic cancer demonstrated that compound **60** has antitumor efficacy both alone and in combination with gemcitabine.



60 (TH-302)

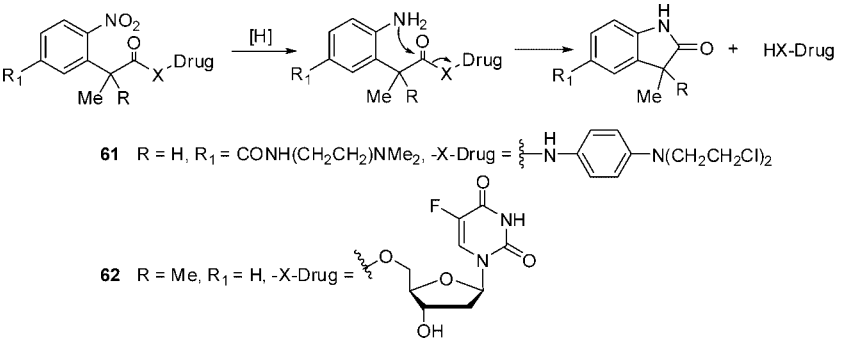
c. Drug Release and Activation Through Cyclization. Cyclization is another strategy that utilizes the change in electron density/nucleophilicity before and after reduction to effectively release and activate the parent drug. When this approach was applied to nitroaromatics in the design of bioreductively activated prodrugs, an amide or ester group can be cleaved intramolecularly by the amine formed after reduction of the nitro group to trigger the release of the parent drug.

Compounds **61** and **62**, as potential bioreductively activated prodrugs, employed the 2-nitrophenylalkanoyl as the promoiety, releasing the parent drug 4-aminoaniline mustard [142] and 5-FUDR,[143,144] respectively, upon reduction of nitro to amino group *via* "cyclization-extrusion" mechanism, that is, cleavage of amide or ester bond by cyclization as shown in Scheme 19. The nucleophilicity of amino group on the benzene ring and the geometry of the compound were found to be important while the leaving groups with varying substitutions were shown to have little effect on the rate of cyclization.[142] Methyl groups on the benzylic carbon restrict the side chain to the conformation required for rapid cyclization and to sterically hinder hydrolysis by serum esterase for increased stability. Cyclization of both compounds **61** and **62** occurs rapidly upon reduction of the nitro to amino group; however, these compounds were not good substrates of nitroreductase probably due to steric hindrance or low reduction potential that prevented efficient enzymatic reduction.

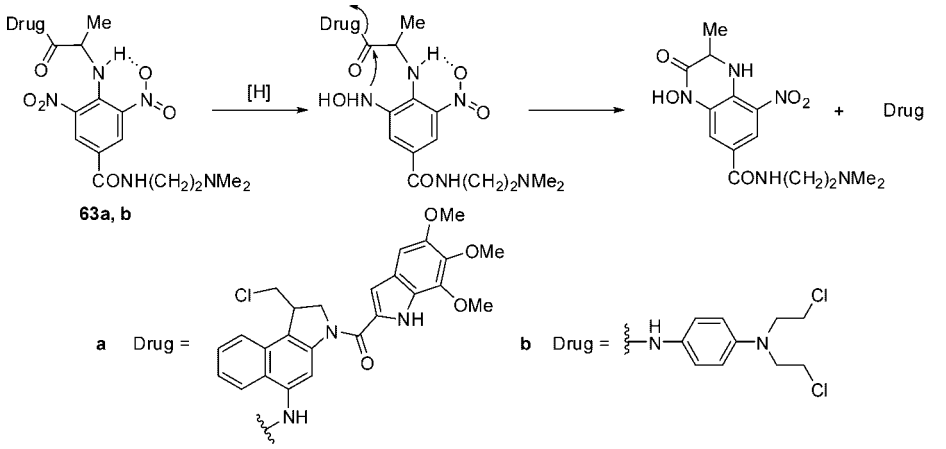
To obtain prodrugs that can be efficiently reduced by nitroreductase, further optimization of this promoiety led to the development of 2,6-dinitrophenyl conjugates in the form of **63** featuring higher reduction potential and conformation restriction through intramolecular H-bonding between aniline NH group and the adjacent 2-nitro group[145] as shown in Scheme 20. 5-Amino-1-(chloromethyl)benz[e]indoline and 4-aminoaniline mustard alkylating agents were attached to this system and the resulting conjugates **63a** and **63b** showed cell killing by radiation-induced reduction. However, they were not activated efficiently by cellular nitroreductases.[145]

C. N-Oxides

Compounds containing N-oxides were described as cytotoxic agents as early as the 1960s[146] and have drawn interests as bioreductively activated prodrugs[7,8,14,147,148] due to their potential to be selectively



Scheme 19. Bioreduction and subsequent cyclization activation of 2-nitrophenylalkanoyl conjugates of amine or alcohol-containing drugs.



Scheme 20. Bioreduction and subsequent cyclization activation of 2-nitrophenylalkanoyl conjugates of amine or alcohol-containing drugs.

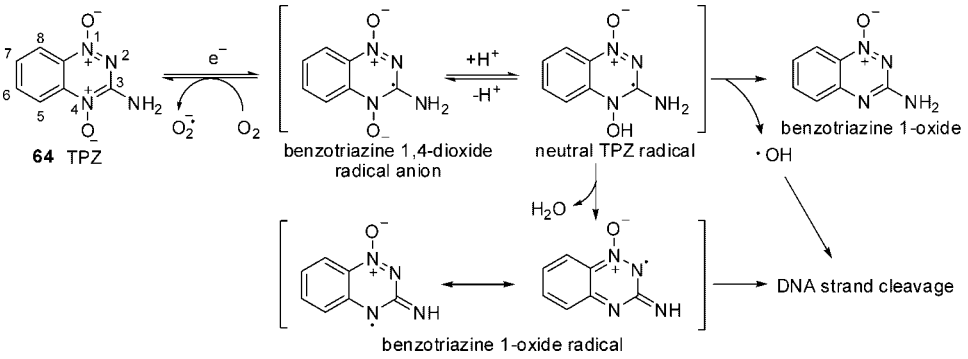
reduced under hypoxic conditions or by certain reducing enzymes. Two representative drugs, tirapazamine (TPZ, **64**) and AQ4N (**68**), have advanced into clinical trials and showed promising results in combination with radiotherapy and other chemotherapies.[18] Based on structures, N-oxide containing compounds can be divided into two classes, each with different mechanisms of activation.[148]

1. Aromatic N-Oxides

Aromatic N-oxides can be reduced by a one-electron process to generate a possible cytotoxic radical species (nitroxide radical), which undergoes radical-mediated oxidative DNA strand cleavage, primarily at DNA C'-4 ribose site, without covalent binding to DNA and protein. Their hypoxia selectivity is believed to be due to the back oxidation of radical species by molecular oxygen during the initial reversible stage of their metabolism.[148]

Tirapazamine (TPZ, **64**), one of the benzo-1,2,4-triazine dioxide derivatives, was first emerged as a hypoxia-selective cytotoxic agents in the 1980s.[149] TPZ can be activated primarily by cytochrome P450 and cytochrome P450 reductase[150] as well as by aldehyde oxidase, xanthine oxidase, and nitric oxide synthase under hypoxic conditions *via* one-electron reduction to form oxidizing radicals that can damage DNA.[8] TPZ shows a hypoxia selectivity as high as 200 depending on the cell lines used, drug exposure time, and oxygen tension; however, the short-lived nature of the oxidizing radicals means limited diffusion to surrounding tumor tissues after activation. One special property of TPZ is that it does not require low oxygen tension to be activated, unlike nitroaromatics or quinones; TPZ can, thus, kill hypoxic cells over a much wider range of oxygen level.[151]

Detailed mechanism of TPZ activation has been investigated by several groups focusing on oxidizing species responsible for DNA cleavage and the partial aerobic cytotoxicity distinct from other bioreductive prodrugs.[18,152] It was initially proposed that neutral TPZ radical can abstract hydrogen atoms from the DNA backbone, which is known to be required for DNA strand scission. It was later identified that the well-known DNA damaging agent hydroxyl radical was released from this neutral TPZ radical after one electron reduction and might be the active species for DNA cleavage.[153] More recent studies revealed that the benzotriazinyl 1-oxide radicals from dehydration of the neutral TPZ radical instead of the hydroxyl radical might be responsible for oxidative DNA cleavage as shown in Scheme 21.[154,155] The one electron reduction potential of the benzotriazinyl 1-oxide radicals correlated well with cytotoxicity under hypoxic conditions while the one electron

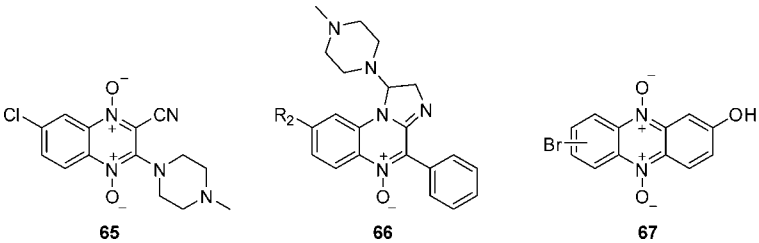


Scheme 21. Reductive activation of TPZ (**64**): detailed mechanism and active species responsible for DNA strand cleavage.

reduction potential of the TPZ metabolite, benzotrazine 1-oxide, accounted for the aerobic cytotoxicity. It was suggested that balancing these two reduction potentials during the drug design process can provide more selective bioreductive prodrugs.[156,157]

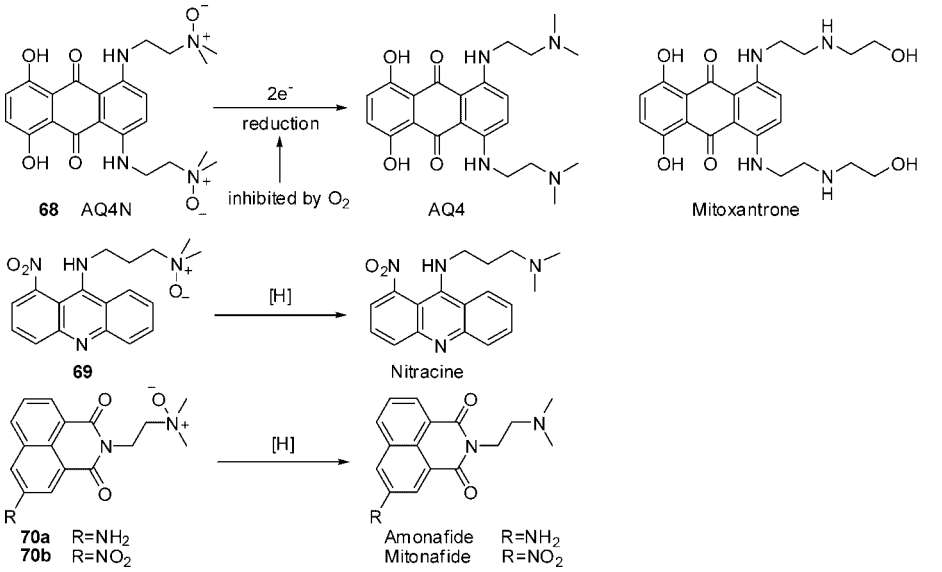
A large number of TPZ analogues with improved solubility, potency and therapeutic indices have been studied.[158–160] Basic functional groups at 3-position were found to be preferred for increasing hypoxia cytotoxicity and solubility/lipophilicity. Electron-withdrawing substituents at the 7-position of TPZ increased in vitro potency by modulating the reduction potential of N-oxides. DNA-targeting moieties have also been incorporated to TPZ analogues to increase its cytotoxicity.[161,162] However, high hypoxia selectivity and potency *in vitro* did not translate into *in vivo* antitumor activity in most cases. Poor drug penetration and diffusion was likely one of the key factors affecting the *in vivo* activity.[163] Recently, pharmacokinetic/pharmacodynamic modeling was used in efforts to facilitate this translation from *in vitro* cytotoxicity to *in vivo* activity during lead optimization.[158,164]

In addition to benzo-1,2,4-triazine 1,4-dioxide derivatives, other heterocyclic systems bearing aromatic N-oxide have been synthesized and tested as antitumor agents targeting hypoxic conditions. These include quinoxaline 1,4-dioxide **65**,[165–172] imidazoquinoxaline N-oxides **66**,[173] imidazopyridopyrazine N-oxides,[174–177] oxadiazole N-oxides,[178–180] triazine N-oxides,[181–183] and phenazine 5,10-dioxides **67**.[184–186]



2. Alkyl Tertiary Amine N-Oxides

Alkyl tertiary amine N-oxides, as exemplified by AQ4N (**68**), can undergo two electron reduction by cellular reductive enzymes, primarily CYP3A isozyme of NADPH:cytochrome C P450 reductase, generating the corresponding tertiary amines. AQ4 is structurally similar to mitoxantrone, which is an anthracycline antineoplastic agent used in the treatment of certain types of cancer, including metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma. Both AQ4 and mitoxantrone act as DNA intercalating and topisomerase-II inhibitors. The tertiary amine side chains



Scheme 22. Reductive activation of the alkyl tertiary amine N-oxides.

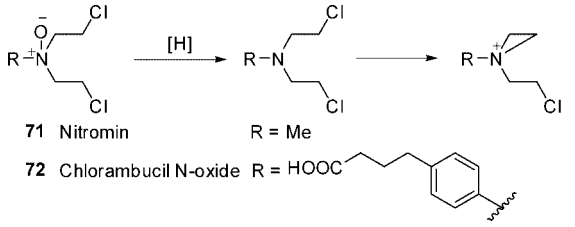
in these agents are critical for the electrostatic binding to DNA in addition to ensuring good uptake into cells.[187] N-oxidation to N-oxides abolished the DNA binding affinity of the tertiary amines and dramatically decreased its cytotoxicity; the N-oxides can be efficiently reduced to the corresponding amines in hypoxic tumor cells by cytochrome P450 3A enzymes. Thus, the less toxic alkyl tertiary N-oxides are transformed upon reduction into the more cytotoxic tertiary amines that possess superior diffusion properties than the short-lived radical species generated from the aromatic N-oxides. Their hypoxia selectivity results from the inhibition of the irreversible enzyme reduction process by oxygen as shown in Scheme 22.[8] Besides acting as bioreductive prodrug targeting hypoxia, AQ4N was also used in gene therapy in combination with cytochrome P450 enzymes like 1A1, 2B6, and 3A4.[116,188–191]

Nitracrine (**28**) is one of the antitumor acridine derivatives capable of DNA intercalation. It showed potent cytotoxicity and moderate selectivity toward hypoxia through reductive activation of the nitro group. The nitroacrine **69** with the tertiary amine side chain being oxidized to N-oxide was evaluated in an attempt to develop a bioreductive activated prodrug possessing dual reduction sites. Similar to the development of AQ4N, the N-oxidation dramatically lowered the DNA binding and resulted in excellent hypoxia selectivity greater than 1,000-fold. However, poor *in vivo* activity was observed probably due to rapid metabolism and poor diffusion to hypoxic tissues. Approaches to modulate its reduction potential of the nitro group and the steric environments failed to significantly improve the *in vivo* activities.[192]

Amonafide and mitonafide are two naphthalimide-type anticancer agents bearing tertiary amino side chains that inhibit the activity of topoisomerase and act as DNA intercalators. To avoid side effects such as CNS toxicity, myelosupression, vomiting, and erytherma side effects related to these naphthalimides, they were prepared into their N-oxide prodrugs **70a** and **70b** in a similar manner as mitoxantrone and nitroacrine. However, only low hypoxia selectivity was observed for these compounds in *in vitro* assays.[193]

3. N-Oxides of Other Cytotoxic Agents for Bioreductive Activation

Because nitrogen mustards are not selective and are unstable, there was considerable interest in developing nitrogen mustard N-oxide derivatives as tumor targeting agents. Nitromin (**71**) was first



Scheme 23. Reductive activation of nitromin and chlorambucil N-oxide.

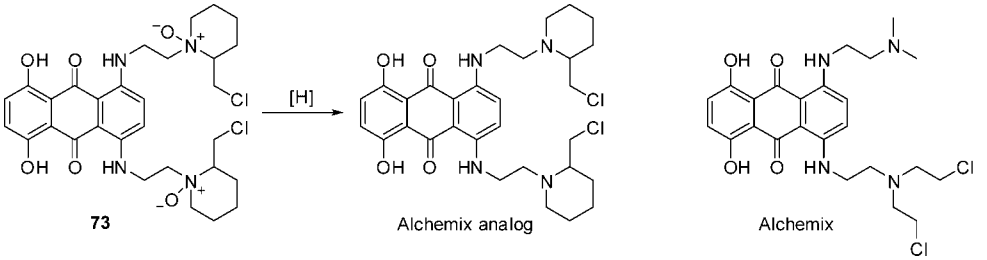
tested as bioreductive prodrug of simple nitrogen mustard and it showed moderate hypoxia selectivity due to bioreductive release of the mustard.[194] Chlorambucil N-oxide **72** provided another example of this type of prodrug releasing aniline mustard upon reduction[195] as shown in Scheme 23. However, N-oxide **72** failed to show any hypoxia selectivity.

Alchemix is an anthraquinone DNA intercalating agent bearing a bis(chloroethyl)amine side chain and was designed to overcome the drug resistance due to the reversible DNA binding related to conventional anthraquinone-based anticancer drug.[196] The piperidinyl alkylamino analogue of alchemix was shown to crosslink DNA in the low nanomolar range[197] and was converted into its N-oxide **73** resulting in total loss of DNA unwinding, DNA crosslinking, and cytotoxicity.[198] These results suggest that the N-oxide **73** could potentially be used as a bioreductively activated prodrug targeting hypoxia as shown in Scheme 24 but its cytotoxicity under hypoxic conditions has yet to be evaluated.

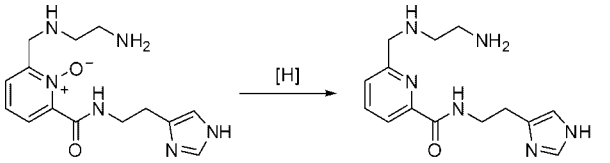
An analogue of bleomycin has also been modified to pyridine N-oxide **74** as a bioreductively activated prodrug as shown in Scheme 25. It was believed that bleomycin forms a chelate with Fe^{2+} together with oxygen occupying all six coordination positions and the chelate converts the oxygen to a reactive species causing cell killing through DNA breakdown. The N-oxide in prodrug **74** would block the chelating site for the bleomycin analog making it incapable of activating O_2 into the reactive species. Such non-toxic N-oxide could potentially be reduced under hypoxic conditions generating parent active drug. Indeed, N-oxide **74** showed similar cytotoxicity against Chinese hamster V79 cells as the reduced bleomycin analog after 2-hr exposure in air following a 2-hr exposure to hypoxic conditions, suggesting that the N-oxide **74** was activated under hypoxic conditions.[199] However, the concentrations required to inhibit the V79 cell growth were high in the millimolar range. Further optimization of the parent bleomycin analog would be needed to render the prodrug useful.

D. Metal Complexes as Prodrugs for Bioreductive Activation

Metal complexes, particularly those of cobalt, have been exploited as potential prodrugs to target tumor hypoxia.[200] The rational behind this is that the metal complexes with cytotoxic ligands at



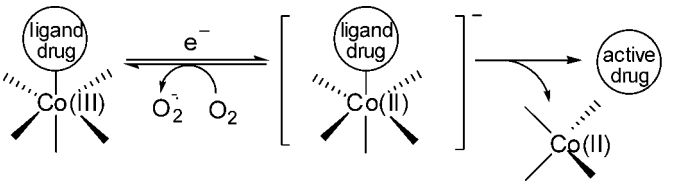
Scheme 24. Reductive activation of the N-oxide prodrug to give an alchemix analog.



Scheme 25. Reductive activation of N-oxide prodrug to give a metal-binding analog of bleomycin.

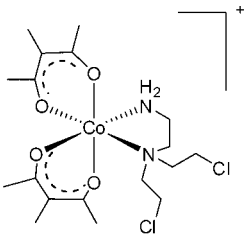
higher oxidation state would either stabilize the active cytotoxic agents or protect them from being rapidly metabolized and that the complexes upon reduction would become relatively unstable at the lower oxidation state releasing the corresponding cytotoxic ligands. Thus, the design of such prodrug requires that the metal ion possesses two accessible oxidation states and a large difference in stability of the complexes between the two oxidation states for efficient release of active cytotoxic agent. Although there are still some controversy over the mechanism of reductive activation of metal complexes, the hypoxia selectivity is believed to be derived from the redox cycling in the presence of oxygen between the oxidation states of metal complex similar to other types of hypoxia-targeted prodrugs discussed above. In the instance of Co(III) complexes, the labile Co(II) complexes formed upon the reduction may be re-oxidized by O_2 back to Co(III) complexes, while in the absence of oxygen the labile Co(II) complexes would rapidly dissociate to release the ligand as the active drug as shown in Scheme 26.[201]

However, in cases where such reoxidation was too slow, another mechanism was proposed to explain hypoxia selectivity related to cobalt complexes.[201–203] A series of cobalt-mustard complexes, $[Co(Racac)_2(DCE)]^+$, was initially synthesized using neutral bidentate mustard ligand N,N-bis(2-chloroethyl)ethylenediamine (DCE) and the R-substituted acetylacetonato (Racac) anion as the ligands. Their lead compound $[Co(Meacac)_2(DCE)]^+$ (SN24771, **75**, R = CH_3) exhibited a 5- to 30-fold selectivity for hypoxic cells in cell culture assays. The reduction of cobalt (III) to cobalt (II) by biological reductants was found to be inhibited by the presence of oxygen and thus the hypoxia selectivity of this complex was initially assumed to be the result of reoxidation of cobalt (II) to cobalt (III) prior to the release of cytotoxic mustard ligand. A later study on the kinetics of this reduction by pulse and steady-state radiolytic methods revealed that oxygen reoxidation reaction was too slow relative to the ligand release to make the redox cycling possible. Another mechanism was suggested based on the competition between the Co(III) complex and oxygen.[202] Although SN 24771 exhibited significant hypoxia selectivity in culture, no selectivity for hypoxic cells *in vivo* was observed. A new series of Co (III) complexes **76** were synthesized by replacing the methyl-substituted acetylacetonato (Meacac) ligand in SN24771 with R-disubstituted thiol-containing ligand dithiocarbamato (R_2dtc-) in an attempt to modulate the electron transfer properties which may allow reoxidation of Co(II) intermediate fast enough to enable redox cycling. However, this new type of complexes was

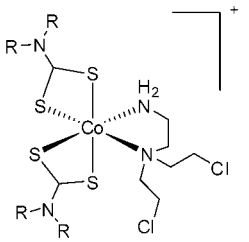


Scheme 26. Redox cycling of cobalt complexes in the presence oxygen and release of the active drug under hypoxic conditions.[201]

found to be unstable in cell culture medium and no hypoxia selectivity was observed *in vitro*.[204–206]



75 SN24771



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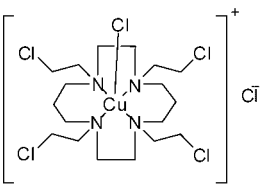
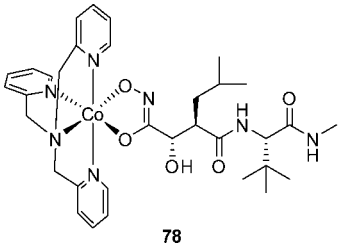


77

Recently, novel Cu(II) complex **77** with a mustard derivative of 1,4,7-tetraazacyclododecane (cyclen) as the ligand exhibited 24-fold higher cytotoxicity under hypoxic conditions *in vitro*, and its cytotoxicity under normoxic conditions was approximately 10-fold lower than SN24771. Reversible redox behavior and stability of the cyclen-Cu(II) complex **77** in aqueous solution correlated well with the hypoxia selectivity, making this complex a promising lead for the development of hypoxia-selective cytotoxic agents.[207]

Marimastat is a synthetic broad-spectrum matrix metalloproteinase inhibitor and is able to bind to the Zinc ion in the active site of matrix metalloproteinases thereby inhibiting the proteinase function. It has been tested in several cancer models;[208] however, clinical development of marimastat has been discontinued due to the lack of therapeutic benefits in patients. The lack of drug efficacy was partially attributed to the presence of hydroxamic acid functional groups in this compound which has strong inherent affinity to metal ions, especially Fe (III).[209] Co (III) and Fe (III) were used to form complex with the tetradendate ligands, tris(2-methylpyridyl)-amine (tpa) and N,N-bis(salicylidene)ethane-1,2-diimine (salen), respectively. Complex **78** showed lower cytotoxicity and higher stability than **79** against A2780 human ovarian cancer cells and has the potential to deliver the MMP inhibitor to tumor cells. *In vivo* assay of Co (III) complex **78** showed higher level of growth inhibition against 4T1.2 murine mammary tumor than the free marimastat and the control groups.[209–211] However, a real time PCR assay showed that complex **78** and marimastat potentiated metastasis of the murine mammary tumor in this *in vivo* model.[209]



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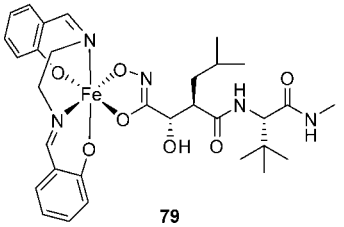

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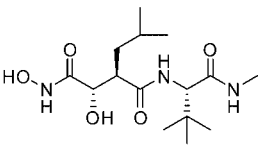
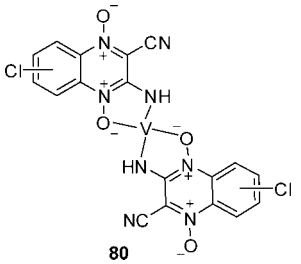


Marimastat

Quinoxaline N,N-dioxides have been developed as bioreductively activated drugs as discussed earlier. They were shown to have excellent *in vitro* activities; however, these quinoxaline N,N-dioxides have too short a half life and insufficient aqueous solubility to be useful.[171] Complexes with vanadium (V) were shown to have improved selectivity and cytotoxicity. Although medicinal applications of vanadium complexes have focused on their *in vitro* and *in vivo* activity as the treatment of diabetes, vanadium complexes **80** were shown to be 3-fold more potent than the free quinoxaline dioxide ligands and 10-fold more active than TPZ with a hypoxia selectivity of >15-fold in *in vitro* assays. Moreover, Vanadium complexes increased significantly the water solubility of the parent

quinoxaline N,N-dioxides.[212] Additional research is needed to evaluate the *in vivo* activity of these vanadium complexes.



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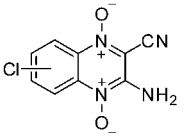
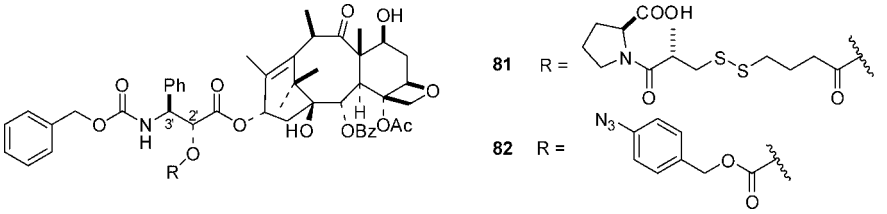


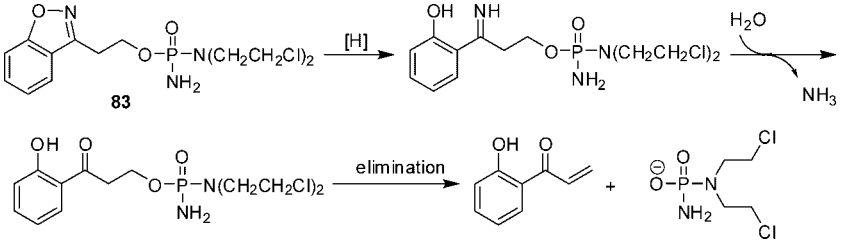
Quinoxaline N,N-dioxide

E. Miscellaneous Prodrugs Designed for Reductive Activation

In addition to the four types of bioreductively activated prodrugs discussed above, disulfide and azido functional groups have also been used in the design of prodrugs to selectively target hypoxic tumor tissues. For example, paclitaxel is one of the most widely used anticancer agents and has activities in many types of cancer. However, its low aqueous solubility, dose-limiting toxicity, and drug resistance have presented major problems in its clinical application. Various prodrugs of paclitaxel have been designed to overcome these problems.[213] Captopril, an ACE inhibitor reported recently to have antiangiogenic effects, was attached to paclitaxel at 2′-OH through a 2,2-dimethyl-4-mercaptobutyric acid linker to give conjugate **81** for reductive activation in hypoxic tumor tissues and for improving of its solubility. Conjugate **81** underwent cyclization with a half-life of 25 min upon reduction by DTT. It showed superior *in vivo* anticancer activity as compared to paclitaxel itself.[214] In another example, aromatic azido group has been introduced to paclitaxel through a self-eliminating linker at 2′-OH to give conjugate **82**. Compound **82** was shown to release paclitaxel under chemical reduction and exhibit a lower cytotoxicity under aerobic conditions.[215]



Recently a novel prodrug **83** as shown in Scheme 27 was designed based on the structure of an anti-epileptic agent, zonisamide.[216] Zonisamide has a benzisoxazole that underwent reductive cleavage of heterocyclic N-O bond followed by hydrolysis to give a ketone metabolite. 1,2-Isoxazole was reduced by cytochrome P450 and liver cytosolic aldehyde oxidase, preferentially under hypoxic conditions. 1,2-Benzisoxazole phosphorodiamidate **83** and analogs with various substitutions on the



Scheme 27. Reductive activation of a 1,2-benzisoxazole phsophorodiamidate prodrug.

benzene ring were synthesized in an attempt to investigate the effect of electron density of aromatic ring on the drug release property and cytotoxicity. It was found that these prodrugs underwent reductive metabolism and generated alkylating phosphoramide mustard; however, no hypoxia selectivity was observed in cell culture assays.[216]

4. PERSPECTIVES AND CONCLUSIONS

Over the last 20 years, many prodrugs have been developed to deliver anticancer cytotoxic agents to tumor cells and some have been to shown to be highly selective in their targeting of hypoxic tumor cells both *in vitro* and *in vivo*. The extensively investigated prodrugs for reductive activation include quinones, nitroaromatics, N-oxides, and metal complexes. In addition to the clinically used mitomycin C, there are several other bioreductively activated prodrugs that are being tested in clinical trials. For example, AQ4N (**68**) is currently in phase 2 clinical trials for brain tumor, chronic lymphocytic leukemia, and non-Hodgkin lymphoma; EO9 (**3**) is in phase 3 clinical trials for bladder tumor as intravesical instillation; PR-104 (**32**) is in phase II clinical trials for solid tumors; tirapazamine (**64**) was in phase 3 clinical trials for uterine cervix tumor and recently abandoned; CB1954 has been tested in human clinical trials for prostate cancer and liver cancer in combination with virally delivered *E. coli* nitroreductase. The diffusion properties of these prodrugs are considered to be a critical factor for a successful targeted cancer therapy exploring bioreduction. Recent advances in the experimental models on the evaluation of prodrugs during the design process have also been shown to help with the development of prodrugs having sufficient potency and desirable pharmacokinetic properties. In gene therapy, enzymes transfected to target cells are an important research area in addition to the prodrugs used in combination. New enzymes are still needed in GDEPT with improved reduction kinetics, minimal immune response, and higher specificity. Metal complexes are also being investigated as a promising type of reductively activated prodrugs to target hypoxic tumor tissues. Metal complexes can act as chaperones, which deactivate active drugs until released upon reduction. Such complexes can be fine tuned to achieve desired physiological and chemical properties through modification of the ancillary ligands. Along with the advances in the knowledge of the tumor microenvironment and better understanding the enzyme/drug interaction, better bioreductively activated prodrugs can be designed to effectively improve the therapeutic efficacy and reduce systemic side effects in the treatment of cancer.

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