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Targeting tumors with hypoxia-activated cytotoxins

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1. ABSTRACT

This review focuses on the recent development of hypoxia-activated cytotoxins. Such drugs are prodrugs activated to cytotoxic products in the hypoxic environment of solid tumors (so-called "bioreductive prodrugs"), but can also be activated by radiation (radiation-activated prodrugs). These compounds grew out of research on hypoxic radiosensitizers, which are compounds that can overcome the radiation resistance of hypoxic cells, and we will discuss this area also. The advantages and limitations of each class of the hypoxia-activated cytotoxins are discussed. In addition we will discuss a novel method of targeting drugs to tumors based on anaerobic bacteria, the so-called "clostridia-directed enzyme prodrug therapy" or CDEPT, which also exploits the hypoxic environment of solid tumors.

2. INTRODUCTION

Tumor hypoxia was first postulated by Thomlinson and Gray based on histological studies of human lung adenocarcinomas (1). They reasoned that because of unrestrained growth tumor cells are forced away from blood vessels beyond the effective diffusion distance of oxygen (O_2), becoming hypoxic and eventually necrotic. Given the typical values for intracapillary O_2 tensions and consumption rates, they calculated that O_2 diffusion distances would be approximately 150 μm and this was consistent with their histological observations (1). This type of hypoxia is termed "chronic" or "diffusion-limited" hypoxia. Acute hypoxia also develops in tumors through temporal (reversible) cessation or reduction of tumor blood flow resulting from highly disorganized tumor vasculature (2, 3). Definitive evidence for acute hypoxia and

fluctuating blood flow has been demonstrated in transplanted tumors in mice injected at some time apart with two different diffusion limited fluorescent dyes by showing mismatch of labeled cells (4, 5). Tumor hypoxia is common in both experimental (6) and human tumors (7), producing a dynamic situation with fluctuating oxygen diffusion in many parts of tumors.

Tumor hypoxia has received growing attention because of its prognostic role in cancer therapies. First, tumor hypoxia is a major factor contributing to the failure of radiotherapy. This is largely because DNA damage produced by ionizing radiation, which would otherwise become fixed and lethal to cells by reacting with O_2 in well oxygenated conditions, can be restored to its undamaged form under hypoxic conditions (8). Clinically hypoxia predicts for poor local control and survival of patients undergoing radiotherapy for carcinoma of the head and neck (9, 10), and cancer of the cervix (11, 12). Second, tumor hypoxia may compromise the outcome of conventional chemotherapy. Because the hypoxic tumor cells are distant from functional blood vessels they receive much lowered concentrations of anticancer drugs than the target concentrations (13). Furthermore, hypoxic tumor cells may be slowly or non-cycling (14), and hence may be relatively resistant to many anticancer drugs that target rapidly dividing cells. Third, hypoxia contributes to tumor progression. This is in large part the result of the hypoxia-inducible factor 1 (HIF-1) transcription factor which results in increased expression of a large number of genes involved in cellular metabolism and survival under hypoxia (reviewed in (15)). Among these, genes such as vascular endothelial growth factor (VEGF) (16, 17) and lysyl oxidase (18) are involved in tumor angiogenesis (19) and metastasis (18), processes contributing to a more malignant phenotype . Clinical evidence consistent with this arises from the fact that primary tumors with low oxygen tension are associated with a high incidence of distant metastases in patients of soft tissue sarcomas (20) and of squamous cell carcinoma of the cervix (21).

Thus, there is substantial evidence that hypoxia interferes with the effective therapy of solid tumors and contributes to a more malignant phenotype. However, hypoxia may also be exploited to give a therapeutic advantage: because it is virtually unique to tumor cells, therapies that target hypoxic regions may have the potential to kill malignant cells while leaving nonmalignant cells relatively unaffected.

One of the earliest attempts to overcome the problem of the resistance of hypoxic cells in tumors to radiotherapy was to increase O_2 levels in the blood stream thereby increasing the diffusion distance of O_2. A number of trials were conducted with patients breathing 100 % O_2 at 3 atmospheres pressure, but the results were mixed (22-24). One potential reason for such failures is that increasing the diffusion distance of O_2 would not be expected to reduce the levels of acute hypoxia. In some systems, the use of carbogen (95% O_2 / 5% CO_2) appears to be more effective than 100 % O_2 in increasing O_2 level in the blood stream (25) possibly by preventing the vasoconstriction caused by high partial pressures of O_2. Other potential approaches to overcome hypoxia include the use of nicotinamide (26-28) in conjunction with carbogen (29), agents to increase tumor blood flow such as flunarizine (30), artificial blood substitutes carrying increased levels of O_2 (31, 32), drugs to reduce the affinity of hemoglobin for O_2 (33, 34), and hyperthermia (35).

This review will discuss some examples of hypoxic cell radiosensitizers, bioreductive prodrugs, radiation-activated prodrugs, and some gene therapy approaches to enhance the reductive activation of hypoxia-activated cytotoxins.

3. HYPOXIC RADIOSENSITIZERS

Adams and his colleagues (36) proposed in the 1960's that electron-affinic drugs might act like O_2, a potent electron-affinic molecule, to sensitize hypoxic tumor cells. These agents, called hypoxic radiosensitizers, mimic O_2 by reacting with the short-lived DNA free radicals generated by ionizing radiation. However, unlike O_2, hypoxic radiosensitizers are not rapidly metabolized by the cells through which they penetrate and are thus able to reach areas beyond the O_2 diffusion distance. Some examples of radiosensitizers are discussed below.

3.1. Nitroimidazoles

Misonidazole and metromidazole (Figure 1) are the prototype members of this class. They were administered to patients in clinical studies in the 1970's but had disappointing results; they had very little efficacy in sensitizing tumors and were limited in their dosing by peripheral neuropathy (37, 38). With the limited dose of misonidazole that could be administered to patients, almost all of the clinical trials of radiotherapy combined with misonidazole were negative (39). However, a recent meta-analysis of 50 randomized trials shows a small but significant benefit of misonidazole and other hypoxic radiosensitizers when added to radiotherapy of head and neck cancers (40).

Attempts to reduce peripheral neuropathy of misonidazole led to the development of etanidazole (41), pimonidazole (42), and nimorazole (39) (Figure 1). Of these, nimorazole showed a significant benefit of loco-regional control when given in conjunction with radiotherapy to patients with invasive carcinoma of larynx and pharynx (43, 44) and is now given as part of the standard of care for radiotherapy of head and neck cancer patients in Denmark (45).

3.2. Mixed function radiosensitizers

The neurotoxicity of misonidazole stimulated the search for drugs not only with less toxicity, but also with increased efficiency as radiosensitizers on a dosage basis. This was achieved by incorporating other functional moieties in the drug molecule.

CB 1954 (2,4-dinitro-5-aziridinylbenzamide; Figure 1) is a hypoxic radiosensitizer bearing DNA alkylating moiety of aziridine and showed more efficient

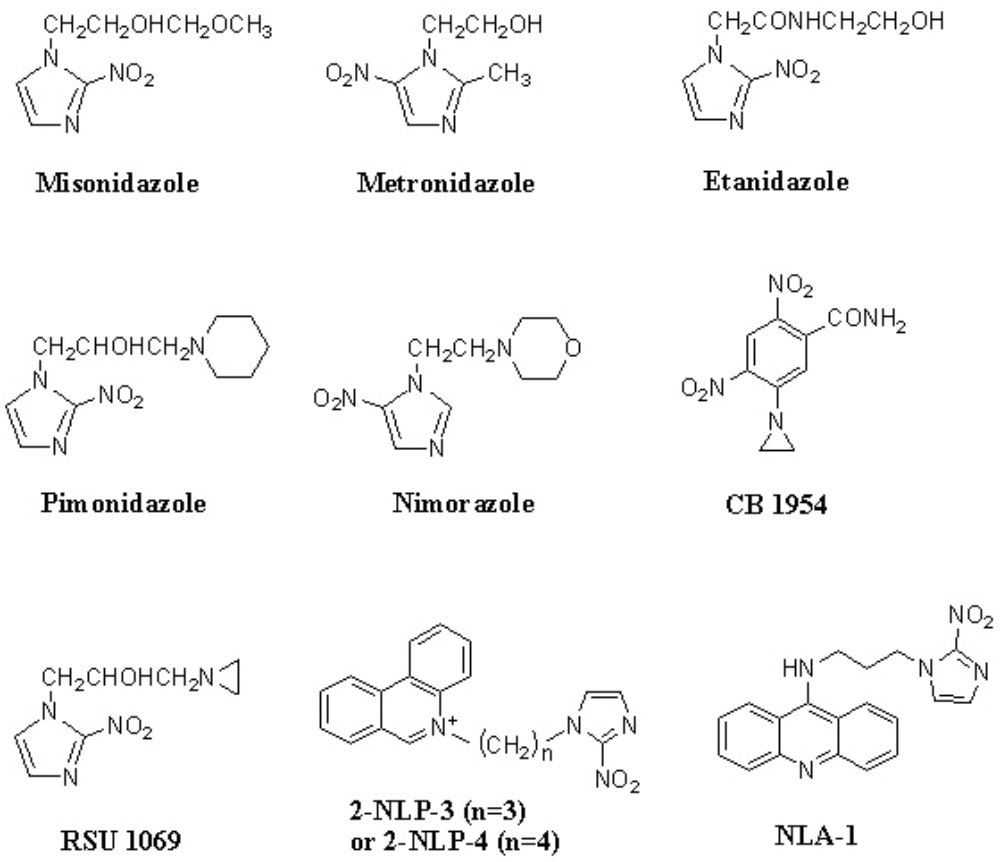

Figure 1. Chemical structures of some radiosensitizers. The name of radiosensitizers is shown with each structure.

radiosensitization than misonidazole (46). Similarly, RSU 1069 (1(2-nitro-1-imidazolyl)3-aziridinyl-2-propanol; Figure 1), the second generation of CB 1954, showed 10-fold higher radiosensitization potency than misonidazole at equimolar doses (47, 48). The more effective sensitizing ability of RSU 1069 was suggested to be due to a greater degree of hypoxic cell cytotoxicity rather than to enhanced radiosensitizing ability (49). Both CB 1954 and RSU 1069 are further discussed as bioreductive prodrugs in the Sections 4.3.1 and 4.3.3, respectively.

3.3. DNA affinic radiosensitizers

This class of the radiosensitizer incorporates a DNA-affinic functional group attracting the drug molecule closer to DNA for the radiosensitizing effect. Nitracrine, 1-nitroacridine derivative (1-nitro-9-(dimethylaminopropylamino)acridine), showed 1,700 times more efficient radiosensitization than misonidazole (50). However the development of nitracrine as a hypoxic radiosensitizer was limited by its rapid cellular metabolism (50) and poor extravascular penetration (51).

2-NLP-3 (5-[3-(2-nitro-1-imidazolyl)-propyl]-phenanthridinium bromide; Figure 1) and 2-NLP-4 (5-[3-(2-nitro-1-imidazolyl)-butyl]-phenanthridinium bromide; Figure 1) are 2-nitroimidazoles attached to the DNA intercalator phenanthridine. They were shown to be 10-100 times more efficient as radiosensitizers than misonidazole both *in vitro* and *in vivo* (52). The structurally similar compound, NLA-1 (9-[3-(2-nitro-1-imidazolyl)propylamino]acridine hydrochloride; Figure 1) is a DNA-targeted acridine-linked 2-nitroimidazole (53). Although NLA-1 is a potent radiosensitizer *in vitro*, it lacked *in vivo* activity (54).

3.4. Limitations of hypoxic radiosensitizers

A major drawback of the hypoxic radiosensitizer is that the radiosensitization efficacy in tumors decreases with the radiation doses used in clinically relevant fractionated irradiation. Experimental results showed that 2 to 3 Gy fractionated irradiation resulted in lower enhancement ratios than single large doses for misonidazole (55-57). One explanation for the lack of radiosensitization at fractionated low doses has been suggested to be due to the reoxygenation occurring between each fractionated dose of irradiation (55, 58). However, an equally likely reason may be that it is not the cells at maximum radiation resistance, but those at intermediate oxygenation and intermediate radioresistance that dominate the response to fractionated irradiation (59). This is a consequence of the logarithmic nature of the curve of radiosensitization versus dose – as cell sensitivity increases it takes geometrically more drug to increase their radiosensitivity still further. Thus a hypoxic cell partially

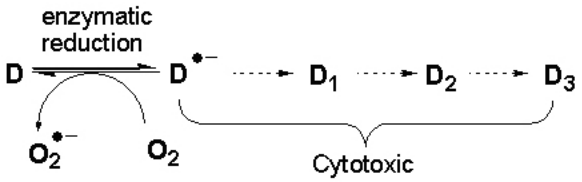

Figure 2. A scheme of activation for bioreductive prodrug (D) by enzymatic reduction. The reduced intermediate of the bioreductive prodrug ($D^{\bullet-}$) can be back-oxidized by the presence of oxygen (O_2) with the formation of superoxide anion ($O_2^{\bullet-}$). $D^{\bullet-}$ may itself be cytotoxic or be further reduced (D_1, D_2, or D_3) to produce cytotoxins killing hypoxic cells.

sensitized by some oxygen takes much more drug to increase its sensitivity by a given amount compared to a fully hypoxic cell (60). Hence it seems likely that the radiosensitizers will only play a major role *in situ*ations where large, single doses of radiation are given such as stereotatic radiotherapy for brain tumors, and less to the outcome of fractionated radiotherapy.

4. BIOREDUCTIVE PRODRUGS

4.1. Introduction

An alternative strategy to overcome the problem of hypoxic tumor cells is to selectively kill them by using bioreductive prodrugs. Typically these are non-toxic compounds that are reduced by enzyme(s) in hypoxic tumor cells. This results in one-electron reduced intermediates, which may themselves be cytotoxic, or further reduction to produce cytotoxins killing the activating cell and, in some cases, the surrounding cells (Figure 2). The development of bioreductive prodrugs was originally stimulated by the findings that nitroimidazoles were more toxic to hypoxic than oxic tumor cells even without irradiation (61, 62).

Selectivity for hypoxia is usually a result of a futile redox cycling in which the presence of O_2 reoxidizes the one-electron reduced intermediate thereby regenerating the non-toxic parent drug (Figure 2; (63)). Superoxide anion ($O_2^{\bullet-}$), a major by-product of this reaction, is capable of producing DNA strand breaks and other oxidative damage, but can be detoxified by superoxide dismutase and catalase (64). Hypoxia selective cytotoxicity therefore occurs when the reduction of the bioreductive prodrug produces a more toxic intermediate than the $O_2^{\bullet-}$ radical (65, 66).

Bioreductive prodrugs are ideally combined with therapies that target relatively well oxygenated tumor cells such as ionizing radiation and chemotherapeutic agents, because they produce a profile of cytotoxicity as a function of distance from blood vessels in tumors that are the opposite of that produced by these conventional therapies (60). In other words, bioreductive prodrugs kill the hypoxic tumor cells that are resistant to the conventional therapies. In addition, they may release stable and diffusible cytotoxins capable of killing the surrounding tumor cells at relatively higher O_2 concentrations, producing a so-called "bystander effect" (67). Unlike radiosensitizers, the efficacy of the bioreductive prodrug is independent of the dose of radiation because there is no interaction between the two agents. In fact the benefit of adding a bioreductive prodrug to fractionated irradiation increases with the number of times the drug is administered and can produce a greater benefit than if all of the tumor were fully oxygenated (68).

4.2. Nitroimidazoles

In addition to their action as hypoxic radiosensitizers, nitroimidazoles can also be bioreductively activated to cytotoxic products and so act as hypoxic cytotoxins. Examples of this class of bioreductive prodrugs are metronidazole, misonidazole (69), bis-nitroimidazole (70, 71), and NLCQ-1 (72). NLCQ-1 is a nitroimidazole-linked chloroquinoline (4-[3-(2-nitro-1-imidazolyl)-propylamino]-7-chloroquinone hydrochloride), a member of NLP-1/NLA-1/THNLA-1 series (73, 74). NLCQ-1 shows time-dependent increase in hypoxic cytotoxicity *in vitro* and is currently under preclinical evaluation (72, 75).

In mammalian cells, aldehyde oxidase, DT-diaphorase, xanthine oxidase, NADPH cytochrome P450 reductase, cytochrome b_5 reductase, NADH dehydrogenase, and succinate dehydrogenase have been reported to be the reductive enzymes, known collectively as nitroreductases (76-78).

A general scheme for nitroheterocycle reduction is that the addition of an odd number of electrons (1, 3, 5) leads to radical intermediates, while an even number of electrons (2, 4, 6) leads to the nitroso (R-NO), hydroxylamine (R-NHOH), and amine reductants ($R-NH_2$), respectively (63, 79). O_2 is able to reverse or inhibit reduction at the one-electron radical anion, although it could in principle act at various stages (80). In the absence of O_2, further reduction occurs primarily via disproportionation reactions of $R-NO_2^{\bullet-}$, ultimately leading to the fragmentation of the imidazole ring (81, 82). Two- and four- electron reduced derivatives may have different stabilities and reactivities depending on the nature of the aromatic ring and its substituents (83).

4.3. Other nitroaromatics

4.3.1. CB 1954

CB 1954 is a monofunctional alkylating agent that can be enzymatically activated to a potent difunctional alkylating agent which crosslinks DNA (8, 84, 85). CB1954 has shown to be a substrate for NADPH cytochrome P450 reductase (86) or DT-diaphorase (84) in mammalian cells. Reduction of CB 1954 produces the potent cytotoxic metabolites, the 2- and 4-hydroxylamines and their corresponding amines (87). The activation of CB 1954 also occurs through a second activation step by a non-enzymatic reaction with a thioester (such as acetyl CoA) to form the final DNA reactive species, which is presumably 4-(*N*-acetoxy)-5-(aziridin-1-yl)-2-nitrobenzamide (88). Currently CB 1954 is used in gene-directed enzyme-prodrug therapy (GDEPT) with *Escherichia coli* B

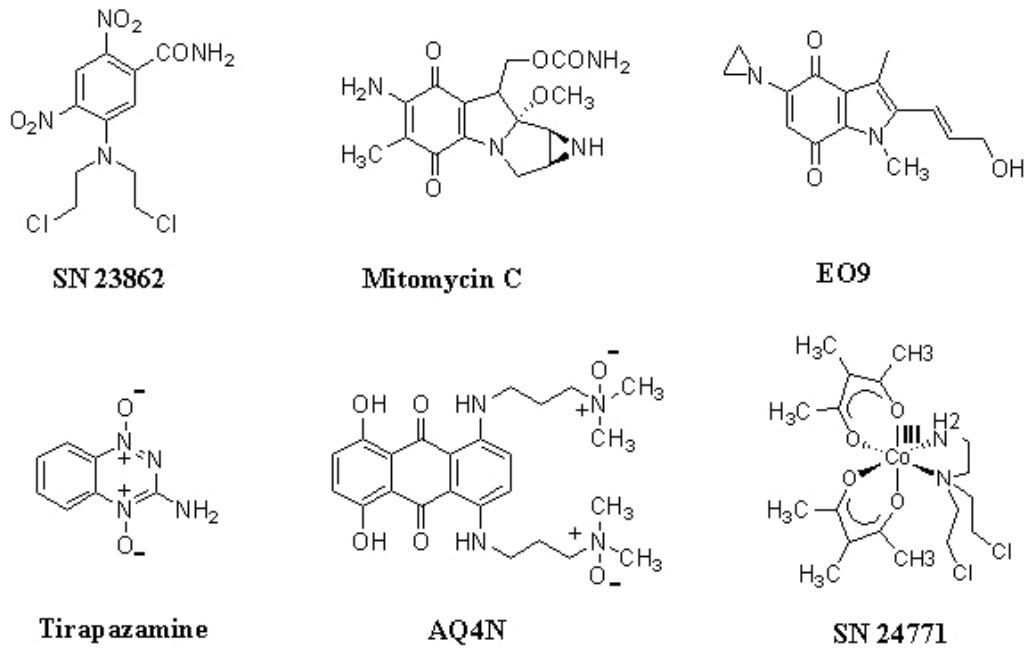

Figure 3. Chemical structures of some bioreductive prodrugs discussed in this review.

nitroreductase (89, 90), and is in currently phase I clinical trials (91, 92).

4.3.2. SN 23862 and PR-104

Structurally similar to CB 1954, a nitrogen mustard compound, SN 23862 (5-[*N,N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide; Figure 3) exploits the nitro-group as an electronic switch in that nitro-to-amine conversion shifts electron density modifying the reactivity of a drug molecule (87, 93). The chemical reduction of each of the 2-nitro and the 4-nitro group of SN 23862 showed an increase in cytotoxicity by 160- and 9-fold in AA8 cell line, and by 4,400- and 83-fold in UV4 cell line, respectively, and that the reduction of both nitro groups led to further increase in cytotoxicity (87, 94). Good bystander effect in both *in vitro* and *in vivo* systems together with substrate specificity to *Escherichia coli* B nitroreductase warrants development of SN 23862 for GDEPT (95-97).

The newest bioreductive prodrug PR-104 is a phosphate pre-prodrug of the dinitrobenzamide mustard PR-104A that is activated in hypoxia to become a potent bifunctional alkylating agent producing DNA interstrand crosslinks (98, 99). Moreover, PR-104 has demonstrated a substantial bystander effect, killing aerobic as well as hypoxic cells in solid tumors (100), and producing significant antitumor activity as a single agent alone. It is also superior to tirapazamine (see Section 4.5) in combination with fractionated irradiation in preclinical models (99). PR-104 entered clinical phase 1 trials in January 2006.

4.3.3. RSU 1069 and RB 6145

RSU 1069 is bioreductively activated by NADPH cytochrome P450 reductase forming a bifunctional crosslinking agent (101, 102). In air, RSU 1069 functions as a monofunctional alkylating agent due to the presence of the aziridine group (101). It shows *in vivo* antitumor activity against the KHT sarcoma and RIF-1 tumor when given before or after irradiation (49).

The bromoethylamine derivative RB 6145 was developed as a prodrug of RSU 1069 because of irreversible gastrotoxicity observed in early phase I trials of RSU 1069 (103). RB 6145 showed slightly less hypoxia selective cytotoxicity than RSU 1069 *in vitro* (104) but was active against hypoxic cells *in vivo* with lowered toxicity compared to RSU 1069 (104, 105). However, further development of this compound was stopped because preclinical studies showed irreversible cytotoxicity toward retinal cells in mice and rats (106, 107).

4.4. Quinones

4.4.1. Mitomycin C

Mitomycin C (Figure 3), isolated from *Streptomyces caespitosus*, was introduced into the clinic in 1958, and was subsequently shown to have a moderate *in vitro* hypoxia selective cytotoxicity (hypoxic cytotoxicity ratio[1] of about 1 to 5; (108, 109)). The cytotoxicity of mitomycin C is associated with formation of monofunctional alkylation and the more potent intra- and inter-strand DNA crosslinks, all of which require bioreductive activation (110-112).

The one-electron reduction of mitomycin C results in a semiquinone, which under hypoxic conditions activates the aziridine ring and results in binding of the drug to DNA (113). Following the initial covalent attachment of mitomycin C to DNA, the drug can undergo further reductive activation to form a second alkylating site (113). The one-electron reduction pathway can be catalyzed

by any of several enzymes including NADPH cytochrome P450 reductase (113, 114) and xanthine oxidase (113), in a process that can be reversed by O_2 (115-117). Mitomycin C can also be reductively activated via two-electron reducing DT-diaphorase generating an O_2-insensitive hydroquinone (114, 118).

Despite variable results in hypoxia selective cytotoxicity observed in preclinical studies (108, 114, 119), clinical trials have reported that mitomycin C in combination with radiation showed a significant benefit in local regional control rates for patients with head and neck cancer (120-122), squamous cell carcinoma of the cervix (123), and laryngeal and hypopharyngeal cancer (124).

4.4.2. Porfiromycin

Porfiromycin, a second-generation version of mitomycin C, showed superior *in vitro* hypoxic selectivity over mitomycin C as a result of lowered aerobic cytotoxicity (109, 125) and an improved *in vivo* therapeutic index as a result of higher LD_{50}[2] in mice (126). Although a phase I trial showed an acceptable toxicity profile, a recent phase III randomized trial showed that porfiromycin was inferior to mitomycin C as an adjunct to radiotherapy in patients with squamous cell cancer of the head and neck (127). This suggests that the efficacy of mitomycin C as an adjunct to radiotherapy is unlikely to be the result of any preferential cytotoxicity to hypoxic cells.

4.4.3. EO9

EO9 ([3-hydroxymethyl-5-aziridinyl-2-methyl-2-(H-indole-4,7-indione)-propenol]; Figure 3) was originally developed as a synthetic analog of mitomycin C (128). The reductive bioactivation of EO9 occurs in a similar manner to mitomycin C such that one- and two-electron reduction processes yield the corresponding semiquinone and hydroquinone, respectively (129). The semiquinone is believed to be more cytotoxic than the hydroquinone based on its ability to produce DNA interstrand crosslinks and strand breaks (129-131).

Although partial responses were observed in a small number of patients in phase I trials (132), no apparent antitumor activity by EO9 alone was demonstrated in phase II studies in patients with advanced breast, gastric, pancreatic, and colorectal carcinoma (133). However, some concerns about the design of the trials were raised in that enzymatic activity in patients' tumors was not measured routinely (134) and that hypoxic cytotoxins such as EO9 should be combined with other treatment modalities such as radiotherapy or chemotherapy to demonstrate detectable clinical responses (135).

4.4.4. Aziridinylbenzoquinones

AZQ (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone; diaziquone), DZQ (2,5-diaziridinyl-1,4-benzoquinone), MeDZQ (2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone), and a more recent compound, RH-1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone) are quinone-containing bioreductive prodrugs that are reduced by DT-diaphorase (136-138) to alkylate DNA via aziridine group activation (139, 140). Although there is much interest in RH-1, which recently entered in phase I trials, further discussion of this class is beyond the scope of this review (reviewed elsewhere (139, 141)) because the two-electron reduction by DT-diaphorase is less likely to be influenced by O_2 futile cycling.

4.5. Benzotriene di-*N*-oxides

Tirapazamine (SR 4233; 1,2,4-benzotriazin-3-amine 1,4-dioxide; Figure 3) is the prototype of this class of bioreductive prodrugs. Under hypoxic conditions, tirapazamine is reduced to an O_2-sensitive tirapazamine radical (142). The tirapazamine radical then eliminates a water molecule to form a nitrogen-centered oxidant, a benzotriazinyl radical, which can mediate the initial cytotoxic process by abstracting a hydrogen atom from the deoxyribose backbone of DNA (143). The tirapazamine radical may also lead to the formation of the two-electron reduced product, SR 4317, a major non-toxic metabolite detected in cultured hypoxic cells (144, 145), in mice (146), and in humans (147). Formation of SR 4317 can also occur by a number of different routes, including the direct two-electron reduction of tirapazamine by DT-diaphorase (148), by radical disproportionation reaction, or by hydrogen abstraction from macromolecules other than DNA (149).

Tirapazamine has a unique O_2 concentration dependency such that its cytotoxicity does not level off at high concentrations, but gradually decreases as the O_2 concentration increases (150, 151). Tirapazamine shows hypoxic cytotoxicity ratios of up to 200 in murine and 50 in human cell lines (152).

The hypoxic cytotoxicity of tirapazamine is due to the formation of DNA strand breaks resulting in chromosome aberrations (153-155). The chromosome breaks produced by tirapazamine were shown to be less easily repaired than those produced by X-rays and this has been suggested to be a result of probable metabolism by reductases located close to DNA (154). Although a large proportion of tirapazamine is metabolized in the cytoplasm by enzymes such as cytochrome P450 (156-158), NADPH cytochrome P450 reductase (142, 156, 157, 159-161), xanthine oxidase (162), and DT-diaphorase (148, 157), it is the nuclear metabolism of tirapazamine (approximately 20 % of the overall cellular metabolism) that accounts for essentially all of the tirapazamine-induced DNA damage under hypoxic conditions (163, 164). Recently, tirapazamine in hypoxic conditions has also shown to markedly inhibit DNA replication (165) and to poison topoisomerase II activity (166) *in vitro* leading to the suggestion that the DNA double strand breaks produced by tirapazamine result from its poisoning topoisomerase II (166).

Tirapazamine potentiates cell killing with fractionated irradiation in mouse tumors (167), and with cisplatin in a highly schedule-dependent manner (168). Tirapazamine is currently in phase III evaluation and appears particularly effective in combination with cisplatin (169-172) or as an adjunct to cisplatin/radiotherapy treatment (173, 174).

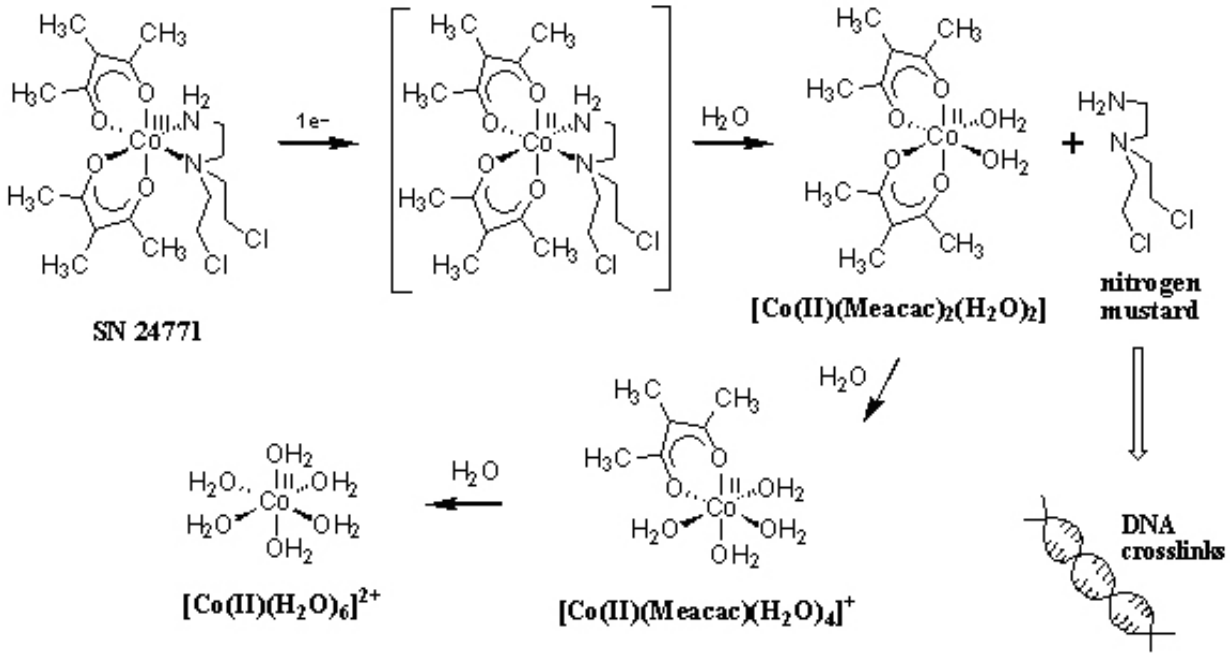

Figure 4. A reductive activation scheme of SN 24771 releasing nitrogen mustard. The resulting cobalt(II) complex ($[Co(II)(Meacac)_2(H_2O)_2]$) sequentially loses Meacac auxiliary ligands, giving rise to a final cobalt containing product, $[Co(II)(H_2O)_6]^{2+}$.

4.6. Tertiary amine *N*-oxides

4.6.1. Nitracrine *N*-oxides

Nitracrine *N*-oxide was developed as a prodrug of nitracrine, a hypoxic cytotoxin whose cytotoxicity is due to nitroreduction that is inhibited by O_2 (51). While nitracrine has hypoxic selectivity similar to that of misonidazole (approximately 10-fold) *in vitro*, its metabolism is too rapid to provide selective killing of hypoxic cells *in vivo* (51). Derivatization with tertiary amine *N*-oxide lowered DNA binding (15-fold), cell uptake, and aerobic cytotoxicity compared to nitracrine while its hypoxic selectivity was greatly increased (1,000-1,500-fold; (175). The very high hypoxia selectivity was suggested to be due to a requirement for both the nitro and *N*-oxide moieties for full activation in an O_2 inhibitable manner (175). Despite some improvement of extravascular diffusion properties observed *in vitro* (175), *in vivo* activity against KHT tumors was only observed at doses lethal to the host (176).

4.6.2. AQ4N

AQ4N (1,4-bis-{[2-(dimethylamino-*N*-oxide)ethyl]amino}5,8-dihydroxyanthracene-9,10-dione; Figure 3) is a prodrug of AQ4 designed to prevent its binding to DNA (177, 178) until metabolized in hypoxic cells to give AQ4, a stable, O_2 insensitive metabolite (179). AQ4 is a DNA intercalator and potent inhibitor of DNA topoisomerase II (177, 178, 180). Early development of AQ4N has been confounded by the lack of hypoxia selective activity in several cell lines. However, a large (> 100-fold) increase in cytotoxicity of AQ4N was observed when cells were incubated under hypoxic conditions with liver microsomes (177). It was later shown that AQ4N is activated by cytochrome P450 (CYP) isoforms (181). Several studies showed that the metabolism of AQ4N correlates with levels of CYP1A1, 2B6 and 3A in humans (182), 3A in mice (183), and 2B and 2E in rats (184). Reduction of the two *N*-oxide functionalities of AQ4N has been suggested to be involved in an oxygen atom transfer from the *N*-oxide side chains in a process that is O_2 sensitive (182). Moreover, heme-containing systems other than CYP such as nitric oxide synthase have also shown to reduce AQ4N (185). AQ4N has antitumor activity in mice when combined with radiation (186, 187) or when combined with methods inducing additional tumor hypoxia, for example by hydralazine (183), dimethylxanthenone acetic acid (188), or clamping (183). It is currently in phase I clinical trials.

4.7. Cobalt(III) complex

A number of transition metal complexes such as cobalt (III) (Co(III)) (SN 24771; $[Co(III)(Meacac)_2DCE]^+$; Figures 3 and 4 (189)) and copper (II) (190) have been reported as hypoxia selective bioreductive prodrugs. Both classes of the complex incorporate nitrogen mustard as the cytotoxin and show the hypoxic cytotoxicity ratios of 20-25 in cell suspensions primarily due to the release of the nitrogen mustard ligand under hypoxic conditions. The reduction of the Co(III) complex, SN 24771, occurs via one-electron reduction resulting in the one electron reduced Co(II) intermediate, followed by the sequential loss of Meacac ligands leading to the formation of the $[Co(II)(H_2O)_6]^{2+}$ complex (Figure 4). The one electron reduction causes inert d^6 electron spin state of Co(III) to become labile d^7, which dramatically weakens the coordination bond due to electron repulsion induced by an added electron hence releasing the cytotoxin (191).

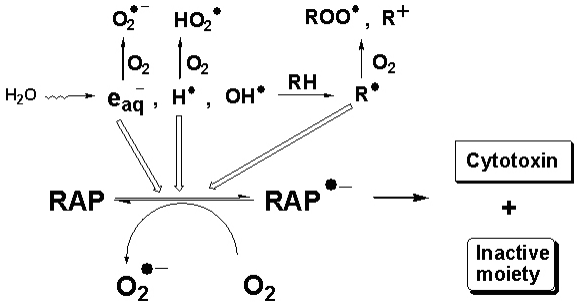

Figure 5. A scheme of reductive activation for radiation-activated prodrugs (RAP) by ionizing radiation. The radiolysis of water (H_2O) give rise to primary radicals, e_{aq}^-, $H^{\bullet}$, and $OH^{\bullet}$. e_{aq}^- is the major reductant activating the process. $H^{\bullet}$ and $R^{\bullet}$ (a reducing radical formed from endogenous molecules (RH) oxidized by $OH^{\bullet}$) can also contribute to the reductive activation process. Activation can be inhibited by the presence of oxygen (O_2) rapidly scavenging the reductants, e_{aq}^-, $H^{\bullet}$, and $R^{\bullet}$, or back-oxidize the reduced RAP ($RAP^{\bullet-}$). $RAP^{\bullet-}$ subsequently releases a cytotoxin and the rest of the drug molecule becomes biologically inactive.

Although it was originally suggested that the initial one-electron reduced intermediate of SN 24771 is back-oxidized by O_2 conferring hypoxia selectivity (67), it was later shown in a pulse radiolysis study that the rate of back-oxidation was too slow to account for the hypoxia selectivity and that the O_2 inhibitable metabolic reduction occurs by competition between SN 24771 and O_2 for biological reductants (192). A study investigating the O_2 dependence in cell killing by SN 24771 showed that the complex was very sensitive to trace amounts of O_2 (C_{50}[3] value of approximately 0.01 %) well below those required for maximal radiosensitization (189). This suggests that SN 24771 is less likely to be effective in targeting hypoxic cells at intermediate oxygen concentrations. SN 24771 was later shown to be metabolically unstable in mice resulting in a large fraction of the injected dose to be secreted in urine in the form of final cobalt-containing product $[Co(II)(H_2O)_6]^{2+}$ (193).

4.8 Limitations of bioreductive prodrugs

One potential weakness for bioreductive prodrug therapy may be the need to know the type and levels of the reductase(s) in individual tumors necessary for reduction. Assays to monitor the type, activity, and spatial distribution of specific reductase(s) in the tumor are currently not well developed in order to prospectively select patients who will benefit from therapies using bioreductive prodrugs. However, a way to overcome this limitation may be to measure the cytotoxic lesion produced bioreduction from a fine needle aspirate from individual tumors as has been suggested for PR-104 (99).

Another potential limitation of some bioreductive agents is that two-electron reductases such as DT-diaphorase can bypass the O_2-sensitive one-electron reduced intermediate and lead to some unwanted host tissue toxicity. Systemic toxicity of proteinurea in patients treated with EO9 (132), a substrate for DT-diaphorase, may well be the result of high activity of DT-diaphorase in human kidney (194). However, even one-electron reduced bioreductive prodrugs, such as CI-1010 (the R-enantiomer of RB 6145) and tirapazamine, may not be completely suppressed in enzymatic reduction in normal tissues. These drugs were shown to cause a dramatic loss of rods and cones in the retina of mice (107, 195) as a consequence of metabolic activation of the drugs by O_2 inhibitable reductase(s) in the retina as the severity of the lesion was dependent on the respiratory gas O_2 content (195). However, clinical studies of tirapazamine have shown that muscle cramping and reversible hearing loss are the main drug-related systemic toxicities (170) and that retinal toxicity is not a clinical issue.

A very sensitive O_2 dependence of the bioreductive prodrug for the inhibition of the hypoxic selective cytotoxicity may also be a problem because this means that acutely hypoxic cells will be spared. A steep O_2 dependency has been demonstrated with most bioreductive prodrugs including quinones (196, 197), nitroaromatics (198, 199), and the Co(III) complex (189). However the KO_2 value[4] of tirapazamine is higher than other bioreductive drugs and better complements that of radiation (151). This is the likely reason tirapazamine is effective when combined with fractionated irradiation.

The rate of cellular metabolism can also affect the efficacy of bioreductive prodrugs. This is because of the nature of bioreductive prodrugs: they are consumed (by being activated by metabolic reduction) as they diffuse, hence may be compromised in their extravascular transport (145). Tirapazamine suffers from this problem in that its metabolism is too rapid in hypoxic cells as demonstrated by the fact that cellular layers are more resistant in cell killing by tirapazamine than single cell suspensions under the same conditions (145).

5. RADIATION-ACTIVATED PRODRUGS

5.1. Concepts of radiation-activated prodrugs

Another strategy to target hypoxic tumor cells is to use ionizing radiation, rather than enzymes, to effect the reduction of the prodrug. Radiation-activated prodrugs (RAP) are reduced by e_{aq}^- (aquated/hydrated electron) generated from the radiolysis of water, offering two distinct mechanisms strongly inhibited by O_2 (Figure 5). These mechanisms involve either back-oxidization of the one-electron reduction intermediate of RAP ($RAP^{\bullet-}$) or to scavenge e_{aq}^- at diffusion controlled rate (200). Other primary radicals formed by radiolysis of water ($OH^{\bullet}$ and $H^{\bullet}$) are expected to be scavenged at very high rates by other biomolecules; some of the resulting organic radicals also have reducing properties so might make a further contribution to prodrug reduction (200) (Figure 5).

The RAP approach offers a number of potential advantages such as selective activation within the radiation field (tumor bearing volume); exploitation of necrotic regions lacking reductase activity through release of a stable cytotoxin with a good bystander effect, no requirement for expression of specific reductase(s); lack o

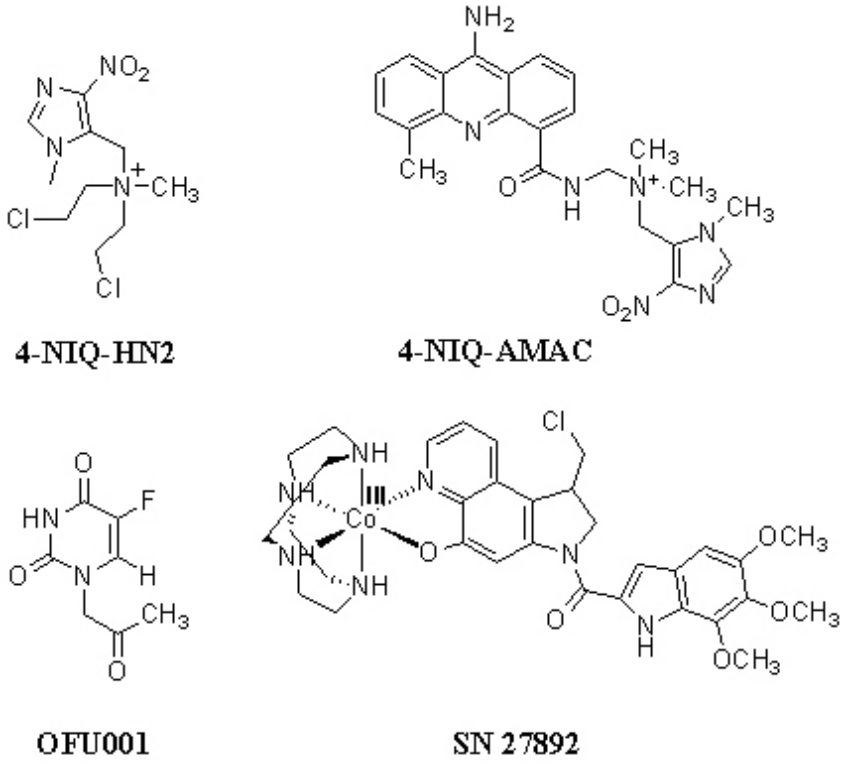

Figure 6. Some examples of radiation-activated prodrugs discussed in this review.

two-electron activation; and improved extravascular transport by designing prodrugs that are not substrates for reductase(s) in tumors (200). However one challenge is the low yield of e_{aq}^- formed from clinically relevant radiation dose (approximately 20 micromoles per kilogram over a typical fractionated course of 70 Gy) (200). To compensate for this low yield of the major reductant, RAP should release a potent cytotoxin upon reduction, that has an ability to efficiently kill cells in a variety of pH environments and proliferative states, particularly those slowly cycling cells that predominate in hypoxic regions of the tumor (201). Some of the main classes of the compounds that have been considered as candidates for RAP are discussed below.

5.2. Nitro(hetero)cyclic methylquarternary ammonium (NMQ) salts

NMQ ammonium salts of 4-nitroimidazole (*N,N*-bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-5-imidazolyl)methyl]ammonium chloride; 4-NIQ-HN2; Figure 6) and 5-nitropyrrole (*N,N*-bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-1-pyrrolyl)methyl]ammonium chloride; 5-NPQ-HN2) were originally developed as bioreductive prodrugs (202). They are also shown to be RAP that are reducible with one-electron stoichiometry by pulse and steady-state radiolysis (200, 203). Irradiating 4-NIQ-HN2 in anoxic human plasma at 20 Gy, followed by exposure to UV4 cells showed that the apparent IC_{50}[5] was markedly decreased with radiation approaching a value approximately equal to that of the free cytotoxin, mechlorethamine (HN2) (200). However, a relatively high one-electron reduction potential[6] of the prodrug (hence susceptible for enzymatic reduction) and only modest cytotoxicity of HN2 limits their utility to that of only model compounds.

To incorporate a more potent cytotoxin within the RAP system, NMQ prodrugs bearing AMAC (aminoacridine carboxamide), a very potent DNA alkylator (204), have been synthesized. 4-NBQ-AMAC, 4-NIQ-AMAC (Figure 6), and 5-NPQ-AMAC showed two orders of magnitude less cytotoxic than the free cytotoxin (AMAC) against a panel of tumor cell lines (200). Irradiation of the prodrugs in anoxic buffer or culture medium released AMAC although the yield of AMAC was lower than that for the HN2 analogs (200). AMAC prodrugs were later shown to cause convulsion in mice by a mechanism unrelated to the cytotoxin release (200), suggesting that they are not likely to be useful as RAP prodrugs.

5.3 5-fluorouracil (5-FU) releasing prodrugs

Nishimoto and colleagues (205) first reported that the N(1)-C(5)-linked dimer of 5-FU (1-(5'-fluoro-6'-hydroxy-5',6'-dihydrouracil-5'-yl)-5-fluorouracil) releases 5-FU by irradiation in anoxic aqueous buffer with approximately three-electron stoichiometry. Recently, OFU001 (1-(2'-oxopropyl)-5-fluorouracil; Figure 6) and OFU101 (1-(2'-oxopropyl)-5-fluoro-2'-deoxyuridine) have been reported to release 5-FU and 5-fluoro-2'-deoxyuridine (FdUrd), respectively, with higher efficiency (two-electron stoichiometry) in anoxic buffer upon irradiation (206, 207).

The mechanism proposed is that e_{aq}^- generated from hypoxic irradiation is attached to the compound to form the corresponding *pi** anion radical, which is thermally activated to the *sigma** anion radical with a weakened N(1)-C(1') bond linking 5-FU or FdUrd to the oxoalkyl side chain. Subsequently hydrolytic dissociation of the N(1)-C(1') occurs intramolecularly, releasing 5-FU or FdUrd (208). Although a significant cell killing was observed when irradiated compounds were added *in vitro* (206, 207), they failed to demonstrate *in vivo* activity (207, 208).

5.4. Cobalt(III) complex

The Co(III) complex such as SN 24771, originally developed as a bioreductive prodrug, can also be activated via one-electron reduction by e_{aq}^- generated by ionizing radiation. One attractive feature of this class of RAP is that the one-electron reduction potential can be fine tuned by varying the auxiliary ligand(s) within the complex so that the mode of reductive activation can be manipulated: the lower the one-electron reduction potential the less susceptibility for enzymatic reduction.

SN 27892 is a newer Co(III) complex of a tetradentate macrocyclic auxiliary ligand bearing a very potent DNA minor groove alkylator cyclopropylindoline (Figure 6). The development of this drug was in attempt to design a metabolically stable Co(III) complex that is not susceptible to enzymatic reduction. Experimental data showed that SN 27892 fulfilled requirements as a RAP including a good masking of the cytotoxicity of the DNA alkylator by more than two orders of magnitudes; an efficient release of the cytotoxin with clinically relevant radiation dose of 2 Gy in anoxic human plasma or buffer; good extravascular diffusion due to the cell exclusion property of the complex; and a metabolic stability in mice when administered systemically (193, 209). Moreover SN 27892 showed a reversible cyclic voltammetric square wave (Ware, D.C., personal communication) indicating that the one-electron reduced Co(II) intermediate is stable enough to be back-oxidized by O_2. However, SN 27892 was metabolically reduced in hypoxic HT29 cells showing a hypoxic cytotoxicity ratio of approximately 20 (209), indicating that SN 27892 is also a bioreductive prodrug. However, further development of SN 27892 as a RAP or a bioreductive drug has failed due to the lack of antitumor activity in RIF-1 bearing mice when given before or after irradiation (193). This failure was suggested to be due to excessive hypoxic metabolism of SN 27892 impeding its extravascular diffusion into hypoxic tumor tissues (210).

5.5. Limitations of RAP

While the concept of this approach is theoretically attractive, no examples with activity in tumors are yet available. One of the biggest challenges is the low yield of e_{aq}^- to significantly effect the reductive activation of RAP. The presence of competing endogenous electron acceptors may in addition compromise the radiolytic activation of prodrugs in tissues (200).

Another potential problem is that the radiolytic activation of RAP may be too sensitive to O_2. While this was also problematic for bioreductive prodrugs as mentioned earlier, the problem becomes more severe for RAP approach. This is because O_2 will inhibit activation of RAP by competing for cellular reductants as well as by redox cycling (200). This problem can be overcome by designing RAP, which releases a stable cytotoxin to exert 'bystander' effects.

6. GENE-DIRECTED ENZYME PRODRUG THERAPY (GDEPT)

GDEPT for cancer therapy commonly utilizes a suicide gene to express the enzyme in the tumor so that systemically administered non-toxic prodrugs become converted to active cytotoxins to kill tumor cells. Some examples of enzyme/prodrug combinations include herpes simplex virus thymidine kinase/ganciclovir, cytosine deaminase/5-fluorouracil (5-FC), cytochrome P450/cyclophosphamide, and horseradish peroxidase/indole-3-acetic acid, and reviewed extensively elsewhere (92, 211).

Tumor hypoxia can be exploited in GDEPT approach to selectively express reductases to enhance the efficacy of bioreductive prodrugs (reviewed in (212, 213)). This is achieved by incorporation of a hypoxia-responsive promoter into the reductase expressing cassette. Hypoxia-responsive elements (HREs) within the promoter bind the transcription factor HIF-1 (214), resulting in the transcriptional activation of the reductase gene as well as endogenous up-regulation of HIF-1 target genes under hypoxic conditions. The use of HRE-NADPH cytochrome P450 reductase system demonstrated good antitumor activity in mice with RB 6145 (215) and tirapazamine (216) when tumors were made hypoxic.

7. CLOSTRIDIA-DIRECTED ENZYME PRODRUG THERAPY (CDEPT)

Another approach targeting tumor hypoxia is to exploit the necrotic regions as a target for cancer therapy using a non-pathogenic strain of the bacterial genus *Clostridium,* an obligate anaerobe (217, 218). This Gram-positive, spore-forming bacterium becomes vegetative and grows only in the absence or at very low levels of oxygen (8). Clinical studies in 1970's already have proven the safety of *Clostridium* and its tumor specific germination (219-221). Recently the use of genetically engineered *Clostridium* has shown the tumor specific expression of *E. coli* enzymes of nitroreductase (NTR) (218, 222) and cytosine deaminase (CD) (223) when intravenously injected in mice, demonstrating that CDEPT is a powerful tumor- and hypoxia-specific gene delivery system. Moreover, the systemic administration of prodrugs, CB 1954 or 5-FC, in mice injected with NTR- or CD-expressing spores showed an enhanced CB 1954 metabolism (222) or conversion of 5-FC to 5-FU (224) in the tumor leading to significant cell kill (222, 223). Combining with vascular disrupting agents such as ZD6126 (*N*-acetylcochinol-*O*-phosphate), DMXAA (5,6-dimethylxanthenone-4-acetic acid) (224), combretastatin A-4 disodium phosphate (225), which increase tumor necrosis, have shown to enhance the germination of *Clostridium* and subsequently the efficacy of CDEPT.

8. CONCLUSIONS

This review covers some strategies for selectively targeting hypoxic tumor cells in cancer therapy. The fact that very low levels of oxygen are unique to solid tumors provides an excellent basis for tumor selectivity. However, The clinical opportunity of exploiting tumor hypoxia has yet to be realized. Despite this, the positive clinical results with the combination of the hypoxic cytotoxin tirapazamine with cisplatin in advanced non small cell lung cancer and with chemoradiotherapy with advanced head and neck cancer demonstrate the potential of this approach. There is good reason to expect that future drugs or strategies will do better: in particular, we now know that the efficacy of tirapazamine and other hypoxic cytotoxins is reduced by their limited diffusion through tumor tissue to reach all of the hypoxic cells. Recent advances in experimental models now give us the tools for quantifying the ability of prodrugs, and their activated metabolites, to diffuse through tumor tissue, so designing second generation prodrugs with properly optimized micropharmacokinetic properties is now possible. In summary, there are many new strategies and compounds to be explored in the field of hypoxia-targeting cancer therapy. The use of well validated models and advances in knowledge of the tumor microenvironment promise to deliver agents that will improve current cancer cure rates.

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Footnotes:[1] Concentration of drug in air divided by concentration of drug in hypoxia to produce the same level of cell killing, [2] The dosage required to kill 50 % of the treated population, [3] O_2 required for 50% inhibition of log cell kill, [4] O_2 concentration for 50% inhibition, [5] Concentration of a compound giving 50% growth, inhibition relative to the control cells, [6] Thermodynamic parameter measuring the efficiency of a compound to undergo one-electron reduction

Key Words: Hypoxia, Tumor, Radiosensitizer, Bioreductive Prodrug, Radiation-Activated Prodrug, Review

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