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Hypoxia-targeted drug delivery

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Abstract

Hypoxia is a state of low oxygen tension found in numerous solid tumours. It is typically associated with abnormal vasculature, which results in a reduced supply of oxygen and nutrients, as well as impaired delivery of drugs. The hypoxic nature of tumours often leads to the development of localized heterogeneous environments characterized by variable oxygen concentrations, relatively low pH, and increased levels of reactive oxygen species (ROS). The hypoxic heterogeneity promotes tumour invasiveness, metastasis, angiogenesis, and an increase in multidrug-resistant proteins. These factors decrease the therapeutic efficacy of anticancer drugs and can provide a barrier to advancing drug leads beyond the early stages of preclinical development. This review highlights various hypoxia-targeted and activated design strategies for the formulation of drugs or prodrugs and their mechanism of action for tumour diagnosis and treatment.

1. Introduction

A global increase in cancer diagnoses, combined with moderate clinical response rates of conventional chemotherapies, necessitates the discovery of novel drugs and delivery vehicles that modulate their anticancer effects *via* tumour specific mechanisms. Characterized by rapid cellular proliferation, cancer malignancies can be triggered through a variety of mechanisms, including exogenous agents, altered gene expression, or protein dysfunction. For instance, a single genetic alteration may lead to the development of a malignant tumour. Consequently, current therapeutic regimens are largely focused on genetic-based cancer taxonomy in addition to basic morphological aspects. Advances in cancer genomics, proteomic biomarker technologies, and cancer therapeutics have enabled the progression from a “one drug for all” approach to an expanding cadre of “personalized medicines”. This

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Conflicts of interest

There are no conflicts to declare.

evolution of drug discovery reflects progress towards a new paradigm that has been proposed as a therapeutic ideal.^{1–4} In January 2015, the introduction of the Precision Medicine Initiative (PMI) by the then-President Barack Obama served to broaden the concept of personalized medicine to encompass an extensive range of determinants that could lead to improved health impacts.⁵

Tumour hypoxia refers to solid tumour regions characterized by low oxygen levels. The origins of hypoxia can be traced in large measure to the abnormal vascularization, which results in an insufficient oxygen and nutrient supply to interior regions of the tumour.^{6,7} The existence of hypoxia within human tumours was first postulated almost 60 years ago by Thomlinson and Gray,⁸ who observed that tumour hypoxia imparted resistance to chemo- and radiation therapy (Fig. 1). Owing to the highly dynamic tumour microenvironment, the oxygen concentration can vary from 0.02–2% O₂ (below 2.5 mmHg pO₂), as compared to 2–9% in normal cells (40 mmHg pO).⁹ The recent development of analytical tools, such as the Eppendorf electrode for the assessment of molecular oxygen levels in tumours and biomarkers for non-invasive hypoxia imaging, have helped to underscore the common existence of hypoxia in human solid tumours.^{10–14} Relative to oxygenated healthy cells and tissues, hypoxic tumours are generally characterized by a lower pH (acidic environment) as a result of increased anaerobic respiration,¹⁵ high levels of reactive oxygen species (ROS),^{16,17} enhanced local invasiveness,^{18–20} altered metabolism,^{21,22} unregulated angiogenesis,^{23,24} incipient metastases,²⁵ and down-regulated DNA repair pathways.²⁶ Hypoxia also provides conditions favourable for the spread of cancer stem cells.²⁷ Moreover, it can result in the suppression of innate and adaptive immune response mechanisms in the tumour microenvironment.^{28,29} Hypoxia-related intratumoural heterogeneity plays a pivotal role in metastatic tumour progression and can lead to different dose–response profiles for drugs used in clinical practice.

Many of these deleterious effects are ascribed to (a) an insufficient supply of nutrients and anticancer drugs,³⁰ (b) the development of multidrug-resistant proteins,³¹ (c) cellular proliferation, which is increased after re-oxygenation of hypoxic cells,³² and (d) enhanced expression of various genes responsible for the upregulation of angiogenesis, tumour invasion, and meta-stases.^{9,25,27,33–35} As normal cells do not typically contain hypoxic regions, these unique and unfavourable features of hypoxic tumours may be exploited as an approach to developing cancer-selective therapies. In addition, hypoxic regions represent an environment in which the normal antioxidant pathways are off balance. This results in a higher level of oxidative stress. A number of agents can accentuate exogenous ROS levels *via* redox cycling or other mechanisms thus increasing oxidative stress. In this review, we summarize the most promising strategies being pursued in the context of hypoxia-targeting drug discovery and development. Not covered are approaches to hypoxia-selective sensors. However, systems that show promise for both therapy and sensing, so-called theranostics, are discussed where hypoxia is critical to effective function.

2. Hypoxia-targeted and activated prodrugs

Continuous changes in the field of pharmacoeconomics and a growing understanding of cancer biology have led the US Food and Drug Administration (FDA) and the European

Medicines Agency (EMA) to encourage drug developers to identify new diagnostic tools based on predictive biomarkers with the goal of achieving specific and selective personalized cancer treatments.^{36–39} This effort has *inter alia* generated an increased understanding of the role hypoxia plays in tumour metastatic progression and in the regulation of drug action. Against this background, hypoxia-targeted therapeutics have emerged as a promising strategy in the development of personalized medicine, particularly in the area of cancer. The drug release profile and pharmacokinetics of various prodrugs in hypoxic environments have been monitored by their conjugation to diagnostic units including fluorophores, PET, or MRI agents, which are termed as “all-in-one” theranostic agents.^{40–42} The theranostic strategy is a “smart” combination of diagnosis and therapy, which provides positive and negative feedback information after cancer therapy, through visualization of disease status; it can lead directly to increases in drug efficacy. For an overview from a clinical perspective, the reader is referred to several excellent reviews and monographs.^{43–45} In this Review, hypoxia-activated/bioreductive prodrugs, sometimes termed hypoxia-selective cytotoxins, will be discussed in detail. Based on their design and activation mechanism, these strategies have been divided into four categories, which are treated in turn.

3. Quinone-based therapeutics and theranostics

Quinones are common subunits found in numerous natural and synthetic compounds that include anticancer and antimicrobial agents, dyes, vitamin Ks, and cofactors (topoquinone, pyrroloquinone).^{46–49} Because of their propensity to undergo facile reduction, quinones play fundamental roles in various cellular functions, including redox cycling^{50–52} and energy transduction.^{53,54} Quinones can undergo a one-electron reduction mediated by various reductive enzymes, such as cytochrome reductase (P-450R, CPR), NADH-cytochrome b5 reductase (CB5R), and NADPH:ubiquinone oxidoreductase (complex I). This results in the formation of semiquinone radicals. These radical anions can react with molecular oxygen, which results in enhanced ROS production and increased oxidative stress. Generally, the formation of semiquinone radical anions is suppressed in normoxic environment. In hypoxic environments, the semiquinone radicals can be further reduced to hydroquinones (Fig. 2). However, the resulting hydroquinone species are not benign. They are, for instance, associated with toxicity related to DNA cross-linking. Two-electron reduction processes are often correlated with the presence of DT-diaphorase [also referred as NAD(P)H:(quinone-acceptor)oxidoreductase (NQO1) in later discussions], an enzyme that promotes two-electron reduction and which is commonly overexpressed in many human solid tumours, such as breast, ovarian, thyroid, and colon cancer.^{55–61} The use of special design strategies has enabled the production and selective activation of quinone-based prodrug systems, either in a hypoxic tumour environment, through a one-electron reduction pathway, or by DT-diaphorase (NQO1), a two-electron reduction enzyme.

3.1. Quinone as a drug core scaffold

The first quinone-based drug, mitomycin (**1**, MMC),^{62,63} was recognized in the 1960s and proposed to induce hypoxia-selective cytotoxicity through a reductive mechanism that was followed by DNA alkylation (Fig. 3).^{64,65} NQO1 proved capable of activating **1**. However, the tumour response towards **1** proved unrelated to NQO1 expression levels as inferred from

many studies that failed to demonstrate a correlation between these two variables.⁶⁶ On the other hand, **1** was found to be responsive to several cancer-associated reductases, including NADPH: cytochrome P450 reductase (P-450R), NADPH:cytochrome b5 reductase (b-5R), xanthine oxidase (XO), and NADPH:cytochrome b5 reductase (b-5R), and NAD(P)H:quinone oxidoreductase 2 (NQO2). Moreover, activation of **1** by NQO1 was found to be pH dependent,⁶⁷ providing a rationale for why it was less sensitive towards hypoxic cells.⁶⁸

The above findings prompted further development of an MMC analogue, porfiromycin (**2**, Fig. 3), as a hypoxia-sensitive quinone-based drug; its sensitivity was confirmed through clinical evaluation.^{69–71} Further efforts were also made to develop aziridinylquinone-based prodrugs, namely EO9 (**3**),^{72,73} RH1 (**4**),^{74–78} and AZQ (**5**),^{79–81} as analogues of **1** with enhanced selectivity toward hypoxic tumour environments. In these agents, the thought was that the aziridine ring attached directly to indolequinone and benzoquinone would become activated upon bioreduction, and could serve as a potent alkylating agent that reacts with nucleophilic sites within DNA strands (Fig. 3).⁸²

The mode of action of **3** has been extensively investigated under both hypoxic and normoxic conditions.^{72,73,83} Three major pathways are believed to be involved: (i) ROS generation by redox cycling to induce DNA strand breaks followed by quinone moiety reduction promoted by a one-electron reductase, (ii) DNA alkylation by the electrophilic centres at C3 and C2 indole position upon quinone reduction by one- and two-electron reductase, and (iii) DNA alkylation by aziridine ring opening after protonation.⁸⁴ Under normoxic conditions, tumours expressing NQO1 displayed good response towards **3**. However, under hypoxic condition, one-electron reductase (P450R) activity was predominant and resulted in enhanced sensitivity to **3** in tumours expressing low levels of NQO1. These results led to the suggestion that the cytotoxicity of **3** under both aerobic and anaerobic conditions reflects the NQO1 expression levels.⁵⁵ Animal studies confirmed that **3** exhibited activity against solid tumours with minimal bone marrow toxicity. Lead **3** was evaluated in a phase II clinical trial for the intravesical treatment of advanced breast, bladder, colorectal, gastric and non-small lung cancers. Ultimately, **3** failed to demonstrate a significant therapeutic response in humans, a result ascribed to its poor pharmacokinetic properties.⁸⁵ Recent phase III clinical trails (NCT00461591 and NCT00598806) with **3** for nonmuscle invasive bladder cancer (NMIBC) resulted in a minimal difference in the two-year recurrence rate between the treatment and placebo group. Consequently, significant efforts have been made to improve the pharmacokinetic features of **3** through examination of structure–activity relationships (SARs) involving the indolequinone scaffold.^{86–90}

One effort to prepare an improved quinone-based cytotoxic agent led to the synthesis of the bis-aziridine, RH1 (2,5-diaziridinyl-3-(hydroxymethyl)-6-methyl-1,4-benzoquinone, **4**). This water-soluble compound was expected to display particular selectivity toward NQO1 reductase (Fig. 3).^{74–78,91,92} Compound **4** was found to exhibit nanomolar activity *in vitro*, with its cytotoxicity ascribed to the formation of the semiquinone form upon reductive activation and the subsequent formation of DNA inter-strand cross-linked adducts.⁹³ Compared with the corresponding species derived from **1**, the semiquinones formed after bioreduction of **4** are quite stable. Both *in vitro* and *in vivo* cytotoxicity of **4** was found to

correlate well with NQO1 activity.^{93,94} A formulation of compound **4** with cyclodextrin (20%) was selected for consideration by the National Cancer Institute (NCI) and Cancer Research UK (CR-UK) (NCT00558727) for clinical dose-escalation studies (phase I) in advanced solid tumour patients. However, the paired biopsy data was not encouraging as the DNA cross-linking was observed in even low NQO1 level regions.⁹⁵ It was proposed that activation of **4** at non-cancerous sites was facilitated by alternative activation mechanisms, including those involving P-450R and NQO2.^{96,97}

Also, subject to initial clinical development was 2,5-bis-(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone (AZQ, **5**), a second-generation analogue of **4**.⁷⁴⁻⁷⁷ In phase I clinical studies, **5** was found to produce partial and complete responses in patients with malignant astrocytomas and meningeal leukemia. Further, phase I studies with **5** revealed promise of activity in patients with CNS and recurrent or resistant brain tumours. Unfortunately, phase III studies of brain tumour patients confirmed that compound **5** was less effective than other tested chemotherapeutic agents (nitroureas, BCNU (1,3-bis(2-chloroethyl)-1-nitrosourea), or PCNU(1-(2-chloroethyl)-1-nitrosourea)), although activity in resistant brain tumours and nonlymphocytic leukemias was seen. Eventually, clinical studies were terminated due to poor pharmacokinetics, severe toxicity, and lack of sufficient efficacy relative to existing approved anticancer agents.^{98,99}

3.2. Quinones as a trigger for targeted chemotherapeutic drug delivery

Quinone based moieties have been exploited in a number of drug release scenarios due to their reductive capabilities. Reduction to the hydroquinone can facilitate internal electronic rearrangements resulting in covalent bond cleavage and drug release. To date, two major release mechanisms have dominated the quinone-based drug release literature. These limiting approaches are discussed below.

3.2.1 Hypoxia selective drug release via tandem benzoquinone reduction and iminium ion generation.—Indolequinones have been extensively explored to effect delivery of cytotoxic agents.^{85,100-102} Typically, the cytotoxic agents in question are tethered at the C-3 position. Upon bio- or radiolytic reduction, the tethered cytotoxic agent is released as the indolequinone is converted to an electrophilic iminium species (Fig. 4). The iminium daughter product can promote DNA alkylation or other cellular damage resulting in synergistic cytotoxicity. Nishimoto *et al.* employed indolequinone prodrug **6**, to deliver an active drug, 5-fluorodeoxyuridine, upon radiolytic reduction (G value $0.38 \times 10^7 \text{ mol J}^{-1}$) under hypoxic conditions (Fig. 5). Prodrug **6** exhibited minimal cytotoxicity under aerobic conditions toward EMT6/KU cells. However, under hypoxic irradiation, prodrug **6** undergoes a one-electron reduction leading to biomolecular disproportionation of the semiquinone radical anion intermediate. The resulting electrophilic iminium cation and active drug displayed synergistic activity and potency in a radiation dose-dependent manner that was superior to that seen for the parent drug alone.¹⁰¹

Camptothecin (CPT, an irinotecan derivative) is a quinolone alkaloid that acts as a potent DNA enzyme topoisomerase I inhibitor. It displays anticancer activity that is ascribed to lethal DNA strand breakage that occurs during the replication process.^{103,104} Despite

promising preclinical results, the unfavourable physiochemical properties of camptothecin, including poor water solubility and lactone ring instability, have restricted its clinical use. Nevertheless, considerable effort has been devoted to developing new camptothecin derivatives (Topotecan, SN-38). Work has also focused on creating prodrug forms and using it to produce drug delivery vehicles. For instance, Nishimoto *et al.* synthesized the water-soluble prodrug **7** (Fig. 5) with a CPT and an indolequinone that is conjugated at the C-3 position through an *N,N'*-dimethyl-aminoethylcarbamate side chain.¹⁰² Relative to what was seen with camptothecin, prodrug **7** exhibited slightly improved activity in HT-29 tumour cells under hypoxic conditions (1.8 fold) compared to normoxic conditions. This result was ascribed to fast bioreductive activation followed by cyclization of the chemical linker, a chemical reaction that serves to release the parent drug camptothecin in its active cytotoxic form.

In separate work, Chakrapani *et al.* employed the indolequinone motif (Fig. 5) to improve the hypoxia-selective delivery of nitric oxide (NO), a potent tumour-static species.¹⁰⁵ Conjugate **8** (R = H) exhibited a hypoxia-selective antiproliferative effect in various cell lines, including DLD-1 (IC₅₀ = 0.25 μM), HeLa (IC₅₀ = 0.96 μM), and human urinary bladder cancer cells T-24 (IC₅₀ = 0.56 μM). When methyl substituents were introduced onto the C-2 position of the active agents containing a 4,7-dioxindole moiety (**8**, R = Me), the inhibitory potency of the conjugates was diminished by 10–16 times. This result was ascribed to a reduction in the metabolic rate.^{106,107} Mechanistic studies revealed that conjugate **8** induced nuclear DNA double-strand damage with the simultaneous formation of γ-H2AX foci.

In an effort to improve further the efficacy of CPT, its analogue SN-38 was conjugated to a peptide bearing an indolequinone subunit. This gave structure **9**, which incorporates an integrin-selective peptide (shown in pink in Fig. 5). Integrins are highly expressed in tumour vasculature during angio-genesis;¹⁰⁸ thus, conjugate **9** containing both an α_vβ₃ integrin-selective cyclic tetrameric peptide c(RGDfK) and a CPT subunit was expected to target angiogenic tumour cells over normal proliferating endothelial cells. In the absence of DT-diaphorase, conjugate **9** was nontoxic up to concentrations of 300 nM in human cervical carcinoma KB cells. However, in the presence of DT-diaphorase reductase, about 50–70% inhibition in cell growth was observed when cells were exposed to 300 nM of **9**. This was taken as evidence that the activity of the conjugate was mediated by recognition of α_vβ₃ integrin receptors on the cancer cell surface and that CPT drug release is achieved upon reduction.¹⁰⁹

Xu *et al.* reported a series of C-3 functionalized indolequinone-based diterpenoids (orisonin analogues) as hypoxia selective prodrugs. Oridonin is an *ent*-kaurene diterpenoid natural product that is extracted from *Radosia rubescens* and which was found to exhibit antitumour activity in various cancer cell lines.^{110,111} For instance, compared to taxol (used as a positive control), various orisonin analogues were found to exhibit higher toxicity profiles in NQO1-overexpressing human colon carcinoma (HT-29) cell and lung cancer (A549) cell lines. Moreover, relatively low toxicity was seen in the NQO1-deficient lung adenocarcinoma (H596) cell line. A lead compound (**10**, Fig. 5) was found to have good antiproliferation activity in NQO1-overexpressing HT-29 and A549 cells. In the presence of dicoumarol (an NQO1 inhibitor), the activity of **10** in A549 cells was largely repressed.

Further, **10** exhibited synergistic cytotoxicity in these cells when compared with equimolar combination of oridonin and 10-hydroxy indolequinone. Detailed mechanistic studies, including DCF-ROS staining, mitochondrial membrane potential (MMP) measurement, DNA-based cell cycle analysis and western blotting analysis (caspase 3/9, cytochrome *C*, Bax, Bcl-xl) revealed that compound **10** induced NQO1-dependent apoptosis through the ROS-triggered mitochondrial apoptotic pathway. Efficacy was also verified in liver cancer (H22) xeno-grafted mouse models.¹¹²

In 2003, Borch *et al.* demonstrated that the C-2, as well as the C-3, position of indolequinone scaffolds could be modified to deliver phosphoramidate mustards after DT-diaphorase reductive activation (Fig. 6).^{100,113} It was observed that the C-2 and C-3 substituted indolequinone analogues **11** and **12** (Fig. 6) underwent rapid activation following two-electron reduction and exhibited excellent selectivity toward human DT-diaphorase isoenzymes. In contrast, the C-3-substituted indolequinone derivative **11** was preferentially activated *via* a one electron reduction pathway. It was also found that prodrug **11** would undergo nucleophilic activation to release a phosphoramidate anion when exposed to glutathione (GSH) or dimethyldithionatecarbamate (DDTC) as monitored by ³¹P NMR spectroscopy. In contrast, the corresponding C-2 substituted analogue **12** displayed an extremely low rate of activation when exposed to GSH.¹¹³ In model experiments that involved reacting sodium dimethyldithiocarbamate (DDTC) with **11** and **12**, direct displacement of the bromide substituents present in the parent molecule was also observed. However, substitution of the chloro analogue was not observed, a finding that was taken as highlighting the choice of chloro analogues for prodrug development. *In vitro* cytotoxicity assays revealed that both C-2 and C-3 substituted indolequinone-derived prodrugs exhibited nanomolar toxicities against the HT-29 and BE human colon cancer cell lines. As a general rule, the C-2 substituted systems (*i.e.*, **12**) displayed a cytotoxicity profile consistent with DT-diaphorase activity, while no such correlation was observed in the case of the C-3 substituted analogues. Furthermore, comparative cytotoxicity studies involving **11** and an acetoxy functionalized derivative led to the suggestion that the indolequinone moiety and phosphoramidate anion act in synergistic fashion. No evidence of such a benefit was seen in the case of the C-2 substituted compounds.

Conjugate **13** (Fig. 7), a benzoquinone scaffold linked to a nitrogen mustard analogue, was investigated in the context of hypoxia-responsive drug activation. This agent was designed to function as a dual mode of action prodrug. After bio-reduction in hypoxic cells, conjugate **13** was expected to release the active mustard, 4-[bis(2-chloroethyl)amino]benzoic acid, along with an intermediate bearing an electrophilic site that was expected to be susceptible to nucleophilic attack by DNA leading to alkylation-based DNA damage.¹¹⁴

3.2.2 Hydroquinone assisted cyclization-based release mechanisms.—

Benzoquinones incorporating three methyl groups (one at the *ortho*-position of the quinone ring and two at the β -carbonyl positions) can be considered as possessing “trimethyl locks” that result in restricted conformational motion. These “locked” cores have been studied in the context of drug release applications. Under hypoxic conditions, the quinone form is reduced to the corresponding hydroquinone, which leads to severe steric repulsion between

the three methyl groups. This is a design feature that can favour release of tethered alcohols or amines.^{115–118} Taking advantage of this strategy, Carpino *et al.*¹¹⁵ introduced a tripartite quinone-based prodrug approach, which was further developed by other research groups.^{116–118} One embodiment is the melphalan-quinone derivative **14**. Upon reductive activation, the parent alkylating agent is released *via* lactonization (Fig. 7).¹¹⁴

Phillips *et al.* reported a 4-aminophenyl nitrogen mustard-benzoquinone conjugate **15**, which proved to be a good substrate for DT-diaphorase with $V_{\max} = 11.86 \pm 3.09 \text{ mmol min}^{-1} \text{ mg}^{-1}$ and $K_m = 2.70 \pm 1.14 \text{ mmol L}^{-1}$ (Fig. 7).¹¹⁶ The nitrogen mustard released upon bioreduction displayed significant toxicity towards T47D cells overexpressing both DT-diaphorase under aerobic conditions and cytochrome P-450 enzyme under hypoxic condition. A favourable hypoxia cytotoxicity ratio (HCR) relative to normoxia of about 15.8 was noted.¹¹⁶

Moody *et al.* utilized the same strategy to create the trimethyl quinone derivatives **16** and **17** (Fig. 7) containing semaxanib (SU5416) and its corresponding 6-hydroxy derivative, both of which are potent VEGF receptor tyrosine kinase Flk-KDR inhibitors.¹¹⁷ Preliminary *in vitro* assays revealed that derivatives **16** and **17** (at 10 μM) exhibited VEGF-stimulated angiogenesis inhibition upon bioreduction, comparable to equimolar treatments of the parent SU5416 (semaxanib) and 6-hydroxy SU5416 drug systems in cultured human umbilical vein endothelial cells (HUVECs). These results provided support for the suggestion that both derivatives **16** and **17** release the active drugs in these biological milieus.

Another investigation involved structure **18** wherein a CPT is conjugated to a quinone propionic acid (Fig. 7). The resulting system displayed significant cytotoxicity in DT-diaphoraseoverexpressing cells and permitted the simultaneous tracking of the drug activation process *in vitro*.¹¹⁸ To improve the cancer-targeting ability of this system, Kim *et al.* developed prodrug **19**, composed of three moieties, an anticancer drug SN38 (a topoisomerase I inhibitor), Q3PA (trimethyl-locked quinone propionic acid), and a biotin moiety as a cancer-targeting unit. After preferential uptake in cancer cells (presumably biotin-mediated), prodrug **19** is activated by NQO1. This leads to the release of active SN38 and results in cancer cell apoptosis. Drug release and the determinants of apoptosis of cancer cells were further studied in the case of cells expressing high levels of NQO1 and biotin receptors.¹¹⁹

Borch *et al.* reported a related class of compounds, namely structures **20** and **21** (Fig. 7). Here, a phosphoramidate mustard is tethered to either a naphthoquinone or benzimidazole quinone scaffold.¹²⁰ Upon bioreduction by human DT-diaphorase, the naphthoquinone prodrugs were found to release the parent drugs rapidly ($k_{\text{cat}}/K_m = 3 \times 10^7 - 3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$). Clonogenic assays provided support for the notion that the naphthoquinone derivative **21** exhibited potent cytotoxicity in both the HT-29 (DT-diaphorase +ve) and BE (DT-diaphorase -ve) colon cancer cell lines. However, the cytotoxicity profile did not correlate well with the DT-diaphorase activity. Nucleophilic attack by GSH at the C-3 position of naphthoquinone, followed by parent drug release, was proposed as an alternate prodrug activation pathway for these derivatives. The corresponding benzimidazole quinone analogues **20** did not display

appreciable cytotoxicity *in vitro*. This could reflect the stability of the reduced quinone form and a lack of promoted release of the active phosphorodiamidate species.

4. Nitro/azo-based therapeutics and theranostics

Over the past few decades, nitroaromatics have been extensively utilized in the development of non-invasive probes for imaging tumour hypoxia. Nitroaromatics are readily reduced to corresponding amines by nitroreductases (NTRs), a family of flavin-containing enzymes. NADH and NADPH can provide the requisite reducing equivalents (Fig. 8). Under hypoxic conditions, several intracellular reductases such as NTRs, DT-diaphorase, and azoreductase are overexpressed.¹²¹ A direct correlation between NTR levels and solid tumour hypoxia has been established. In this section, the focus is on hypoxia-responsive nitroaromatic (pro)-drugs, with a particular emphasis on their specific biological and medicinal features. The discussion is organized in terms of the proposed mode of action.

4.1. Nitroaromatics as “oxygen-mimetic radiosensitizers”

Radiation therapy (RT) has a time-honoured role in cancer therapy. Utilization analyses have revealed that about 30% of the cancer patients received RT either alone or in combination with other treatments.¹²² In RT, ionizing radiation is used to control or kill the malignant cells. Oxygen, being an extremely electron-affinic molecule, plays a key role in the chemical reactions that lead to DNA damage after a given dose of ionization radiation.^{123,124} The resulting DNA lesions are thought to result in cell death. Typically, hypoxic tumour regions are 2–3 times more resistant to radiation damage than cells in normal oxygenated environments.^{125,126} In clinical practice, this results in so-called fraction of the radiation dose, administering it in a discontinuous fashion over considerable time. This is done in part so as to allow oxygenation of erstwhile hypoxic regions.

In 1976, Adams *et al.* suggested that certain compounds, characterized by a high electron affinity, could improve the efficacy of RT¹²⁷ by acting as mimetics of molecular oxygen. These compounds, termed “oxygen mimetic radiosensitizers,” were found to stabilize the DNA lesions induced by RT.¹²⁸ Nitroimidazoles have received considerable attention as oxygen mimetic radiosensitizers. Metronidazole (**22**) was the first nitroimidazole derivative to be explored in this context (Fig. 9).^{129,130} It was found to provide radiosensitization both *in vitro* and *in vivo*, exclusively under hypoxic conditions, and became the subject of various clinical studies.^{131–133} Initially positive results were seen and this prompted a search for other high electron-affinity agents with enhanced radiosensitizing ability, *e.g.*, misonidazole (**23**, Fig. 9). Compound **23** exhibited high potency in various animal tumour models and human malignancies after the administration of a single dose of radiation. Extensive clinical trials were conducted worldwide. Unfortunately, phase II trials revealed severe dose-limiting encephalopathy and peripheral neurotoxicity.^{134–138}

In an effort to reduce the neurological toxicity associated with nitroimidazole, Brown *et al.*¹³⁹ reported a more polar amide analogue of misonidazole (**23**), namely etanidazole (**24**, Fig. 9). Etanidazole proved more effective than misonidazole for two major reasons. First, it displayed reduced toxicity, presumably due to its shorter half-life and relatively rapid excretion rate. Second, the more hydrophilic nature of etanidazole resulted in a slower

uptake by neural tissues and a subsequent reduction in neurotoxicity relative to **23**.^{139,140} As reported by Coleman *et al.*,^{141,142} etanidazole (**24**) was well tolerated at a dosage level three times higher than that of misonidazole (**23**). However, etanidazole (**24**) was not developed as it failed to meet clinical endpoints in trials involving head and neck cancer.¹⁴³

Another hypoxia sensitizer, nimorazole (**25**, Fig. 9),^{144,145} was found to exhibit considerably lower clinical toxicity and has been registered for use in the treatment of head and neck cancer in Denmark.^{146–150} Another agent, doranidazole (**26**, Fig. 9) demonstrated reduced neurotoxicity, presumably owing to its impermeability to the blood–brain barrier (BBB).^{151,152} This agent demonstrated radiosensitization under hypoxic conditions, both in mouse tumour models and human tumour xenografts.^{153–155} To date, **26** has been evaluated clinically in Japan, where it showed encouraging results in phase I/II studies for advanced non-small-cell lung cancer (NSCLC).¹⁵⁶

Further developments in RT have produced a highly precise and potentially more effective radiation therapy modality termed stereotactic body radiotherapy (SBRT). In SBRT, advanced imaging technologies and sophisticated computer programs are used to guide the delivery of high doses of radiation directly to tumour site. Clinical results utilizing SBRT to treat various solid tumours revealed efficacy profiles equal or superior to fractionated RT.¹⁵⁷ Moreover, SBRT offers many advantages over conventional methods, such as a shortened treatment course, faster pain relief, fewer patient visits, and reduced need for surgical operations. This evolution in the standard of care (SOC) has provided an incentive to test novel putative radiation sensitizers in conjunction with SBRT. A summary of these efforts is given below. Unfortunately, to the authors' knowledge, there are no reports where a putative radiation sensitizer has been tested in a comparative fashion under conditions of both RT and SBRT.

Hay *et al.*¹⁵⁸ reported the alkyl sulfonamide substituted nitroimidazole derivatives **27**, **28**, **30**, and **31** (Fig. 9) and tested them as possible novel radiosensitizers in conjunction with SBRT. Preliminary *in vitro* clonogenic assays revealed that derivatives bearing 2-nitroimidazole functionality induced significant hypoxia-selective cytotoxicity (6 to 64-fold) against a panel of human cancer cells lines (HT29, H1299, HCT116, PC3, and 22RV1) relative to analogues based on a 5-nitroimidazole unit. Compared with **23**, compounds **27** and **28** displayed only modest sensitization, while compounds **30** and **31** displayed sensitization that was equal or superior to that of **23** in HCT-116 human colorectal cell lines. The enhanced radiosensitization observed in **30** and **31** was ascribed to the presence of basic side chains, which was thought to lead to an increase in cellular uptake.

Recently, these researchers utilized a phosphate prodrug strategy to improve the aqueous solubility of conjugates **28** and **32** and compared their equimolar cytotoxicity to **24**, as well as the prodrugs **29** and **33** (Fig. 9).¹⁵⁹ Although **29** possess similar polarity to **24** (log *D* values = 1.53 and 1.37, respectively), it displayed enhanced HCR (40) compared to **24** (HC = 19) in HCT116 colon cancer cells. Compound **33**, having properties similar to those of **25**, displayed only a modest HCR (4). Relative to **24** and **25**, prodrugs **29** and **33** showed improved drug delivery, enhanced peak tumor drug concentration, and substantial

radiosensitization in combination with RT and delayed the tumor regrowth (3 folds) as compared to radiation (12.5 Gy) alone in HCT116 xenografts.

4.2. Nitroaromatic subunits as integral components of hypoxia-activated prodrugs

During the development of radiosensitizers, a number of compounds were identified as being substantially more cytotoxic than would be predicted based on their radiosensitization properties alone.^{160,161} The hypoxia-selective toxicity of these agents, among the first of which was metronidazole (**22**), was initially noticed by Sutherland *et al.*¹⁶² This observation is considered a watershed moment in the development of second-generation drugs and triggered the initial development of what are now referred to as “hypoxia-activated prodrugs (HAPs)” or “hypoxia-selective cytotoxins (HSCs)”. The rationale for the development of these drugs is based on the concept that hypoxia-selective metabolic reduction of the nitro group by an intracellular nitroreductase *via* stepwise electron transfer converts the compounds into a cytotoxic species as illustrated in Fig. 8. These cytotoxins can form strong DNA inter-strand cross-links and ultimately lead to cancer cell death. Although the concepts underpinning HAPs were established over 40 years ago, it was only over the course of time that refinements in structural design led to the development of a number of bioreductive drugs. Some of these later systems moved into clinical trials. An example is RSU1069 (**34**, Fig. 10),^{163–165} which contains an aziridine moiety on the N1 side chain of the nitroimidazole core and acts as weak monofunctional alkylating agent. In light of the promising *in vitro* and *in vivo* results obtained in combination with RT,¹⁶⁶ compound **34** was evaluated clinically in 1985. However, it caused severe emesis and was withdrawn from the further clinical trials.¹⁶⁷ RB6145 (**35**, Fig. 10), a prodrug of compound **34**, emerged as a more suitable hypoxia-selective candidate^{168,169} since it displayed a systemic toxicity roughly 2.5-times lower than **35**. However, further development of **35** was limited due to the high retinal toxicity of its *R*-enantiomer (compound **36**, also known as C-1010, Fig. 10).^{170,171}

In an effort of develop more acceptable agents, compounds **37** and **38** were developed (Fig. 10). These agents are relatively electron-affinic due to the incorporation of 5-nitrofurans moieties in lieu of 2-nitroimidazole cores. In fact, compounds **37** and **38** proved effective as radiosensitizers in tissue culture under hypoxic conditions.^{172,173} Naylor *et al.* conducted detailed SAR investigations to understand the effect of varying the electronic character of the side chains on the radiosensitizing and HAP activity within a series of 5-nitrofurans, 2-carboxamide, and 3-carboxamide derivatives.¹⁷⁴ It was found that in hypoxic V79 Chinese hamster cells, compared with analogues **34** and **35**, the derivatives with greater electron affinity exhibited higher radiosensitization levels *in vitro*. However, these trends were not translated into *in vivo* studies; in fact, across the board these systems exhibited low sensitizing activity in murine KHT tumours. This was attributed to several factors including, poor distribution and tumour uptake, rapid metabolism, and undesired interactions with other noncritical targets in animal models.

In 1992 Wilson *et al.* suggested¹⁷⁵ that the hypoxia selectivity and cytotoxicity of this class of bioreductive drug might be further enhanced by incorporating two redox centres within the same molecule to produce so-called “bis-bioreductive drugs”. This approach relies

mainly on the principle that both redox centres must be reduced independently by oxygen-inhibitable processes for full activation. As a consequence of this design principle, it was expected that the bis-bioreductive drugs in question would display particularly high hypoxia selectivity compared to their non-activated congeners. This led to the preparation of bis-nitroimidazole *N*-[2-(2-methyl-5-nitroimidazolyl)ethyl]-4-(2-nitroimidazolyl)butanamide (NSC639862, **39**)¹⁷⁶ (Fig. 10). Compound **39**, with 2-nitro and 5-nitroimidazole units connected through a carboxamide linker, showed 200-fold higher toxicity towards AA8 Chinese hamster cells compared with mononitroimidazoles. *In vivo* studies revealed that compound **39** was active against KHT¹⁷⁷ and MDAH-MCa-4¹⁷⁶ tumours when administered in combination with radiation.

An unfortunate corollary of producing bioreductive drugs that are only fully active under conditions of extreme hypoxia,^{178,179} is that oxygenated tumour regions might remain resistant.¹⁸⁰ One conceivable approach to circumventing this problem would be to develop bioreductive drugs that, upon reduction, produce cytotoxic species with sufficiently long half-lives to diffuse into the relatively well-oxygenated cells that surround the chronically hypoxic region. The strategy is known as the “bystander effect” and was investigated in detail by Wilson *et al.*¹⁸¹ They used nitrogen mustard **40**¹⁸² (Fig. 10) as the cytotoxin. A number of bioreductive drug candidates based on nitrogen mustards,¹⁸³ such as CB1954 (**41**)¹⁸⁴ and SN23862 (**42**),¹⁸⁵ are now known (Fig. 10). Compound **42** was found to be a more promising HSC than the corresponding aziridine analogue **41**.¹⁸⁶ The limited hypoxia-selective cytotoxicity of **41** was attributed to oxygen-insensitive bioreduction by NAD(P)H:quinone oxidoreductase (DT-diaphorase), unlike **42**, which undergoes preferential reductive activation by cytochrome P-450 reductase. The active meta-bolite of **42** (*i.e.*, the 2-amine product), was reduced under hypoxic conditions and displayed exceptional bystander activity in multicellular layer (MCL) cultures and was found to be 2000-fold more potent than the parent prodrug.¹⁸⁷ Although **41** displayed limited hypoxia selectivity, promising results were obtained when used in combination with gene-dependent enzyme prodrug therapy (GDEPT). It was found to serve as an efficient substrate for *Escherichia coli* nitroreductase (NTR). This enzyme converts the prodrug into a bifunctional alkylating agent, resulting in DNA cross-links and cell apoptosis.^{188–191} In combination with RT, the combination of NTR and **41** was found to induce synergistic cytotoxicity in HeLa cells. Phase I/II clinical trials for virus directed enzyme-prodrug therapy (VDEPT) with **41** were carried out in patients with prostate-specific antigen (PSA). Unfortunately, only 7 out of 19 patients showed greater than 10% PSA reduction.¹⁹²

Denny *et al.*^{193,194} conducted extensive SAR studies on **42** in order to find a compound with improved cytotoxicity and improved aqueous solubility that simultaneously retained hypoxic selectivity. Structural optimization resulted in the development of a promising HAP, namely PR-104 (**43**, Fig. 10),¹⁹⁵ designed to serve as a water-soluble phosphate prodrug. Mechanistic studies¹⁹⁶ revealed that rapid hydrolysis of the phosphate group by systemic phosphatases serves to generate the alcohol prodrug PR-104A. The nitro group of compound **43** is then reduced by one- or two-electron oxidoreductases. This results in the formation of the hypoxia-selective DNA interstrand cross-linking metabolites, hydroxylamine PR-104H and amine PR-104M (Fig. 10).^{197,198} Various oxidoreductases were considered responsible

for the reduction of **43**, including FAD-dependent oxidoreductase, cytochrome P-450 reductase, and aldoketoreductase 1C3 (AKR1C3).^{199–202} The majority of the activity ascribed to **43** under hypoxic conditions is considered to be the result of a one-electron pathway catalyzed by cytochrome P-450 reductase. In contrast, the activity observed under aerobic conditions was believed to result from a two-electron process catalyzed by AKR1C3.

Recently, Wilson *et al.*²⁰³ demonstrated the potential of compound **43** in the inhibition of triple-negative breast cancers *via* the presumed dual targeting of hypoxia and homologous recombination dysfunction repair. *In vitro* studies revealed excellent hypoxia selectivity in human tumour derived cancer cell lines (SiHa, HT29, and H460) relative to tirapazamine (TPZ, Fig. 29) or conventional mustards.²⁰⁴ Compound **43** showed promising results in various *in vivo* preclinical models. For instance, Konopleva *et al.*²⁰⁵ demonstrated the high efficacy of **43** in murine models for human leukemia. A report by Lock *et al.*²⁰⁶ also revealed that compound **43** had significant efficacy in T-ALL xenografts and was superior to a combination regimen consisting of dexamethasone, vincristine, and L-asparaginase; furthermore, the activity of **43** in T-ALL cells was well correlated with AKR1C3 expression. In clinical phase I/II studies conducted in acute lymphoblastic leukemia patients with **43**, a significant decrease in leukemic hypoxic cells was observed. However, toxicities, such as myelosuppression and gastrointestinal effects, led to investigations of **43** at lower doses than used originally and in combination with other cytotoxins.²⁰⁷ Unfortunately, a first combination trial of **43** with docetaxel in non-small cell lung cancer (NSCLC) was terminated due to the unfavourable nature of the clinical results (NCT00862134). Recent preclinical studies of compound **43** revealed efficacy against breast cancer xenografts in combination with RT. The sensitization induced by **43** was particularly noticeable in *BRCA2*-knockout mutants. To date, no further clinical trials of **43** in combination with RT have been reported.

To enhance the potency of hypoxia-selective cytotoxins, Papadopoulou and Bloomer tethered DNA intercalating moieties to nitroimidazoles, as exemplified by NLCQ-1 (**44**, Fig. 10).²⁰⁸ Compound **44** displayed significant *in vitro* hypoxic selectivity (30-fold) in several rodent (V79, EMT6, and SCCVII) and human (A549 and OVCAR-3) tumour cell lines as compared with normoxic conditions. Perhaps the most striking feature of **44** to emerge from these and other studies was the finding that an increase in exposure time led to dramatically enhanced hypoxic selectivity (up to 386-fold). *In vitro* studies with isolated rat-liver microsomes and NADPH/NADH led to the suggestion that cytochrome P-450 and b5 reductases were primarily involved in the reductive activation of **44**;²⁰⁹ however, other enzymes were also implicated.²¹⁰ Under conditions of radiation therapy, **44** displayed a synergistic benefit against hypoxic V79 cells both *in vitro* and *in vivo*.²¹¹ More importantly, **44** was found to enhance the antitumour effect of various chemotherapeutic agents^{212–217} in murine tumours and human xenografts, and did so without increasing bone marrow suppression or retinal toxicity. These latter adverse events represent two common dose-limiting toxicities associated with this class of therapeutics.

Thymidine phosphorylase (TP), a platelet-derived endothelial cell growth factor (PD-ECGF), plays an important role in angiogenesis, tumour growth, and metastasis. Various immunohistochemical and TP-activity studies have served to confirm its elevated expression

in a wide range of solid tumours compared to normal tissues; this is particularly true in hypoxic regions.²¹⁸ Appreciating this, Jaffar *et al.*^{219,220} reported that the 2-nitroimidazolymethyl uracil derivative **45** and **46** (Fig. 10) acts as novel hypoxia-activated prodrugs. Bioreduction of the nitro group in these prodrugs by cytochrome P-450 reductase produces the corresponding amine forms. The reduced forms of **46** (X = Cl, Br) were found to be potent inhibitors of *E. coli* and human TP (IC₅₀ = 20 nM) at 40 nM enzyme concentration, while the corresponding amino-**46** species (X = Cl, Br) proved 350 fold less reactive (IC₅₀ = 7 μM). In contrast, the 5-unsubstituted analogues (**45** and **46**, X = H) were less reactive than the corresponding 5-halo analogues. The reduced forms of **45** (X = Cl, Br) were roughly 1000 times more potent than the corresponding parent system. An evaluation of TP inhibition and comparison with other known TP inhibitors, such as 6-amino-5-bromouracil (6A5BU) and 5-chloro-6-[(2-iminopyrrolidin-1-yl)methyl]uracil hydrochloride (TPI), led to the conclusion that under conditions of administration, prodrug **45** gives rise to a TP inhibition level that is comparable to that of TPI and 100 times greater than 6A5BU.

Although most of the bioreductive drugs discussed above are promising HAPs, most of the clinical trials conducted with the drug candidates discussed above did not meet their target endpoints.²²¹ It has been proposed that one important limitation is the failure to identify target patient populations likely to benefit from HAP therapy. Thus, to the extent it is possible, developing a correlation between tumour hypoxia and HAP clinical response appears warranted. Other means for improvement would include achieving the selective delivery of HAPs to target cells. It would also be beneficial to create conjugates whose active payloads released after activation, currently species such as, *e.g.*, nitrogen mustards and topoisomerase II inhibitors, are not subject to multidrug resistance.

Addressing these limitations has led efforts to the discovery and development of new classes of cytotoxic agents. In this context, Duocarmycin A (**47**, Fig. 11) has received considerable attention. It has been identified as being a DNA alkylating agent with remarkable cytotoxic potency.^{222,223} Mechanistic studies revealed that the extreme cytotoxicity of this agent arises from sequence-selective alkylation at the N3 position of adenine within the minor groove of DNA. To date, several analogues of **47** were found to demonstrate promising antitumour activity in preclinical studies.²²⁴ Nonetheless, clinical evaluations of **47** and its derivatives have been limited by myelotoxicity, a dose-limiting side effect to which humans appear to be more sensitive than mice.^{225,226}

A desire to circumvent these limitations has led to the development of new prodrug systems that could undergo selective action at the intended tumour site. For instance, Tercel *et al.*²²⁷ reported naphthyl-based alkylating agents obtained by replacing the phenolic hydroxyl group in **47** with a nitro group (giving **48** and **49**; *cf.* Fig. 11). Under conditions of bioreduction, the nitro group is converted to the corresponding amino-derivative followed by a subsequent spiro-cyclization to form the potent DNA alkylating agent **50** (Fig. 11). The nitro-chloromethylindolines derivatives **48** and **49** differ from one another in that they incorporate a neutral 5,6,7-trimethoxyindole (TMI) side chain and a basic 5-[(dimethylamino)ethoxy]indole (DEI) side chain, respectively.²²⁸ They were both found to be promising HAPs with the cytotoxic potency (measured as nitro/amino IC₅₀ ratios) of the amino analogues being approximately 480-fold (for **49**) and 3000-fold (for **48**) higher than

the corresponding nitro analogues. *In vitro* clonogenic survival assays revealed that both **48** and **49** produced HCR values on the order of 300-fold and 200-fold in the case of UV4 and RIF-1 cells, respectively. However, much lower potency and selectivity was observed against HT29 cells.

An investigation of the bystander effect in multicellular layer (MCL) cultures^{229,230} grown from HCT116 cells revealed that an active metabolite is produced from **49** that is highly diffusible, and which induces toxicity in the surrounding cells. Furthermore, the combination of **49** and RT gave rise to two-fold increase in hypoxia-selective toxicity *in vivo* against RIF-1 tumours compared with the prodrug itself.

Tercel *et al.*²³¹ subsequently reported a series of analogs of **49** wherein different electron withdrawing groups (EWGs) were introduced in an effort to raise the one-electron reduction potentials and improve the cytotoxicity. In an independent investigation, the same researchers conducted detailed solvolysis and NMR spectral studies to validate the formation of short-lived DNA alkylating intermediate **50** (Fig. 11), which is presumably an active species formed during bioreduction of prodrugs **48** and **49**.²³²

The most promising analogue contained a sulfonamide ($-\text{SO}_2\text{NH}_2$) group at the 7-position and a DEI side chain (**51**, Fig. 11). *In vitro* studies with two human tumour cell lines, ovarian carcinoma SKOV3 and colon carcinoma HT29, yielded HCRs of 275 and 330, respectively. These values are quite high relative to those displayed by the well-established HAPs, TPZ (discussed in later sections)²³³ and compound **43** (PR-104)²³⁴ under similar experimental conditions. Unfortunately, poor aqueous solubility limited the study of sulfonamide **49** *in vivo*.

To improve the water solubility, a phosphate analogue of **51** (*i.e.*, **52**, Fig. 11) was synthesized.²³⁵ Prodrug **52** readily undergoes phosphate group hydrolysis to generate the corresponding alcohol, followed by bioreduction to generate a strong DNA alkylating agent. *In vitro* studies revealed that **52** is about 150-fold more potent in eradicating colony-forming human cervical carcinoma cells (SiHa) under hypoxic conditions as compared to normoxic ones. *In vivo* studies performed with mice bearing SiHa tumours revealed that prodrug **52**, when administered in combination with high-dose radiation, completely eliminated the tumour burden in three out of five mice tested. Prodrug **52** was also found to exhibit high selectivity in other human xenografts models (*e.g.*, colon, cervix, and lung).

Denny *et al.*²³⁶ also investigated the effect of the leaving group by preparing a series of nitro-chloromethylbenzindoline (nitro-CBI) prodrugs containing sulfonate leaving groups and comparing their cytotoxicity profiles and HCRs to the corresponding chloro-analogues. In the SKOV3 and HT29 cell lines under both aerobic and hypoxic conditions, the sulfonate analogues with a neutral side chain 5,6,7-trimethoxyindole (TMI) displayed higher cytotoxicities than the chloro derivatives. *In vitro* studies with the most effective prodrug (*i.e.*, **54**, Fig. 11) revealed a large variation in the HCR values that were found to depend upon the choice of the cell line. For instance, the HCR values varied from 246 for the SKOV3 cell line to 3.6 in the HT29 cell line. This difference was first assumed to arise from the different expression levels of P-450R across these cell lines. However, the absence of an

observable correlation between P-450R expression levels and IC₅₀ values led the authors to exclude this possibility.

In another investigation, Stevenson *et al.*²³⁷ reported a series of nitro-CBI-based HAPs (**55** and **56**, Fig. 11) that contain a sulfonate leaving group with an electron-withdrawing substituent at the 7 position of the naphthyl core. Prodrugs **55** and **56**, which contain a 7-substituted primary carboxamide and sulfonamide, respectively, as well as a neutral side chain (TMI), displayed high *in vitro* toxicity in the HT29 human cancer cell lines with HCR values of 370 and 1010, respectively.

Terrel *et al.* reported a series of nitro-duocarmycins analogues bearing amine-containing side chains of varying p*K*_a (9.64 to 5.24) and evaluated their hypoxia-selective cytotoxicity in various tumour models.²³⁸ It was initially proposed that the presence of additional amine functionality would contribute to overall toxicity by binding to the minor groove of the DNA. *In vitro* cytotoxicity assays conducted in hypoxic HT29 cells revealed that most of the derivatives delivered the cytotoxic aminoCBIs to the cell. Moreover, it was found that the toxicity of these analogues was dependent more on the structural features than on the choice of particular basic substituents. The highest hypoxia potencies and largest HCR values were found in the case of the derivatives bearing the most basic chains. However, *in vivo* studies conducted either as a single agent or in combination with chemotherapy (gemcitabine or docetaxel) revealed that the highest cytotoxicities were observed in the case of the analogues carrying weak basic side chains. Compared with compound **52**, compound **53** (Fig. 11) displayed efficacy in a resistant H460 tumour model and also proved efficacious towards a hypoxic prostate cancer 22Rv1 xenograft (14 mmol kg⁻¹) when administered as a single dose in combination with docetaxel (32 mmol kg⁻¹) (*n* = 6–7). Moreover, **53** proved capable of eliminating detectable clonogens of radioresistant SiHa cervical tumours when used in conjunction with a single dose of gamma radiation. Lead **53** was also tested for enhanced therapeutic effects in A2780 ovarian xenograft models with gemcitabine (anticancer drug) in a multi-dose treatment schedule. In this study, 3 out of 7 tested animals were found completely tumour free at 100 days following treatment.

4.3. Nitroaromatics as hypoxia-activated prodrugs with drug elimination

In recent years, a modular concept for HAP design that relies on prodrug activation and active species release under hypoxic conditions has attracted considerable interest in the area of chemotherapeutic drug design.²³⁹ The approach is appealing since in principle it may be used to enhance the favourable biodistribution of drugs and reduce off-target toxic effects by promoting the selective release of an active drug form within tumour tissues.

Denny *et al.*²⁴⁰ reported the benzylic quaternary ammonium mustard, SN 25246 (**57**), as a new potential class of HAPs (Fig. 12). This prodrug releases the highly cytotoxic agent methylbischloroethylamine (**58**, mechlorethamine; an aliphatic nitrogen mustard) upon one-electron reduction of the nitro group under hypoxic conditions followed by fragmentation. *In vitro* studies served to confirm that prodrug **57** displayed significant selectivity (200-fold) against hypoxic UV4 cells. The relatively long half-life of **58** (*ca.* 15 min) allows prodrug **57** to exert a strong bystander effect. Support for this conclusion came from the finding that

prodrug **57** displayed greater cytotoxicity against intact EMT6 spheroids than against dissociated ones.²⁴¹ Unfortunately, the prodrug showed only marginal cytotoxicity *in vivo* against hypoxic cells in KHT tumours.

Wilson *et al.*²⁴² reported a detailed mechanistic study of the release of mechlorethamine (**58**) from nitroarylmethyl quaternary prodrugs that involved the use of steady-state radiolysis combined with high-performance liquid chromatography (HPLC). On the basis of their findings, the authors suggested that **57** undergo multiple electron reduction processes before releasing mechlorethamine (**58**) through fragmentation. As part of this investigation, two heterocyclic analogues **59** (SN 25341, 1-methyl-4-nitro-5-imidazolyl derivative) and **60** (SN 26581, (1-methyl-5-nitro-2-pyrrolyl derivative) were identified (Fig. 12). Unlike **60**, these latter agents undergo clean radiolytic one-electron reductive fragmentation to release a high dose of mechlorethamine. A dinitroimidazole (DNBI) dimer, which arises from the arylmethyl radical, is also produced. Within this set of derivatives, the imidazole **59** was found to display strong hypoxic selectivity in EMT6 cells and RIF-1 cells.²⁴³ Prodrug **59** also produced promising *in vivo* results in RIF-1 tumour models when tested in combination with either RT or cisplatin. However, these findings were tempered by unpredictable systemic toxicities and nonspecific release of **58**.

Another HAP agent to advance into clinical studies is TH-4000 (**61**, Fig. 12). TH-4000, termed tarloxotinib, is a bioreductive pan-HER inhibitor. In most cancers, hyperactivation of HER family receptors is common and leads to downstream upregulation of several signaling pathways, such as MAPK, PI3K/AKT and JAK/STAT, that are closely linked with tumour progression and metastasis. TH-4000 (**61**) undergoes a one-electron reduction under hypoxia conditions resulting in a nitro radical that subsequently releases an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI).²⁴⁴ The tyrosine kinase inhibitor in question, erlotinib, is used clinically for treating NSCLC patients.²⁴⁵ Promising preclinical results were obtained with TH-4000,^{246,247} and mechanistic studies conducted on panel of NSCLC cell lines revealed that **61** is metabolized under hypoxic conditions. It was also found to be more effective than the parent drug erlotinib in both wild-type as well as EGFR-mutant xenografts. Subsequently, clinical trials with **61** were conducted in NSCLC, HNSCC, and skin sarcoma cell carcinoma (NCT02454842 and NCT02449641). Encouraging treatment responses were seen in the case of the skin carcinoma patients. However, the trial was terminated due to insignificant responses in patients suffering from HNSCC and NSCLC.

Nagasaki *et al.*²⁴⁸ proposed two novel HAPs, namely **62** and **63** (Fig. 12). These conjugates contain doxorubicin and 5-FU linked to a 2-nitroimidazole core through an amide linkage designed to prevent undesirable drug release before the hypoxic region is reached. Compared to the parent drugs, these HAPs displayed drastically improved penetration in the hypoxic region, a finding ascribed to their increased hydrophobicity and metabolic stability. In the hypoxic region, cytotoxic drug release from **62** and **63** was thought to involve the formation of a 6-membered cyclic structure. *In vitro* studies led to the suggestion that both pro-drugs are highly cytotoxic in the MIA PaCa-2 cell line, particularly under hypoxic conditions. An investigation of the *in vivo* anti-tumour activity of **62** in colon 26 tumour-

bearing mice revealed an improved survival rate relative to doxorubicin alone. A reduction in adverse events was also noted.

Hay *et al.*²⁴⁹ reported an alternative approach to delivering cytotoxins to hypoxic cells. In this case, a nitroimidazole moiety was conjugated to a 4-carboxamidophenyl nitrogen mustard (as the cytotoxic agent) *via* the N1 side chain; this gave HAP **64** (Fig. 12). Reduction of the nitro group favours intramolecular cyclization, resulting in the formation of a tetrahedral intermediate that collapses and expels the active nitrogen mustard. An *in vitro* cytotoxicity assay using the AA8 cell line revealed that conjugate **64** was 3.3-fold more toxic under hypoxic than normoxic conditions.

TH-302 (**65**, evofosfamide) is a HAP that consists of a brominated analogue of isophosphoramidate mustard (Br-IPM) linked to a 2-nitroimidazole moiety (Fig. 12). The proposed mechanism of action for **65** includes reduction of the nitro group by one-electron oxidoreductases under hypoxic conditions. This is followed by fragmentation to release the bromoisophosphoramidate mustard, a species that is recognized as being a potent DNA alkylating agent. Prodrug **65** was first synthesized and evaluated preclinically by Duan *et al.*,²⁵⁰ and was reported to be 400-fold more cytotoxic in the H460 human non-small-cell lung cancer cell line under hypoxic conditions than normoxic conditions. *In vivo* studies in mouse xenograft models bearing MIA PaCa-2 human pancreatic cancers^{251,252} revealed that **65** possessed high antitumour efficacy when administered either alone or in combination with gemcitabine. It was found to extend survival relative to controls with one out of eight of the test animals remaining tumour free at day 44. Other enhancements in chemotherapeutic benefit were reported.²⁵³ For instance, it was found that treating nude mice bearing human breast MCF-7 or prostate PC-3 tumour xenografts with **65** 4 h before the subsequent administration of doxorubicin or docetaxel significantly enhanced the anticancer effects of these classic chemotherapeutics. This proved true both in the perivascular and hypoxic regions. On the basis of this and other promising preclinical studies, **65** advanced into clinical trials and was tested as both a monotherapy and in combination with other chemotherapy regimens in more than 1500 patients. Early on, **65** provided promising results in phase II clinical trials in combination with doxorubicin (soft tissue sarcoma)^{254,255} and gemcitabine (pancreatic cancer).²⁵⁶ However, hematologic toxicity was observed in the case of the doxorubicin trial. Recently, two large phase III clinical studies of **65** were carried out in combination with other anticancer drugs; these were focused on soft tissue sarcoma (NCT0144088) and advanced pancreatic cancer (NCT01746979) patients. Unfortunately, little improvement in the overall survival was seen.

Lu *et al.* developed compound **66** (Fig. 12) as a hypoxia responsive prodrug of SN-38.²⁵⁷ As compared with SN-38 [HCR = 0.74 (in HT29 cancer cells), 0.83 (in H460 cancer cells)], **66** displayed slightly improved HCR in the HT29 (3.82) and H460 (3.71) cancer cell lines.

Nitroaromatics have been widely used to prepare hypoxia selective prodrugs. In hypoxic environments, the NO₂ unit is readily reduced to the corresponding aniline species. Often this is used to unlock the active cytotoxin. The 4-nitrobenzyl moiety has received considerable attention in this regard. It has been widely investigated as a so-called self-immolative linker in various prodrug designs, as well as in the context of other therapeutic

modalities, including antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT) development. Enzymes that metabolize the aromatic nitro groups are particularly important in the context of these efforts owing to the large electronic changes that are produced as a result. Reduction of the nitro group (Hammett σ_p electronic parameter = 0.78) produces either hydroxylamine ($4e^-$ reduction, $\sigma_p = -0.34$) or aniline ($6e^-$ reduction, $\sigma_p = -0.66$), which then undergo a spontaneous 1,6-elimination to release the active drug (Fig. 13). Most ADEPT and GDEPT studies relying on this mechanism have exploited the nitroreductase codified by the *nfsB* gene of *E. coli*, an oxygen-insensitive flavin mononucleotide (FMN) nitroreductase (NTR) having a close sequence homology to the NTR of *S. typhimurium*.²⁵⁸ Single crystal structural studies revealed that this NTR is a homodimer with one FMN unit per monomer, with two channels leading to the active site. There are few contacts with the ligands that can contribute to its observed substrate selectivity.²⁵⁹ NTRs efficiently reduce the nitroaromatics to hydroxylamines in a two-step ping-pong bi-bi mechanism.²⁶⁰ The following examples illustrate the use of the 4-nitrobenzyl moiety as a basis for creating hypoxia selective prodrugs.

Borch *et al.*²⁶¹ employed a 4-nitrobenzyl linker to generate the nitrophenyl phosphorodiamidate prodrugs **67** and **68** (Fig. 13). Prodrug **68**, with an α -methyl substituent, was found to be selective to HT-29 cells with an aerobic/hypoxic toxicity ratio of approximately 90. An *ex vivo* assay revealed toxicity to murine bone marrow granulocyte/macrophage progenitors (GM-CFC) that was comparable with the hypoxic toxicity against HT-29 cells. This led to the conclusion that another mechanism for drug activation exists in addition to the expected nitro group bioreduction-based pathway the linker was designed to exploit.

Other nitrobenzyl-based HAPs, **69** and **70** (Fig. 13), were described by Sartorelli *et al.*²⁶² Upon enzymatic reduction, both of these agents led to the formation of 90CE (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine), an antineoplastic DNA crosslinking cytotoxin.^{263,264} Mechanistic studies revealed that **69** (PNBC) could reductively release the cytotoxin 90CE by either enzymatic nitro reduction or nucleophilic attack by thiols such as GSH or glutathione *S*-transferase (GST), which are often overexpressed in tumours. In contrast, prodrug **70** (KS119) appeared to be activated exclusively *via* nitro group reduction. Presumably, nucleophilic attack by a thiol is suppressed for steric reasons as the result of the methyl group on the methylene carbon spacer. *In vitro* cytotoxicity studies revealed that prodrug **70** was more potent than **69** with high hypoxia selectivity being seen in the EMT6 tumour cell line. Similar promising results were observed in CHO K1 dhfr parental cells. However, **69** was less efficacious against hypoxic cells and also displayed some toxicity in aerobic cells, presumably as a result of GST thiol activation. An analogue, HAP **71** (Fig. 13),²⁶⁵ was designed to deliver 6-thioguanine (6-TG), an antileukemic drug, deep into solid tumours. *In vitro* studies with A549 cells, which are known to express a high level of P450 reductase,²⁶⁶ led to the suggestion that 6-TG is released efficiently under anoxic conditions in the case of **71**.

The therapeutic effect of a majority of the bioreductive prodrugs described above occurs through the release of DNA-damaging agents. As a consequence, there is a risk that using

these prodrugs in combination with standard chemotherapies may increase the possibility of off-target toxicities. An alternative approach, which represents a significant departure from the above-mentioned mechanism, involves a strategy wherein an active drug that acts as a strong inhibitor of a protein or enzyme required for tumour progression, is masked by a hypoxia-responsive linker.

Hammond *et al.*²⁶⁷ utilized this approach to create the bioreductive prodrug **72** (Fig. 13), which contains a checkpoint kinase 1 (Chk1)/Aurora A inhibitor. Chk1/Aurora A is serine/threonine-specific protein kinase that may promote tumour growth, as inferred from the positive correlation of Chk1 expression and tumour grade.^{268,269} The inhibitor in **72** was rendered inactive by tethering it to a nitrobenzyl group. Reduction of the nitro group prompts release of the active Chk1/Aurora inhibitor. Presumably as a result, prodrug **72** was found to display high potency against severely hypoxic cells *in vitro*.

A number of alkylating agents mediate their antitumour effect by damaging DNA through guanine O-6 alkylation. However, this DNA damage is repaired by the protein O6-alkylguanine-DNA alkyltransferase (AGT). This enzyme transfers alkyl groups from an alkylated guanine O-6 position to the cysteine residue in its active site.²⁷⁰ Therefore, it follows that a high level of AGT increases resistance to guanine O-6 alkylating agents.²⁷¹ O-6-Benzylguanine (O6-BG, **75**, Fig. 13) is a known inhibitor of AGT. It deactivates the enzyme through the formation of an S-benzyl cysteine derivative in its active site. It has also been found to sensitize cancer cells to alkylating agents.^{272,273} Unfortunately, O6-BG not only sensitizes cancer cells, it also potentiates normal cells. To address this latter limitation, Penketh *et al.*²⁷⁴ utilized a prodrug strategy to develop conjugates **73** and **74** (Fig. 13). In **73** and **74**, the 2-amino group, which is essential for the AGT inhibitory activity of **75**, is protected in the form of a carbamate. Bioreduction of the nitro group by NADPH:cytochrome P-450 under hypoxic conditions facilitated the unmasking of the amino group. This allowed the site-specific release of O6-BG within hypoxic regions and served to reduce aerobic toxicity. Furthermore, *in vitro* studies performed against DU145 human prostate carcinoma cells led to the suggestion that **74** (40 μ M) sensitizes DU145 cells to the levels of cytotoxicity displayed by larmustine (an alkylating agent) under hypoxic, but not normoxic conditions.

In another report, Saneyoshi *et al.*²⁷⁵ described the synthesis of an oligonucleotide-based HAP, **76** (Fig. 13), in which the thymine base is protected using a hypoxia-labile nitrobenzyl linker. Under hypoxic conditions, deprotection of the thymine group serves to convert **76** to an active oligonucleotide that is able to form stable duplexes with target oligonucleotides, thereby inhibiting mRNA translation.

In separate work, Minutolo *et al.*²⁷⁶ developed a bioreductively activatable inhibitor **77** (Fig. 13) of lysyl oxidase (LOX). This enzyme is overexpressed in hypoxic tumour cells and promotes the metastatic spread of several solid tumours.²⁷⁷ As inferred from an *in vitro* invasion analysis, prodrug **77** exerts a 6-fold higher anti-invasion effect in breast cancer MDA-MB-231 cells under hypoxic conditions than it does under normoxic conditions.

In an effort to develop a hypoxia-responsive paclitaxel pro-drug, Scheeren *et al.* designed a series of paclitaxel analogues conjugated with various nitro/azo-based functional groups. All the compounds generated in the context of this study exhibited good stability in Tris buffer. Moreover, a selected lead, compound **78** (Fig. 13), showed diminished aerobic toxicity in various human cancer cell lines (H226, MCF7, EVSA-T, WIDR, IGROV, MI9, and A498).²⁷⁸

Recently, Kim *et al.*²⁷⁹ developed the hypoxia-activated conjugate **79** (Fig. 14) for the diagnosis and treatment of solid tumours. This system contains a cancer-localizing biotin subunit, the anticancer drug SN38, and 4-nitrobenzyl (a hypoxia-responsive self-immolative linker). In accord with the design expectations, conjugate **79** showed a preference for cancer cells. This preference was ascribed to the cancer-targeting ability of biotin. Activation of **79** by nitroreductase (NTR, *E. coli*), particularly under hypoxic conditions, was expected to promote release of SN38 through 4- or 6-electron reductive elimination of the 4-nitrobenzyl linker. Prodrug **79** displayed high selectivity for, and greater cytotoxicity against, A549 and HeLa cells (biotin-positive) under hypoxic conditions compared to BJ and WI-38 cells (normal cells). *In vivo* studies involving HeLa-inoculated xenograft mouse models served to confirm that **79** accumulates specifically in these particular solid tumours. Its activation under conditions of hypoxia produced a strong anticancer effect, a finding ascribed to the inhibition of the growth factor HIF-1 α .

Theranostic **80** (Fig. 14), based on a nitrobenzyl trigger, was developed by Zhang *et al.*²⁸⁰ This dual action agent was designed to release selectively both the anticancer drug gemcitabine (GMC) and a coumarin (CM) fluorescent probe following hypoxic bioreduction, photo-activation, and demasking in sequence. Evidence in support of this proposition came from the observation of enhanced CM-derived fluorescence under conditions of expected release. In fact, the levels of discharged drug were found to reflect the extent of hypoxia and the exposure time. Further support for this conclusion came from cell viability data involving the MCF-7 cell line studied under hypoxic conditions.

Angiogenesis, a tightly regulated process for new blood vessel formation in solid tumours, can influence the activation and therapeutic efficacy of hypoxia responsive prodrugs. Hence blocking angiogenic pathways in combination with hypoxia responsive prodrugs could be beneficial. Recently, Kim *et al.* developed a theranostic **81** (Fig. 14), where a nonsteroidal anti-inflammatory drug (NSAID) indomethacin (COX-2 inhibitor) was utilized for two separate and complementary purposes *i.e.*, cancer targeting as well as angiogenesis inhibition.²⁸¹ Compared with a control analogue lacking indomethacin unit (SN-38, **81**), theranostic **81** exhibited preferential targeting ability and cytotoxicity in COX-2 positive cancer cells (HeLa, A549) as compared to normal COX-2 negative cells (WI-38 and BJ) under hypoxic conditions. Further, **81** showed prolonged tumour retention and improved therapeutic response in HeLa inoculated xeno-graft animal models. The improved therapeutic efficacy of **81** compared to SN-38 and other controls was ascribed to its both cancer-targeting ability and COX-2 knockdown mediated anti-angiogenesis ability, as confirmed by decreased expression of angiogenic genes (VEGFA, CD31, ANGPT v2, and ANGPT v3) in tumour tissues.

4.4. Azoaromatics as hypoxia-activated prodrugs with drug elimination

The overwhelming majority of bioreductive prodrugs reported to date have been based on the use of quinones, nitroaromatics, and N-oxides as the initial release triggers. Considerably less work has been devoted to incorporating redox active azo groups into hypoxia-sensitive prodrugs.^{282,283} In contrast, the use of azo moieties to create hypoxia-responsive imaging probes is relatively well developed.²⁸⁴ Baumann *et al.*²⁸⁵ reported 6-(benzyloxy)-2-(aryldiazenyl)-9H-purine **77** (Fig. 15) as an azo-based pro-drug of the AGT inhibitor, O6-BG (**75**, Fig. 13), discussed above.²⁷³ Hypoxia-driven azo-reduction of **82** by AGT-expressing DU145 cells resulted in the release of the active inhibitor O6-BG in excellent yield. This release, in turn, served to enhance the cytotoxic effect of laromustine in DU145 cells.

Kim *et al.*²⁸⁶ explored the use of an azobenzene scaffold to create the hypoxia-selective theranostic **83** (Fig. 15). This prodrug specifically targeted the mitochondria in cancer cells where it was expected that hypoxia-mediated reduction of the azobenzene moiety would result in release of *N,N*-bis(2-chloroethyl)-1,4-benzenediamine, a DNA alkylating agent, as well as a fluorescent rhodamine analogue. Theranostic **83** showed good *in vitro* cytotoxicity against the DU145 and MDA-MB-231 cell lines under hypoxic conditions (3% O₂). Furthermore, *in vivo* and ex vivo studies involving xenografted mouse models revealed a significant reduction in tumour burden, along with a decrease in angiogenic markers and suppressed cell proliferation. As prepared **83** is non-fluorescent. However, hypoxia activation *via* azo-reduction leads to release of rhodamine, a fluorescent moiety that allowed for spectroscopic-based monitoring of the release process. Recently, azo-based prodrugs, **84** and **85** were also developed and tested for both hypoxia selective *in vitro* and *in vivo* anticancer activity (Fig. 15).^{287,288}

Most early efforts centered around prodrug activation strategies involving enzymatic activation. However, in 2008, Robillard *et al.*²⁸⁹ proposed an alternative chemical-based route wherein the selective Staudinger reaction was used to achieve prodrug activation. This strategy is embodied in prodrug **86** (Fig. 15), which undergoes hydrolysis spontaneously under aqueous conditions followed by self-immolation to release free doxorubicin. Prodrug **86** displayed a 176-fold higher IC₅₀ compared to free doxorubicin in tests involving the A431 human vulvar skin squamous carcinoma cell line.

Recently, Cheng *et al.* developed a heteromeric prodrug **87** (Fig. 15) containing two chemotherapeutic drugs, doxorubicin and nitrogen mustard, linked through an azo moiety.²⁹⁰ Compound **87** displayed good hypoxia responsive toxicity in 4T1-tumour bearing mice models as compared with controls (PBS, doxorubicin) with minimal side effects.

In another investigation, Urano *et al.*²⁹¹ has reported the development of activatable photosensitizer **88** (Fig. 15), which relies on an azo subunit linked to a conjugated selenorosamine dye. Compound **88** was not found to generate singlet oxygen when irradiated under normoxia condition, a result ascribed to the fact that in the excited state, the system undergoes a very fast rotation around –N=N– bond thus reducing the intersystem crossing needed to generate the triplet state and promote formation of singlet oxygen. However, even under mildly hypoxic condition reduction of the azo group occurs. This

activates the photosensitizer leading to a significant singlet oxygen quantum yield. *In vitro* cytotoxicity studies further demonstrated that compound **88** is highly selective in that it induces the photo-dynamic ablation of human lung cancer-derived A549 cells much more effectively under hypoxic as opposed to normoxic conditions.

5. Metal-based therapeutics

The majority of dynamic pharmaceutical drug ingredients are organic and biologically driven compounds. Consequently, an enormous effort has been devoted to making and testing potential organic chemistry-based drug leads. However, the serendipitous discovery of cisplatin and its eventual approval as a therapeutic in 1978 has changed the cancer treatment landscape over the last 40 years. It cannot be overstated that cisplatin and carboplatin are two of the most important anti-tumour agents in the clinic.²⁹² They are used as front line therapies by oncologists, with roughly half of all patients undergoing chemotherapy receiving one of these complexes, alone or in combination with other agents.²⁹³ Unfortunately, despite their success and history, small molecule platinum agents suffer from multiple limitations: (1) lack of tumour selectivity, (2) high systemic toxicity, (3) multiple mechanisms of resistance (inborn and acquired) and (4) a narrow spectrum of activity.²⁹⁴ Overcoming such limitations has been an interdisciplinary challenge.^{294,295} In cancers such as NSCLC, mesothelioma, and ovarian, these limitations are manifest in low 5-year survival rates of 5–45% in patients treated with cisplatin and carboplatin as frontline therapies.

The success and limitations of cisplatin has prompted an ongoing search for yet-improved new metal-based drug candidates.^{296–298} In contrast with organic-based drugs, metal-based drug candidates possess potential advantages, such as an ability to adjust their respective geometries, vary their coordination numbers, and undergo changes in redox states. One of the most promising avenues is the use of metal-based prodrugs to target tumour hypoxia. The canonical rationale behind this approach is as follows: cytotoxic agents may be tethered to metal complexes that are stable in relatively high oxidation states under aerobic conditions. This imparts stability to the active cytotoxic agents and shields them from rapid metabolism. Upon bioreduction, the metal complexes are reduced to a lower oxidation state, became relatively unstable, and release the active form of the cytotoxic agent.

5.1. Cobalt-based therapeutics

A number of cobalt complexes have been explored as hypoxia-sensitive prodrugs. In general, Co(III) complexes are moderately inert, adopt octahedral geometries (low spin, d_6). Upon bio-reduction, the complexes are reduced to their more labile Co(II) forms. As a general rule, Co(II) complexes are re-oxidized back to Co(III) complexes in the presence of molecular oxygen. However, in hypoxic environments, the labile Co(II) forms (high spin, d_7) can dominate, allowing release of the active drug.^{299,300} The requisite bioreduction processes are typically mediated by endogenous reductases, such as P-450R and xanthine oxidase (XO). This assumes the reduction potential of the Co(III) complex is compatible with that of the cytosolic environment (–200 mV to –400 mV).³⁰¹ In addition, ligand alterations can

influence the reduction potential of the Co(III) complexes. This fine-tuning has inspired the synthesis of a variety of promising hypoxia selective prodrug frameworks (Fig. 16).

In 1987, Teicher *et al.* reported that Co(III) complexes containing both nitro and amine ligands served as promising radio-sensitizers toward hypoxic mammary carcinoma cells (EMT6).³⁰² Further investigations of Co(III) complexes containing acetylacetonate (acac) and a mustard, bis(2-chloroethyl)amine (bca), revealed significant cytotoxicity towards murine cancer cells.³⁰³ A specific complex of this class, [Co(acac)₂(NO₂)(bca)] (**89**, Fig. 17, Co^{III}-bca), proved inactive in its oxidized state. This inactivity was ascribed to the fact that the lone pair of the nitrogen is coordinated to the cobalt center. This potential nucleophilic center can thus not participate in an intra-molecular cyclization, which is deemed necessary to produce the active aziridinium species considered responsible for DNA alkylation-induced cytotoxicity. Upon reduction, the nitrogen mustard is released from the more labile Co(II) form of the complex, as evidenced by cytotoxicity being observed in hypoxic mammary carcinoma cells (EMT6). Unfortunately, screening assays on SCC-25/HN2 cells revealed that **89** underwent overly facile reduction, obviating its presumed bioreduction-mediated selectivity.

Co(III) complexes containing bidentate mustards *N,N'*-bis-(chloroethyl)ethylenediamine (bce) and *N,N'*-bis(chloroethyl)-ethylenediamine (dce) were also investigated.^{304–306} *In vitro* analyses revealed that the cytotoxicity of Co^{III}-dce and Co^{III}-bce (**90** and **91**, Fig. 17) resulted from the released mustard agents only. Further SAR studies provided support for the conclusion that in addition to mustard agents, the acetoacetate ancillary ligand also played a crucial role in mediating the observed hypoxia selectivity. Compared with the unsubstituted acetylacetonate incorporated Co(III) complexes, the corresponding methyl and ethyl analogues displayed substantially improved hypoxia selectivity. Furthermore, a lead complex that emerged from these efforts, [Co(Meacac)₂dce]⁺, **SN 24771**, was found to exhibit cytotoxicity against Chinese hamster ovary fibroblast cells (AA8, UV4) that was enhanced by 6-fold under hypoxic conditions. Significant selectivity was also observed against multicellular spheroids in solid tumours. The mechanism for hypoxia selectivity was investigated in detail using pulse and steady state radiolysis techniques and led to the suggestion that more complex processes were involved than the expected redox cycling.³⁰⁷ A Co(III) complex with tridentate mustard, *N,N'*-bis(2-chloroethyl)-diethylenetriamine, Co^{III}-dcd (**92**, Fig. 17) was also developed with the hope of enhancing the metabolic stability.³⁰⁸ Despite exhibiting modest hypoxia selectivity, the potency was significantly reduced (up to 20-fold) compared to the corresponding bidentate Co(III) analogs.

A series of Co(III) complexes with various ancillary ligands (bis-tropolonate, anionic carbonato, and oxalato) were used to investigate in systematic fashion the impact of these ligands on the metabolic stability of the complexes (Fig. 18).^{309–311} The cobalt bis-tropolonate complex, Co^{III}-dce-tropolonate2 (**93**), exhibited a relatively high reduction potential but a toxicity profile comparable to that of free mustard against UV4 cells and the Chinese hamster ovary fibroblast subline AA8, without any marked hypoxic selectivity. The corresponding anionic Co(III)-dce complexes [Co^{III}-dcecarbonato2 (**94**) and Co^{III}-dce-oxalato2 (**95**)] exhibited lower toxicity compared with the free *N,N'*-bis(2-chloroethyl)-1,2-ethanediamine (dce) species and possessed lower hypoxia selectivity compared with the

corresponding acac complex. The hypoxia-selective cytotoxicity of these family of analogues (Fig. 17 and 18) was investigated by pulse radiolysis techniques. These studies led to suggestion that upon bioreduction, the bidentate alkylating mustards and the acac ligands are released giving rise to a Co(II)–hexaaqua complex. The observed hypoxia-selective cytotoxicity of the most promising Co(III) complexes was thus considered to be the result of competition between molecular oxygen and the reactivity of the corresponding Co(III) species towards intracellular reductants.

Using the same general reduction-based release strategy, Hambley *et al.*³¹² created a Co(III)–tris-(2-methylpyridyl)amine complex designed to deliver matrix metalloproteinase (MMP) inhibitors specifically at hypoxic tumour sites. MMPs are a diverse class of zinc containing endopeptidases, which play a vital role in many biological processes, including arthritis, cancer progression, and tissue ulcerations. So far, several MMP-selective inhibitors have been reported for the treatment of cancer; however, the mixed preclinical and clinical responses have limited their potential utility.^{313–315} One of the perceived impediments is the presence of hydroxamic functionality in the structural designs of the most potent MMPs inhibitors reported to date. The hydroxamic group, which is exceptionally and non-specifically reactive toward various biomolecules (especially metal centres), is thought to diminish the drug efficacy. To overcome this constraint, Hambley and coworkers coupled a Co(III)–tris(2-methylpyridyl)amine (tpa) complex to MMP inhibitors *via* a hydroxamic acid moiety (**96**, Fig. 19). The goal was to minimize side reactions in well oxygenated regions prior to drug activation at hypoxic tumour sites. Initial structural and electrochemical investigations of Co(III)–tpa complexes with alkyl and aryl hydroxamic acid ligands as MMP inhibitors provided support for the design hypothesis, namely that Co(III)–complexes could be used to transport hydroxamic acid-containing compounds specifically to hypoxic tumour sites.³¹⁶ In the context of these studies, a Co(III)–tpa complex with marimastat (a potent MMP inhibitor) was prepared and tested (Fig. 19). *In vivo* toxicity studies provided results inconsistent with the conclusion that the Co(III)–tpa–marimastat complex, **96** was relatively more effective at reducing the growth of implanted 4T1.2 tumours in mice compared with free marimastat. Later, Co(III)–tpa complexes containing both hydroximate and hydroxamate forms have been developed. Interestingly, both protonated forms exhibited different reduction potentials and thus varying abilities to release bound hydroxamic acid groups. As most tumours are both acidic and hypoxic, this feature was noted as one that could be manipulated to develop new tumour-specific Co(III) complexes for use in delivering drug candidates to cancerous lesions.³¹⁷

Other Co(III) complexes composed of tetradentate ligands, including 8-hydroxyquinoline (8-HQ, **97**, **98**) and azachloromethylbenzindoline (azaCBI, **99**, **100** & **101**) as biologically active agents, have been investigated (Fig. 19).^{318,319} Both Co(III) complexes undergo efficient reduction through radiolytic and nonradiolytic pathways in hypoxic cells. Interestingly, the **99** complex displayed a 20-fold increase in cytotoxicity against HT29 cells in hypoxia environments relative to the same cells cultured under normoxic conditions. Further efforts were devoted to the improvement of hypoxic selectivity, complex stability, and solubility. It was found that the incorporation of the dimethylaminoethanol moiety on the pyrroloquinoline ring (**100**) gives rise to an approximately 30- and 40-fold hypoxia

selectivity relative to normoxic conditions in lung cancer cells (A549) and ovarian cancer cells (SKOV3), respectively.³²⁰ Conversely, the incorporation of a 1,7-substituted cyclen as an ancillary ligand with anionic carboxylate, phosphonate, or sulfonate ligands (**101**) resulted in a reduction in the hypoxic selectivity, along with a slight improvement in solubility. Possibly this may be due to the higher lipophilic character of complexes that allows them to enter the cell *via* passive diffusion through the cell membrane. Alternatively, the authors suggested that these complexes may be subject to reduction by outer membrane nitroreductases, thus accounting for the low observed HCRs (hypoxic cytotoxicity ratios).³²¹

A series of Co(III)-azaCBI complexes with cyclam (1,4,8,11-tetraazacyclotetradecane) ligands were designed with the aim of increasing the electron density at the metal center (**102**, Fig. 20). Coordination with the *trans*-like bridged nitrogen atoms within the cyclam serves to enforce a cis coordination about the metal center while enhancing the relative stability of the Co(III) oxidation state relative to the Co(II) form (*i.e.*, decreasing the reduction potential). *In vitro* cytotoxicity studies against a panel of human cancer cell lines confirmed that Co^{III}-azaCBI-cyclam (**102**, R = H) exhibited high HCRs in the range of 81–212 in anoxic environment (in a Pd/H₂ catalyzed anaerobic chamber) as compared to those containing 20% oxygen. Interestingly, the cytotoxicity to **102** was not found to be correlated with one-electron reductase (POR) expression levels, a well-known reductase for hypoxia selective toxins. Moreover, **102** (R = H) displayed poor toxicity against hypoxic and radiation resistant HT-29 tumour models when compared with tirapazamine (TPZ, Fig. 29).³²² The presumed nonenzymatic reductants responsible for the activation of **102** are still unknown, and this presents a major hurdle in the pharmacological application of **102** as a hypoxia-selective cytotoxin.

Curcumin, a natural product derived from *Curcuma longa* (turmeric), a nontoxic yellow pigment, displays a number of biological effects, including anti-inflammatory, antioxidant, ant-proliferative, and antimetastatic activity, both *in vitro* and *in vivo*. However, poor solubility and rapid metabolism has limited the clinical potential of curcumin.^{323–325} Renfrew *et al.*³²⁶ created a Co(III)-based curcumin complex (Co^{III}-Curcumin, **103**) with the aim of improving the aqueous solubility, stability, and preferential uptake in hypoxic tumour regions (Fig. 20). Both fluorescence lifetime imaging and X-ray absorption spectroscopy revealed its reductive activation and ability to release curcumin in various tumour models. Using the same strategy, the hypoxia-activated, tumour targeted delivery of the EGFR inhibitor (ELK-785) was attempted (Fig. 20).³²⁷ *In vitro* and *in vivo* studies of the agent produced for the purpose, Co^{III}-EGFR (**104**), revealed improved antitumour efficacy against A431 xenografts under conditions of hypoxia as compared with normoxic conditions. Encouragingly, **104** produced distinct and reproducible tumour regression in Calu3 xenograft mouse models.

5.2. Ruthenium-based therapeutics

The chemistry of ruthenium (Ru) complexes has been known since the 1950s;³²⁸ however, their antitumour properties were first observed only in the late 1960s.³²⁹ Several Ru metal-based complexes developed to date have displayed promising toxicity profiles against various cancer cell lines, both *in vitro* and *in vivo*.^{330–335}

Ruthenium can exist in several stable oxidation states (*i.e.*, II, III, and IV) under physiological conditions, and a minimal energy barrier exists for the interconversion between these different oxidation states. This has made ruthenium complexes attractive as potential drug candidates. To date, Ru(II) arenebased complexes and bio-reducible Ru(III) complexes have engendered considerable interest in this context (Fig. 21). Currently, two Ru(III) complexes [ImH][*trans*-RuCl₄(DMSO)Im] (Im = imidazole) (NAMI-A, **105**) and [Na][*trans*-RuCl₄In₂] (Na = sodium) (NKP-1339, **107**) are in phase II clinical trials (Fig. 22). These complexes display significant cytotoxicity in both *in vitro* and *in vivo* models.^{336–342} However, their exact mechanism of action remains unknown. In **105** and KP1019 (**106**), *i.e.*, [InH][*trans*-RuCl₄In₂] (In = indazole), the octahedral Ru(III) complex produced through synthesis is reduced to a more labile Ru(II) form by ascorbate or GSH under hypoxic conditions, a transformation that is thought to result in increased DNA adduct-induced toxicity.³⁴³ However, in spite of being similar in structure, these two complexes exhibit different biological activity.^{344–348} Generally, the antitumour activity of these and other higher valent ruthenium complexes appears correlated with the ease of bio-reduction to the corresponding Ru(II) forms.³⁴⁹

Keppler *et al.* reported a homologous series of [Ru^{III}Cl_(6-n)(ind)_n]⁽³⁻ⁿ⁾ (*n* = 0–4; ind = indazole) complexes designed to improve the hypoxia-sensitive response. Collectively, electro-chemical studies and *in vitro* assays revealed that tuning the reduction potential of the Ru(III) derivative through structural modification resulted in a two-fold enhancement of cytotoxicity in the colon cancer (SW480) and ovarian cancer (CH1) cells.³⁵⁰ Likewise, Clarke *et al.* reported that two ruthenium-based anticancer complexes, *cis*-[Ru(NH₃)₄Cl₂]Cl (CCR) and (ImH)*trans*-[RuCl₄(Im)₂] (ICR), displayed enhanced DNA–Ru adduct formation produced and toxicity against HeLa cells that was 150-fold higher under hypoxic, as compared to normoxic conditions.³⁴⁹

In another comparative study, the chemically reduced form of **105** (produced by ascorbic acid, cysteine, or GSH reduction) was found to display greater tumour regrowth inhibition than the oxidized form of **105** in mammary carcinoma (MCa)-bearing mice.³⁵¹ Further support for the “activation by reduction” hypothesis being critical to the action of Ru(III) antitumour complexes came from the finding that **106** displayed an enhanced affinity for GMP upon treatment with two equivalents of GSH. However, increasing the GSH levels further (up to 10-fold), led to a reduction in the GMP binding affinity owing to the formation of a GSH–Ru adduct.^{352,353}

The promising activity exhibited by **106** led to the development of an analogue, NKP-1339 [sodium *trans*-[tetrachloridobis(1*H*-indazole)ruthenate(III)], **107**], which was prepared in an effort to enhance the water solubility of the parent system (Fig. 22).³⁵⁴ Comparatively strong tumour targeting was seen for NKP-1339, an effect ascribed to an ability to bind to serum proteins (albumin and transferrin) coupled with reductive activation within the reducing tumour environment. Detailed mechanistic and cytotoxicity studies revealed that the reduction of the Ru(III) center was favored under hypoxic conditions and the resulting complex perturbed the cellular redox balance and induced apoptosis.^{355,356} A phase I clinical study involving 16 patients revealed that **107** is well-tolerated (dose = 20–420 mg m⁻²) in patients with various solid tumour types (lung, neuroendocrine, colon, ovarian,

cervical, pancreatic, and head and neck cancer) (NCT01415297). Two patients with stage IV neuroendocrine tumors were noted as having experienced disease stabilization. Complex **107** was found to downregulate GRP78 (protein mis-folding processing regulator) activity. Enhanced GRP78 activity is seen in many tumours and has been associated with both intrinsic and conventional chemotherapy-induced drug resistance. In a study reported in 2012, complex **107** was also found to be potent against many tumour types, including those resistant to standard anticancer drugs.³⁵⁷ Further clinical studies of this agent thus appear warranted.

The preclinical anticancer activity of **107** was further studied in combination with sorafenib (a multi-kinase inhibitor, Nexavar™). Nexavar targets diverse oncogenic and pro-angiogenic receptor tyrosine kinases, including vascular endothelial growth factor receptor (VEGFR-1/2/3), platelet-derived growth factor receptor (PDGFR-1), Raf serine/threonine kinases, and RET proto-oncogene. With NKP-1339, synergistic cytotoxic effects were observed against both sorafenib-resistant (HCC1.1, Hep3B, VL-8 and VM-1) and sorafenib-responsive (PLC/PRL/5, HepG2 and HCC2) cancer cells. Enhanced rates of apoptosis with complete loss of mitotic cell population was seen both *in vitro* and *in vivo* in Hep3B hepatoma and VM-1 xenograft mouse models.³⁵⁸

Mukherjee *et al.* studied a sterically hindered imidazole-based Ru(II)-arene complex [(L)Ru^{II}(η⁶-*p*-cym)(Cl)]PF₆ (L = *N*-((1*H*-imidazol-2-yl)methylene)-2,6-diisopropylaniline), **108** and investigated the mechanism of complex activation, as well as its activity profile in the MCF-7 and A549 cancer cell lines (Fig. 23). This sterically hindered metal complex exhibited high stability in 110 mM NaCl solution, underscoring its expected stability in extracellular matrix and resistance to deactivation through GSH. Interestingly, this complex displayed enhanced cytotoxicity towards the MCF-7 and A549 cell lines under hypoxic conditions, with or without GSH (up to 1 mM) relative to normoxic conditions. This enhanced activity was attributed to the slow rate of complex hydrolysis. Cell cycle arrest in the sub G1 and G2/M phases, as well as apoptosis, was observed after activation.³⁵⁹

Further examples of hypoxia-responsive metal complexes with DNA-based cytotoxicity were reported by MacDonnell *et al.* (Fig. 23).^{360,361} A series of cationic metal complexes in which two ruthenium cations are chelated to tetrapyrido[3,2-*a*:2',3'-*c*:3'',2'''-*h*:2''',3'''-*j*]phenazine (tpphz) (**109**), 9,11,20,22-tetraazatetrapyrido[3,2-*a*:2',3'-*c*:3'',2'''-1:'''',3'''-*n*]pentacene (P⁴⁺, tatpp) (**110**), and 1,10-phenanthroline (phen) (**111**) moieties were synthesized. The monomeric [(Phen)₂Ru(tatpp)]²⁺ (**110**) and dimeric [(phen)₂Ru(tatpp)Ru(phen)₂]⁴⁺ (**111**) complexes, in the form of their chloride salts, exhibited good aqueous solubility. They displayed cytotoxicity with corresponding G2-M cell cycle arrest^{362,363} under both normoxic and hypoxic conditions across a panel of cancer cell lines. *In vivo* studies further demonstrated significant tumour growth inhibition (83%) in human lung cancer H358 xenograft mouse models. Supporting electrochemical studies revealed enhanced hypoxic sensitivity that was attributed to concomitant formation of carbon-based radical species.

Another series of ruthenium and iridium b-ketoiminato complexes exhibiting significant toxicity against a panel of cancer cell lines were reported by McGowan *et al.* (Fig. 23).³⁶⁴

Compared to normoxic conditions, the [Ru(b-ketoiminato)] complexes (**112**, X = F, Br) displayed two-fold higher sensitivity under hypoxic conditions than in normoxic environments. They also were found to inhibit thioredoxin reductase effectively (*i.e.*, at nanomolar concentrations). Detailed mechanistic studies revealed that these complexes exhibited a different mode of action from cisplatin drugs. Specifically, they were believed to induce single-strand DNA breakage and, as a result, trigger apoptosis.

In another report, Chao *et al.* presented a series of ruthenium(II) complexes containing anthraquinone subunits. These systems exhibited significant potency that was ascribed in part to their lipophilicity and good cellular uptake.³⁶⁵ *In vitro* toxicity studies revealed that in contrast with cisplatin, the most active analog, [Ru(pbqy)(adtpy)(ClO₄)] (pdpy = 6-phenyl-2,2'-bipyridine) (adtpy = 2-([2,2':6',2''-terpyridin]-4'-yl)anthracene-9,10-dione) (**113**, Fig. 23), was up to 46-fold more potent in hypoxic HeLa cells and 65-fold more potent in 3D multicellular tumour spheroids than in normoxic controls. Further studies confirmed a correlation between the hydrophobicity and cellular uptake behaviour of the complexes with hypoxic toxicity. Under hypoxic condition exposure (8 h), this agent was found to localize preferentially to both the mitochondria (50%) and the nucleus (32%), which is a slight shift compared to what is seen under normoxic condition (60% mitochondrial and 28% nucleus localization). In contrast, cisplatin exhibited preferential accumulation in the cytoplasm with only 8% of the drug being found in the nucleus under both normoxic and hypoxic conditions. Mechanistic studies revealed that conjugate **113** triggered apoptosis through multiple synergistic pathways, including DNA damage, DNA replication inhibition, HIF- α inhibition, and mitochondrial dysfunction.

5.3. Other metal-based therapeutics

Platinum(II) metal complexes are a mainstay of cancer chemo-therapy (**114–119**, Fig. 24). Three platinum(II) complexes have received FDA approval, cisplatin, carboplatin, and oxaplatin. A number of analogues designed to minimize the toxicities (nephrotoxicity and gastrointestinal toxicity) induced by cisplatin are in the development stage. The currently accepted mechanism of action is that the FDA-approved Pt(II) complexes target fast proliferating cells by binding to DNA after cellular uptake.^{366–371} Although in routine use worldwide, the FDA-approved platinum(II) cancer drugs are far from ideal. They suffer from high intrinsic toxicity and their efficacy in many tumour types is limited by various factors, such as low intra-cellular accumulation, upregulated DNA repair mechanisms, and drug inactivation by various thiol reductants, including GSH and metallothioneins. In part because of these considerations, considerable attention has focused on platinum(IV) complexes in recent years (Fig. 24). Relative to their Pt(II) congeners, Pt(IV) complexes are relatively non-toxic. Several Pt(IV) variations are stable in biological fluids, allowing many undesired deactivation pathways to be overcome and clinically attractive strategies, such as oral administration, to be explored.

In platinum(IV) complexes, the metal center typically adopts an octahedral geometry with a low-spin d⁶ electron configuration. The presence of two extra ligands relative to typical Pt(II) complexes imparts enhanced kinetic inertness towards biological nucleophiles. In addition, the axial ligands offer the possibility of tuning various pharmacokinetic parameters, such as

lipophilicity, reduction potential, and cancer microenvironment-targeting. Although the Pt(IV) complexes can interact with DNA in their oxidized forms, adduct formation is relatively slow.³⁷² Thus, Pt(IV) complexes are generally considered to be prodrugs that need to undergo reduction to produce a more active Pt(II) form. Since Pt(II) complexes are typically square planar, reduction generally leads to expulsion of the two axial ligands.³⁷³ The reduction rates are normally correlated with the reduction potentials. Thus, they can often be tuned through modification of the Pt(IV) complex composition. Once reduced, the active Pt(II) forms are thought to generate a cytotoxic response through DNA binding in analogy to what is proposed for the classic, FDA-approved platinum drugs.

Ormaplatin (tetrachloro(*trans*-1,2-diaminocyclohexane)-platinum(IV), **120**) was the first Pt(IV) complex to undergo clinical trials (Fig. 24). Upon bioreduction, an active dichloro(*trans*-1,2-diaminocyclohexane)platinum(II) agent is produced from ormaplatin. This particular Pt(IV) complex was found to be active against cisplatin-resistant cell lines both *in vitro* and *in vivo*. In phase I clinical studies, various dose patterns and administration modes were investigated. Unfortunately, ormaplatin engendered severe neurotoxicity at the maximum tolerated dose (MTD), presumably reflecting its fast reduction from Pt(IV) to Pt(II) form *in vivo*.^{374,375} Another Pt(IV) complex, iproplatin (*cis,trans,cis*-dichlorodihydroxobis(isopropylamine)platinum(IV), **121**) was also investigated clinically.³⁷⁶ Compared to ormaplatin, iproplatin was found to be less prone to reduction owing to the presence of axial hydroxyl groups. Additionally, the higher water solubility (44.1 mM) allowed explorations of various formulations and modes of administration. To date, a total 38 clinical trials, ranging from phase I to phase III, have been conducted with iproplatin targeting a range of cancer types.^{377–379} However, little benefit was seen for ormaplatin in these trials relative to the FDA-approved Pt(II) agents (*e.g.*, cisplatin and carboplatin).

Another Pt(IV) analogue, satraplatin [*trans,cis,cis*-bis(acetate)-aminecyclohexylaminedichloroplatinum(IV)], **122** was developed as an anticancer platinum agent suitable for oral administration (Fig. 24).³⁸⁰ In the bloodstream **122** undergoes bioreduction to furnish six distinct Pt(II) species with ammine(cyclohexylamine)dichloroplatinum(II) being the major daughter product.³⁸¹ In pre-clinical studies, **122** displayed a better toxicity profile compared to cisplatin in several ovarian cancer cell lines *in vitro*. It also exhibited good activity against the cisplatin-resistant cancer cell lines 41McisR and HX/155cisK.³⁸² *In vivo* studies involving ADJ/PC6 plasmacytoma-bearing mice, revealed an improved antitumour efficacy for satraplatin relative to cisplatin, carboplatin and ormaplatin.³⁸² A phase II clinical study, involving patients with squamous head and neck cancer and small cell lung cancer, revealed a 38% response rate in the case of satraplatin. The response rate was similar to that of cisplatin, but without the severe toxicity (neuro/nephrotoxicity) seen for this and other Pt(II) drugs.³⁸³ In other phase II trials, involving patients with advanced and recurrent squamous cervix cancer, more mixed therapeutic responses were observed.³⁸⁴ Satraplatin was also extensively investigated in patients with metastatic castration-resistant prostate cancer where a 62% response rate (stable disease or partial response) was seen.^{385,386} Bristol-Myers commissioned a phase III trials for satraplatin as a combination anticancer therapy with prednisone (synthetic corticosteroid drug, an immunosuppressant agent). However, the

studies were terminated prior to completion since the combination of satraplatin and prednisone was found to be less efficacious than prednisone alone.³⁸⁷ Another study commissioned by GPC Biotech for the same combination was conducted in 950 patients with refractory cancers. It was found that the combined treatment protocol resulted in a 36% reduction in pain progression and an improvement in patient survival as compared with prednisone alone.³⁸⁸ However, FDA approval was not forthcoming based on a lack of sufficient efficacy.³⁸⁹ Currently, another Pt(IV) agent, LA-12 (**123**, Fig. 24) is being developed.³⁹⁰ It is in phase I clinical studies.³⁹¹

Keppler *et al.* reported^{392,393} the cytotoxic profiles of a series of platinum(IV) complexes **124–127** designed to be active against cisplatin sensitive (CH1) and resistant (A549 and SW480) cancer cell lines, under both hypoxic and normoxic conditions (Fig. 25). *In vitro* studies demonstrated that these complexes exhibited a significant toxicity profile (IC₅₀) in low molecular to nanomolar concentration, which was found to be either better or comparable with cisplatin [SW480/CH1 IC₅₀ values for **126** (13), **127** (4)]. These results were found to correlate with lipophilicity and with drug accumulation in a panel of tested cancer cell lines in a non-hypoxia monolayer (2D), as well as in hypoxic spheroid (3D) models. This set of complexes also displayed significant inhibition of tumour growth in hypoxic xenografted SCID mouse models as compared to satraplatin (**122**).

Texaphyrins are macrocyclic scaffolds capable of coordinating various metal cations, including trivalent lanthanides.³⁹⁴ A particular gadolinium(III) complex, *i.e.*, Gd³⁺-texaphyrin (motexafin gadolinium, MGd), proved water soluble and capable of targeting preferentially cancerous lesions.^{395,396} To improve the cancer targeting of Pt(IV) complexes, Sessler *et al.* developed the MGd–Pt(IV) conjugates by tethering gadolinium–texaphyrin complex with inert Pt(IV) centers prodrug through an axial succinate linker (**128**, Fig. 26).³⁹⁷ The resulting conjugate, complex 128, was found to be light sensitive, resulting in photoreduction upon exposure to glass-filtered daylight. *In vitro* studies confirmed that prodrug **128** exhibited good cytotoxicity in both platinum-sensitive ovarian A2780 cells and cisplatin-resistant 2780CP cell lines. In follow up *in vitro* studies, it was discovered that texaphyrin participated as redox mediators in the reduction and activation of Pt(IV) prodrugs in the presence of cancer cells.³⁹⁸

Over the past several years, a number of other efforts beyond those associated with platinum complex and its analogues have been devoted with a view to meeting the challenge of producing modified platinum agents with targeting capabilities. Generally, one or two biologically active non-DNA targeting moieties are included within the Pt(IV) coordination sphere. Compared with the corresponding Pt(II) complexes, these so-called dual action prodrugs have been found to display enhanced potency in drug resistance cell lines. Much of this work has been reviewed elsewhere.³⁶⁹ Here, we discuss only a few representative recent examples.

Gibson *et al.* reported a quadruple action Pt(IV) prodrug (**129**, Fig. 26) obtained by combining a Pt(IV) analogue of cisplatin-containing an axial dichloroacetic acid ligand (DCA, a pyruvate dehydrogenase kinase (PDK) inhibitor) with another platinum(IV) agent, Pt56MeSS [platinum(1*S*,2*S*-diaminocyclohexane)(5,6-dimethyl-1,10-phenanthroline)]²⁺

containing phenylbutyrate (a histone deacetylase and pyruvate dehydrogenase kinase inhibitor) linked at an axial position through a succinate ligand.³⁹⁹ Upon bioreduction, four different bioactive molecules are produced. Compared with cisplatin and other tested controls (analogues lacking either of PDK inhibitor, phenylbutyrate and Pt56MeSS), conjugate **129** exhibited an improved cytotoxicity profile in both monolayer (2D) and spheroid (3D) cultured cancer cells. Prodrug **129** was found to be 200–450 fold more potent in KRAS (Kirsten rat sarcoma) mutated colon and pancreatic cancer cells as compared to cisplatin itself. It was also found to be 40 times more selective towards KRAS mutated cancer cell than towards normal cells.

Photodynamic therapy (PDT) is a clinically approved noninvasive method for the treatment of various types of cancer. It relies on the activation of molecular oxygen present in the tumour environment to generate singlet oxygen. As the concentration of O₂ in the hypoxic tumour is low compared to normal tissues, the efficiency of singlet oxygen generation is low; this significantly limits the utility of the PDT method for cancer therapy. In an attempt to increase PDT efficiency, a mitochondriallytargeted PDT agent, Ir–P(Ph)₃ (**130**, Fig. 27), was developed.⁴⁰⁰ Its phototoxicity was tested in HeLa cells under both normoxic and hypoxic conditions. Compared with the lysosomal targeted complex Ir–alkyl, **131** (a reference PDT agent), incubation with the mitochondrial-targeted agent **130** resulted in an increased intracellular oxygen concentration in HeLa cells under hypoxic conditions, a finding ascribed to respiration inhibition. Both **130** and **131** exhibited low dark toxicity as determined by methyl thiazolyltetrazolium (MTT) assays. The phototoxicity of these two agents was further tested by flow cytometry. Compared with **131**, HeLa cells pretreated with **130** under hypoxia condition (5% O₂) exhibited a greater level of cell death upon irradiation at 475 nm (xenon lamp, laser power 22 mW cm⁻²). Agent **130** also gave rise to higher cell viability ratios (CVR = cell death without/with light irradiation) of 39 and 30 in normoxic and hypoxic conditions, respectively, compared with the lysosomal targeted control **131** (CVR < 1.5).

In an effort to expand the periodic table of inorganic anti-cancer therapeutics, significant work has been devoted to realizing the therapeutic potential of gold-based agents. Gold complexes were originally developed clinically as anti-rheumatic agents. However, as their mechanism of action was elucidated, their anticancer potential began to be appreciated.⁴⁰¹ Early complexes, such as auranofin (**132**) and aurothioglucose, paved the way for the discovery of the next generation of gold(I) and gold(III) complexes with potential utility in oncology (Fig. 28). The field of anticancer gold-based therapeutics is broad, and the majority of recently published complexes do not fall within the scope of this review. For a recent review on gold based anticancer complexes, please refer to Porchia *et al.*⁴⁰² and an earlier seminal review by Susan Berners-Price.⁴⁰³ However, discussed below are examples of gold complexes designed to target the cancer antioxidant pathway.

The majority of gold-based complexes with therapeutic potential are believed to elicit their antiproliferative response *via* the selective inhibition of the selenoenzyme, thioredoxin reductase (TrxR), which is overexpressed in several cancer indications.⁴⁰⁴ Despite the selectivity of Au towards TrxR, the therapeutic utility of this approach is limited due to the network robustness of the cancer antioxidant pathway. This unfortunate result recapitulates

what has become a recurring finding in drug development; specifically, it has been found that singular random deletions or inhibition of specific proteins often gives rise to poor phenotypic outputs and poor preclinical or clinical outcomes due to the scale-free nature of biological networks.⁴⁰⁵ As a consequence, even the most effective protein targeting small molecular approaches (*i.e.*, targeted therapies) do not always lead to viable drug candidates. For example, it was shown that knockdown of TrxR by 90% (*via* siRNA), provided little to no phenotypic change in cell proliferation.⁴⁰⁶ Further treatment with **132** resulted in no differences in cell proliferation between TrxR knockdown A549 and A549 cells treated with mock siRNA. It was proposed by Arumugam and Arambula that this finding and the inferred robustness of TrxR is consistent with a highly networked endogenous antioxidant system that would require multiple modes of drug targeting to be suppressed in a therapeutically useful manner (Fig. 28a).⁴⁰⁷

To test this hypothesis, N-heterocyclic carbene (NHC) Au(I) systems were developed that contain the redox active moieties ferrocene and naphthoquinone (Fig. 28b).^{407,408} Both systems were characterized by submicromolar potency levels across several human cancer cell lines, including A549 lung, A2780 ovarian, 2780CP ovarian, and PC-3 prostate. These fused systems were found to be significantly more potent than the constituent Au(I)-NHC complex or redox active cores. Furthermore, both **133** and **134** were found to be synergistic as regard their anticancer potency as compared to control combinations that were equimolar in the respective components (*i.e.*, 1 equiv. Au(I)-NHC and 2 or 4 equiv. of the appropriate redox active core). These systems were also found to accentuate mitochondrial ROS in a synergistic fashion relative to their constituents and inhibit TrxR effectively. Differential gene expression of A549 cells treated with the ferrocenylated gold complex **133** showed activation of several genes associated with endoplasmic reticulum (ER) stress and unfolded protein responses. Such activation profiles are consistent with prior findings with other systems that led to the suggestion that metal-based therapeutics could act as inducers of cancer-specific immunogenic cell death.⁴⁰⁹

To examine further the selectivity towards the cancer cellular environment over healthy tissue, complex **134** was evaluated in zebrafish embryos bearing A549 lung cancer xenografts. This fluorescence based *in vivo* assay allows for the detection of drug-induced cellular death. It was found that complex **134** induced xenograft specific cell death at drug levels that were well tolerated by healthy tissue (*i.e.*, no healthy tissue specific death was detected). Taken in concert, the results obtained with **133** and **134** provide support for the notion that a good anti-cancer response may be obtained by using a dual targeting approach to attack the cancer antioxidant network.

6. N-Oxide-based therapeutics

Compounds bearing N-oxide units have been developed as potential HAPs. N-Oxide prodrugs are susceptible to enzyme-mediated reduction. This reduction can be used as activation strategy to release cytotoxic drugs.⁴¹⁰ Based on their specific structures and mode of activation, N-oxide drugs may be divided into three main categories. These are discussed below.

6.1. Aromatic *N*-oxide containing aromatics

Tirapazamine, TPZ (**135**, R = H, Fig. 29)⁴¹¹ a benzotriazine-1,4-dioxide, is an extensively studied prodrug that generates reactive radical species by intracellular reductases under hypoxic conditions.⁴¹² The formation of TPZ radicals was found to be mediated by one-electron oxidoreductases. The radicals generated in this way are thought to cause irreversible damage to DNA through single and double strand breaks, thereby triggering apoptosis. Gates *et al.* conducted an extensive study in an effort to determine whether $\bullet\text{OH}$ radicals are the major DNA-damaging species.⁴¹³ Separately, Anderson *et al.*⁴¹⁴ employed spin trapping agents, such as 5,5'-dimethylpyrroline 1-*N*-oxide (DMPO) and 5-diethoxyphosphoryl-5-methyl-pyrroline *N*-oxide (DEPMPO), to show that TPZ produces C- and N-centered radicals, but not the $\bullet\text{OH}$ radical, upon reduction with P-450R. Further support for this latter conclusion came from electron paramagnetic resonance (EPR), pulse radiolysis (PR), steady-state radiolysis, and theoretical calculations (DFT).⁴¹⁵

TPZ (**135**) exhibited promising activity against different tumors, including cervical cancer, head and neck cancer, non-small cell lung cancer, metastatic melanoma, and ovarian and primary peritoneal cancer in a number of phase I and phase II clinical trials alone or in combination with other antitumor agents or photodynamic therapy (PDT).^{416,417} Unfortunately, TPZ yielded poor outcomes in phase III clinical trials when tested as a combination regimen (chemo/radiotherapy) against HNSCC (head and neck squamous cell carcinoma).^{418–420} In fact, little if any improvement in patient survival, whether or not TPZ was used alone or in combination with radio- or chemotherapy.^{421–423} This failure was attributed to the low aqueous solubility, poor extravascular transport, and modest radiotherapeutic quality control.

To improve the hypoxic selectivity and efficacy of TPZ, various derivatives of 3-amino-1,2,4-benzotriazine-1,4-dioxide with enhanced solubility and lipophilicity have been synthesized and evaluated under hypoxic and normoxic conditions.^{424,425} *In vitro* analyses demonstrated that substitution at the 3- and 7-positions was particularly effective under hypoxic conditions in terms of modulating the overall potency. In the most favorable cases, HCR values as high 35 and 23 were seen in HT-29 and SCCVII cells, respectively.

Among the TPZ analogues reported to date are the morpho-line based benzotriazine di-oxide HAPs, SN30000 and SN29751 (**136** and **137**, Fig. 29). These derivatives were found to display promising pharmacokinetic behavior.^{424,426} HAP **136** showed better activity than TPZ^{427,428} and was considered promising. During subsequent preclinical assessments, **136** was found to be light-sensitive under normal laboratory conditions.⁴²⁹ Since this could have adverse implications in terms of clinical development, the photo-degradation behaviour of **136** was investigated in aqueous solution.⁴³⁰ First-order photo-degradation kinetics were seen in dilute aqueous solution with a quantum yield of 0.016. Compared with **136**, the degradation product formed (morpholine *N*-oxide analogue of **136**) displayed reduced cytotoxicity towards human cancer cell lines (HT29 and H1299) under both hypoxic (>2000-fold) and aerobic conditions (6 fold).

Wouters *et al.* utilized genome-scale shRNA screens and oxidoreductase-focused libraries to identify the flavoprotein POR (cytochrome P-450 oxidoreductase) as a potential determinant

29). Monge *et al.* conducted an extensive SAR study to develop the potent hypoxia selective quinoxaline 1,4-dioxide based derivatives. The presence of electron withdrawing groups at the 6- and/or 7-position of the 3-amino scaffold significantly improved the reduction potential of derivatives as compared with 135. Among the analogues tested, derivatives **144a** and **144b** exhibited the greatest hypoxia selectivity. They also displayed a 30-fold higher toxicity than 135.⁴⁴⁹ In a separate study, the water-soluble 3-[[*N,N'*-dialkyl-amino)alkyl]amino]-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides derivatives **144c** and **144d** bearing 7-chloro and 7-trifluoromethyl substituents, respectively, provided higher potency and great selectivity (HCR values of *ca.* 250 and 340, respectively).⁴⁵⁰ Similarly, compound **145** showed selective toxicity against hypoxic V79 cells *in vitro* and MCF breast cancer xenograft models. An alkaline comet assay confirmed the exclusive production of $\bullet\text{OH}$ radicals under hypoxic conditions and corresponding DNA damage.⁴⁵¹

Hori *et al.* developed a series of hypoxic-neoplastic cells targeted hybrid analogues of 1-methyltryptophan (1MT, 2,3-dioxygenase inhibitor) and tirapazamine linked through flexible alkyl chains.⁴⁵² The lead compounds in question, **146** and **147**, displayed improved HCR values of about 4.9 and 5.0, respectively against EMT6/KU cells as compared with 135 alone (HCR = 3.2). The improved HCR values seen for these prodrugs was ascribed to their dual biological function *i.e.*, hypoxic cytotoxin (TPZ) and IDO inhibition by IMT respectively. Other hybrids of 135 with DNA interchelators have also been developed and found to exhibit enhanced hypoxic cytotoxicity (1–56 fold), but with HCR values (8–51) that are more modest than those of **135** (HCR = 62) in HT-29 cancer cell lines. Several analogues with a different range of DNA-binding affinities are currently the subject of further investigation.^{453,454}

Gates *et al.*⁴⁵⁵ reported the TPZ analogue di-*N*-oxide **148** that contains a nitrogen mustard subunit (Fig. 30). This derivative was found to be 30-fold more potent than the mono-*N*-oxide **149** under hypoxic condition. Based on control experiments, the difference in potency was found to be due to overlapping mechanisms of action. Bioreduction of **148** produces the daughter system **149** (Fig. 30). The TPZ subunit in **149** was thought to be more susceptible to hydrolytic release of the reactive mustard as the result of the change in the electronic structure. As a result, under hypoxic conditions, compound **148** (as a prodrug for **149**) was expected to induce oxidative DNA damage *via* the triazine ring, whereas the nitrogen mustard it carries would act as a strong DNA-alkylating agent.

6.2. Aliphatic *N*-oxides

Tertiary alkyl amine *N*-oxides have also shown cytotoxicity toward hypoxic cells. The oxygen sensitive two-electron reduction of an alkyl tertiary amine *N*-oxide in the presence of cytochrome CP-450 reductase or the CYP3A isozyme of NADPH produces a tertiary amine. Alkylamines are protonated under physiological pH and within the nucleus bind to DNA as the result of electrostatic interactions; this can trigger apoptosis.^{456,457} As *N*-oxidation masks the cationic nature of amines, *N*-oxide prodrugs usually exhibited low toxicity and DNA binding ability. Under hypoxic conditions, these prodrugs get activated to produce corresponding amine drugs, hence imparting a degree of selectivity.⁴⁵⁸

A well-investigated alkyl *N*-oxide prodrug is AQ4N (**150**, Fig. 31). In reducing environments, it is activated to produce AQ4, (**151**, Fig. 31). This bis-amine is a strong topoisomerase II inhibitor with DNA-intercalating properties similar to those of mitoxantrone (**152**).⁴⁵⁹ AQN4 (**150**) exhibits low cytotoxicity in hypoxic cells; however, its conversion to **151** under hypoxic conditions leads to *ca.* 100× higher toxicity. Preclinical and phase I investigations provided support for the notion that **150** produces antitumor effects when used as a single agent or in combination with radio/chemotherapy.^{460–463} Unfortunately, development of **150** has apparently not progressed past a phase II trial initiated in 2006 that compared **150** against temozolomide in glioblastoma in conjunction with RT (NCT00394628).

Multi-ring scaffolds, such as 9-aminoacridine (**153**),^{464–466} naphthalamide (**155** and **156**),⁴⁶⁷ anthrapyrazole (**159**),⁴⁶⁸ azaanthrapyrazole (**160**),⁴⁶⁹ and mitoxantrone (**161**),⁴⁷⁰ have been explored as hypoxia-selective prodrugs. These systems were generally prepared by appending tertiary *N*-oxides to the corresponding amines (*viz.*, **154**, **157**, **158**, and **162**; Fig. 31). The cancer cell selectivity of the *N*-oxides, relative to their amino counterparts was found to be 10–1000-fold greater under hypoxic conditions across a number of cell lines. This was taken as an early indication that topoisomerase II inhibitors may be directed towards hypoxic regions *via N*-oxide prodrug formation.

6.3. Discrete *N*-oxide prodrugs

Recognizing the canonical redox chemistry of *N*-oxides, several HAPs containing this moiety were developed in an effort to obtain prodrugs that would be activated under hypoxic conditions. As above, agents of this class have generally been obtained by tethering an *N*-oxide moiety to a cytotoxic agent. Nitromin (**163**, Fig. 32) represents the first example of this type of putative prodrug. This agent showed a 4× increased inhibition of Walker cells growth under conditions of hypoxia than normoxia. Activity was ascribed to the bioreductive production of nitrogen mustard, a bifunctional alkylating agent.⁴⁷¹

Another example of this type of HAP, namely chlorambucil *N*-oxide (**164**, Fig. 32), was reported by Denny *et al.*⁴⁷² Compared to chlorambucil, prodrug **164** displayed a lower level of hypoxic toxicity in AA8 cells; however, it showed some toxicity in the presence of rat liver microsomes and NADPH.⁴⁷³

In separate study, Wardman *et al.*⁴⁷⁴ reported a potential HAP prodrug **165** (Fig. 32), which upon activation under hypoxic conditions forms a complex with the Fe(II) cation. Even under conditions of low oxygen tension, this promotes production of hydroxyl radicals, thereby accounting for the cytotoxicity seen *in vitro* (Chinese hamster V79 cell line). This mode of action mirrors that proposed for bleomycin (an antibiotic that has been used to treat skin, head, and neck cancers).⁴⁷⁵ Prodrug **165** itself contains a hypoxia-responsive pyridine *N*-oxide moiety and *per se* is inactive towards DNA damage. In contrast, its reduced form proved very active, as noted above.

7. Summary and outlook

Cancer is caused by various factors that disrupt the balance of cell survival, proliferation, and differentiation. Over the past 60 years, the influential role of tumour hypoxia in oncology, and its potential as a therapeutic target have become increasingly well recognized. Exploiting tumour hypoxia as a means of targeting tumours and releasing active drugs selectively may allow new drug discovery strategies to be pursued that could in due course lead to improved cancer therapy regimens. In this Review, we have highlighted chemical and preclinical research developments in the area of hypoxia-responsive therapeutics. Particular emphasis has been placed on bioreductive approaches since these currently appear to have the greatest promise for success.

As the reductive pathways involved for prodrug activation are hypoxia dependent and are in turn inversely correlated with available oxygen concentrations, normal cells are largely spared from the side effects that might be expected in the absence of hypoxia targeting and reductive drug release. The hope is that the present summary will prove a useful guide to established researchers, allowing them to extend their understanding while inspiring investigators new to the area to take up the challenges and opportunities associated with hypoxia as a drug discovery strategy. Ultimately, these efforts are expected to lead to new and more effective therapeutic strategies while advancing the rapidly emerging area of personalized cancer medicine.

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Biographies



From left to right: Seyoung Koo, Prof. Jong Seung Kim & Dr Amit Sharma

Seyoung Koo has been working at the Department of Chemistry, Korea University as a PhD student under the supervision of Prof. Jong Seung Kim since 2014. Her research interests involve the design and synthesis of imaging agents and drug delivery systems for cancer therapy.

Jong Seung Kim was born in Daejeon, Korea in 1963. He received PhD from the Department of Chemistry and Biochemistry, Texas Tech University and completed a one-year postdoctoral fellowship at University of Houston. In 2007, he moved to the Department of Chemistry at Korea University in Seoul as a full professor.

Amit Sharma was born in Nagrota Bagwan, Kangra, H.P., India in 1983. He received his PhD from Guru Nanak Dev University, Amritsar, India under the supervision of Prof.

Kamaljit Singh. Thereafter, he joined Sphaera Pharma Pvt. Ltd, India as a research scientist (2011–2014). In 2014, he joined Prof. J. S. Kim's research group as research professor. His research interests concern the development of smart biomarkers and drug delivery systems for chemotherapy.



From left to right: Prof. Jonathan L. Sessler and Dr Jonathan F. Arambula

Jonathan L. Sessler received a BSc degree in Chemistry in 1977 from the University of California, Berkeley. He obtained his PhD from Stanford University in 1982. After postdoctoral stays in Strasbourg and Kyoto, he accepted a position as an Assistant Professor of Chemistry at the University of Texas at Austin, where he is currently the Doherty-Welch Chair. Prof. Sessler was a co-founder of Pharmacyclics, Inc. and Cible, Inc. He was also a WCU Professor at Yonsei University and recently accepted a laboratory directorate at Shanghai University. Prof. Sessler has published > 720 papers (cited > 37 000 times) and his h-index is 100.

Jonathan F. Arambula received his PhD in Chemistry from the University of Illinois and was an American Cancer Society Postdoctoral Fellow at the University of Texas at Austin and M.D. Anderson Cancer Center. Following this, he held an assistant professor position in the Department of Chemistry at Georgia Southern University. In 2016, he co-founded Cible Inc., whose focus is the development of tumor localizing therapies that overcome drug resistance. With research efforts focused on early stage translational science, he is the co-inventor and author of multiple metal-based discovery platforms focused on overcoming cancer drug resistance.



From left to right: Dr Hardev Singh & Dr Rajesh Kumar

Hardev Singh was born in Amritsar, India, in 1982. He received his PhD degree in Organic Chemistry under the supervision of Prof. Vandana Bhalla, Department of Chemistry, Guru Nanak Dev University, Amritsar, India. After a two-year post-doctoral fellowship at Ajou University with Prof. Hwan Myung Kim, he joined Prof. Jong Seung Kim's lab as a research professor in 2015. His current research interests include the development of novel molecular imaging probes and targeted drug delivery systems (DDS's) for cancer therapy.

Rajesh Kumar was born in Pathankot, India in 1978. He received his PhD degree in organic chemistry under the supervision of Prof. Manoj Kumar from the Department of Chemistry, Guru Nanak Dev University, Amritsar, India. From Sept 2011 to June 2015, he worked in Prof. Jong Seung Kim's lab, Korea University, Seoul as a research professor. In June of 2015, he moved to the University of Calgary as a postdoctoral fellow and then Canam

Bioresearch as a research scientist. His research interests concern the development of small molecular biomarkers, drug delivery systems, and nanomedicines for cancer therapy.

Abbreviations

BBB	Blood brain barrier
BCNU	1,3-Bis(2-chloroethyl)-1-nitrosourea
CYP450	Cytochrome P450
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DDTC	Sodium dimethyldithiocarbamate
DMSO	Dimethyl sulfoxide
DEI	5-[(Dimethylamino)ethoxy]indole
EMA	European Medicines Agency
ECGF	Endothelial cell growth factor
FDA	Food Drug and Administration
FMN	Flavin mononucleotide
GSH	Glutathione
GST	Glutathione S-transferase
GDEPT	Gene-directed enzyme prodrug therapy
HSCs	Hypoxia selective cytotoxins
HAPs	Hypoxia activated prodrugs
HCR	Hypoxia cytotoxic ratio
HIF	Hypoxia-inducible factor
HDR	Homology-directed repair
MRI	Magnetic resonance imaging
1MT	1-Methyltryptophan
NTR	Nitroreductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NCI	National Cancer Institute
NHC	N-Heterocyclic carbene

NSCLC	Non-small-cell lung cancer
PET	Positron emission tomography
PCNU	1-(2-Chloroethyl)-1-nitrosourea
P450	Cytochrome P450
PDT	Photodynamic therapy
Q3PA	Trimethyl-locked quinone propionic acid
RT	Radiation therapy
ROS	Reactive oxygen species
SRBT	Stereotactic body radiotherapy
NaCl	Sodium chloride
SCID	Severe combined immunodeficiency
SOC	Standard of care
SAR	Structure–activity relationship
Trx	Thioredoxin
TrxR	Thioredoxin reductase
TMI	5,6,7-Trimethoxyindole
US	United States
VEFG	Vascular endothelial growth factor
XO	Xanthine oxidase

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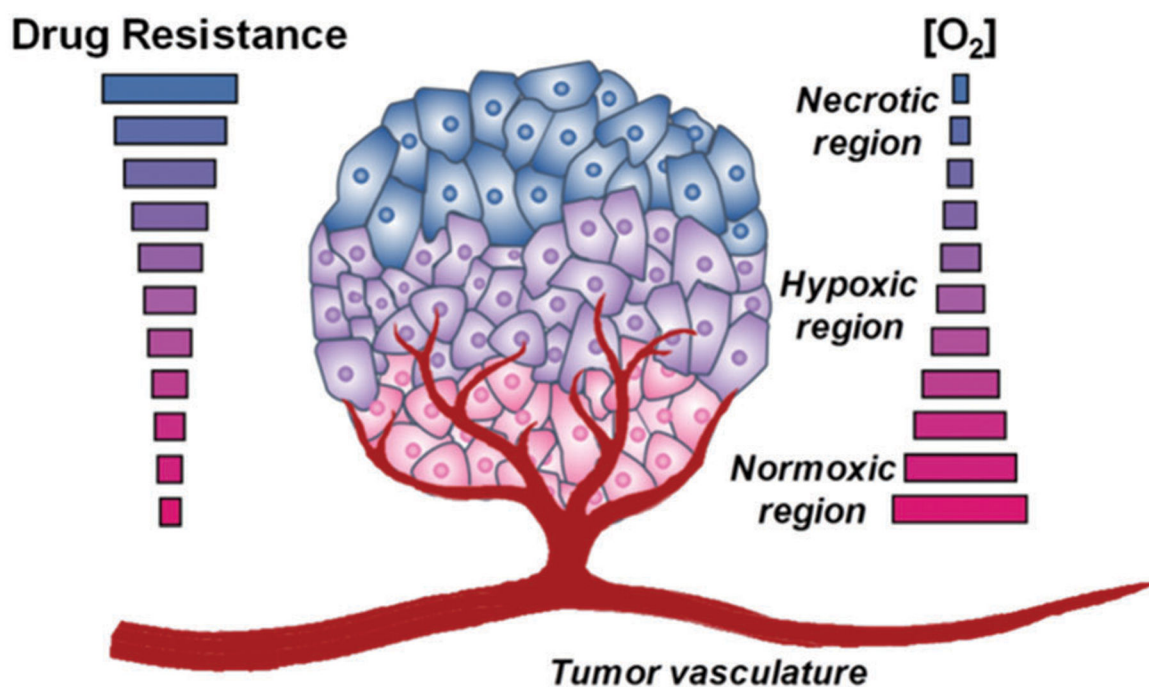


Fig. 1. Hypoxia-associated tumour microenvironment. Generally, solid tumours are characterized by unusual tumour vasculature. When the rate of angiogenesis cannot support the increased rate of tumour growth, the oxygen supply to tumour cells is restricted, and regions of hypoxia develop. Hypoxic tumours exhibit enhanced aggressiveness, metastasis, and resistance to radiation and chemotherapy.

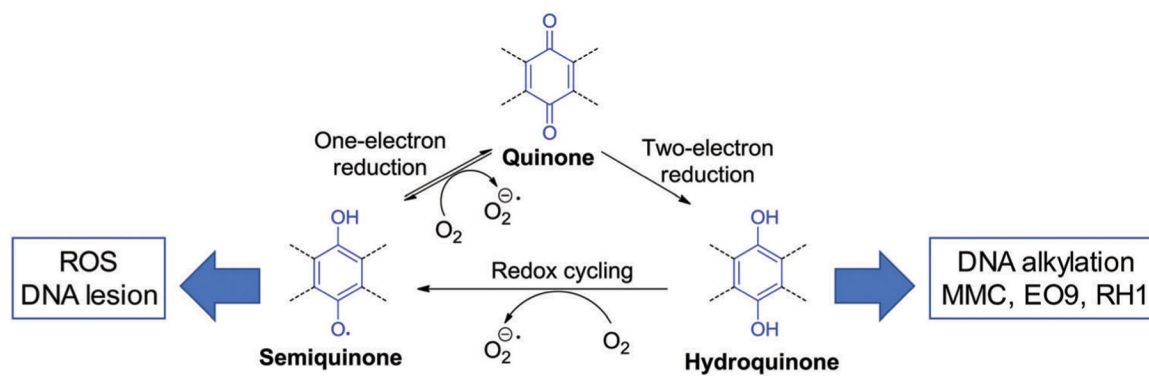


Fig. 2. Quinone reduction routes mediated by cytochrome reductase, ubiquinone reductase, and DT-diaphorase.

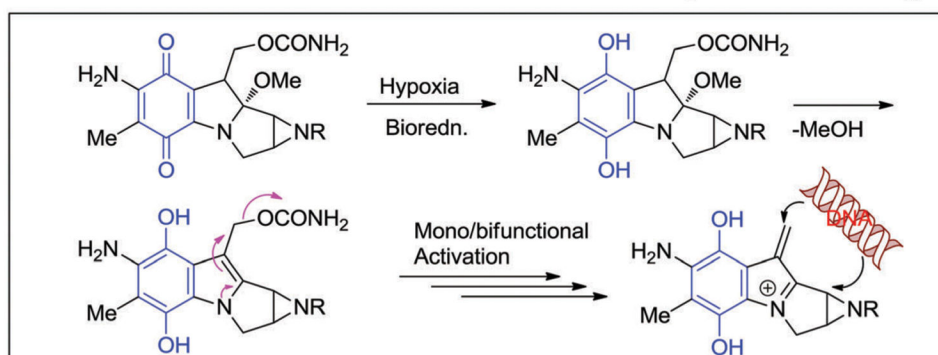
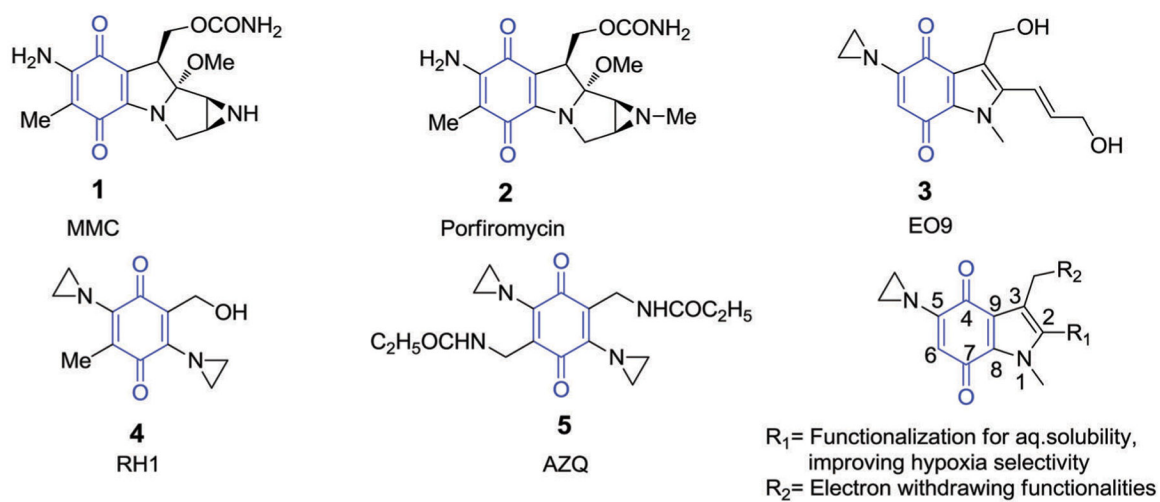


Fig. 3. Quinone-based bioreductive compounds and proposed activation mechanism for MMC (**1**) and porfiromycin (**2**). As shown schematically, this activation results in the formation of a DNA alkylating agent.

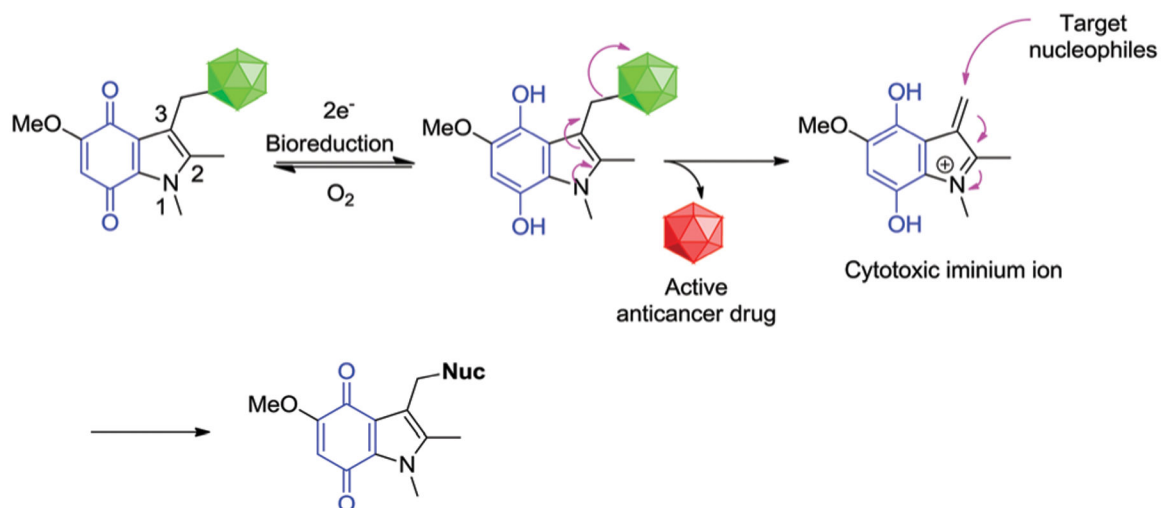


Fig. 4. Bioreductive activation of a generalized indolequinone prodrug and proposed mechanism of drug release under hypoxic conditions.

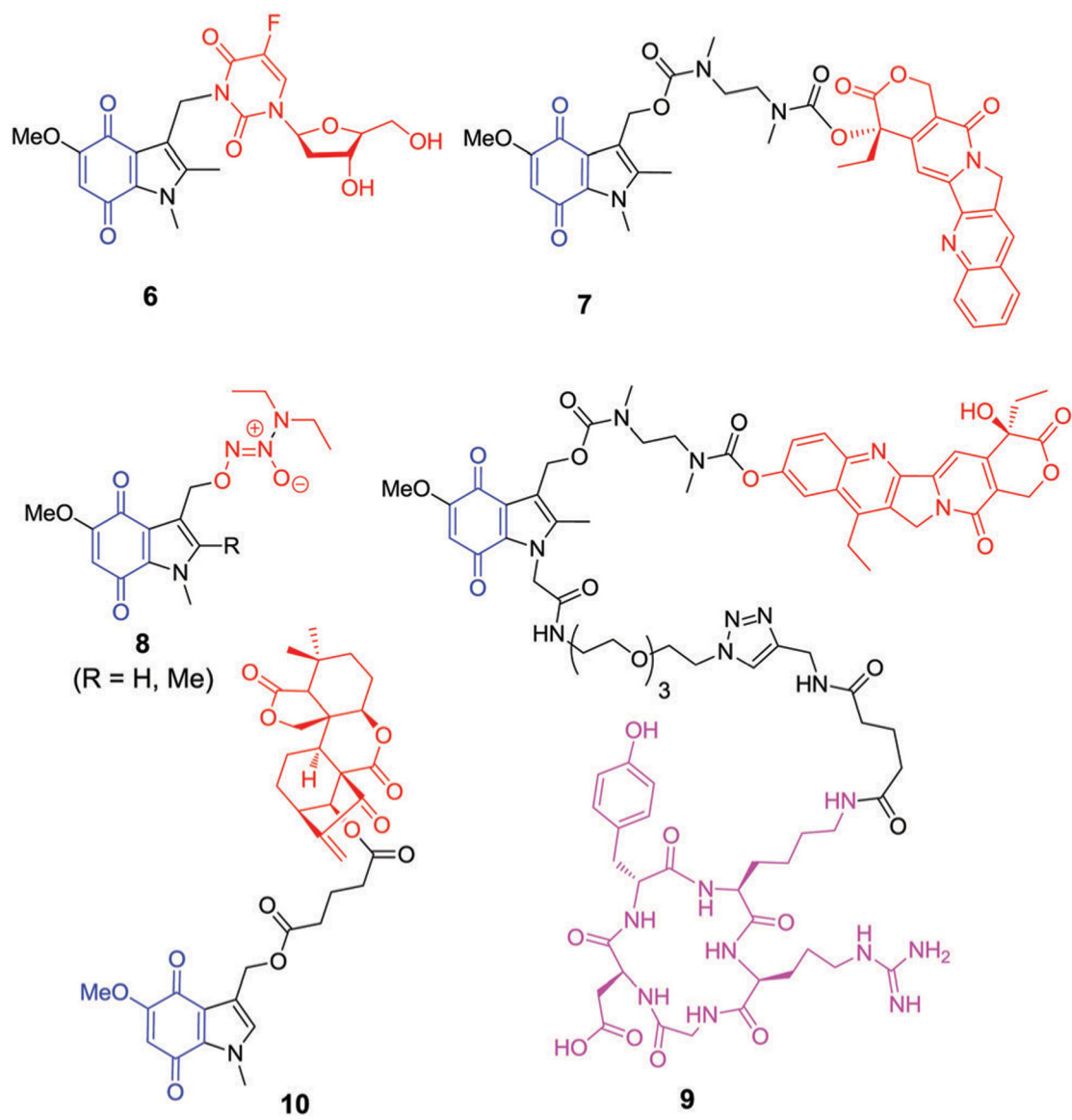


Fig. 5. Chemical structures of indolequinone-tethered drug conjugates. Hypoxia selective indolequinone = blue, conjugated drug = red, integrin selective cyclic peptide = pink.

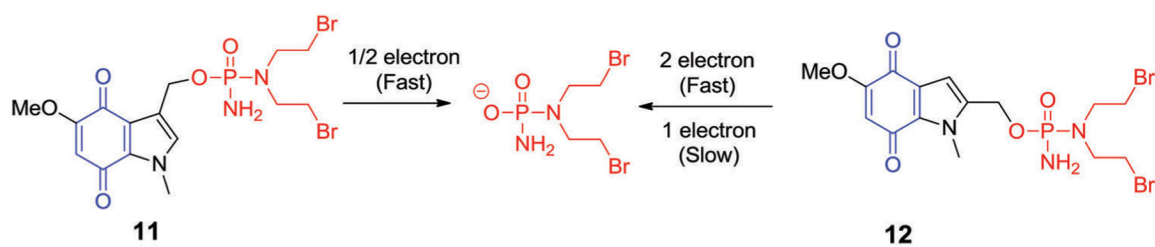


Fig. 6. C-2 and C-3-substituted indolequinone phosphoramidate prodrugs and their proposed modes of activation.

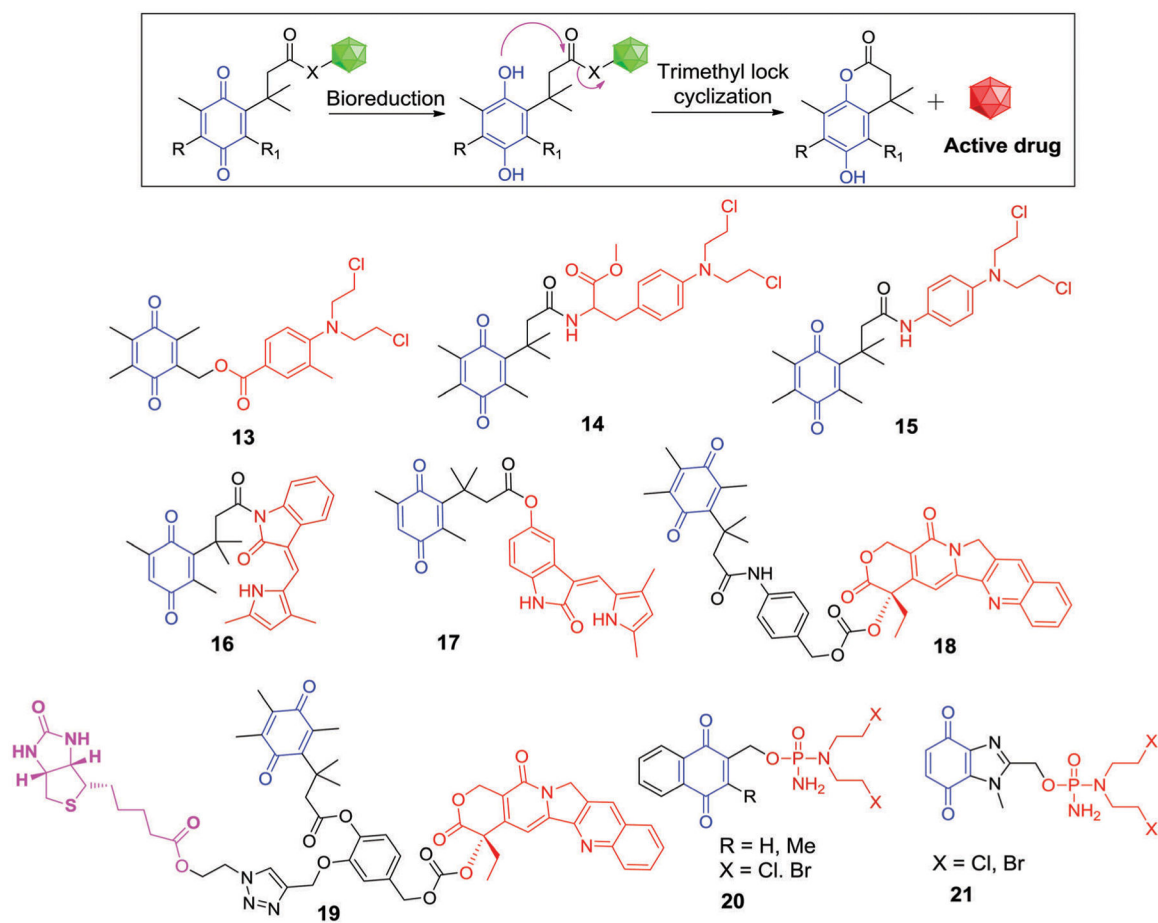


Fig. 7. Tripartite prodrug activation pathway and quinone/trimethyl tripartite quinone-based prodrug structures. Hypoxia selective indolequinone = blue, conjugated drug = red, tumour localizing biotin = pink.



Fig. 8.
Stepwise reduction of nitroaromatics under hypoxic conditions.

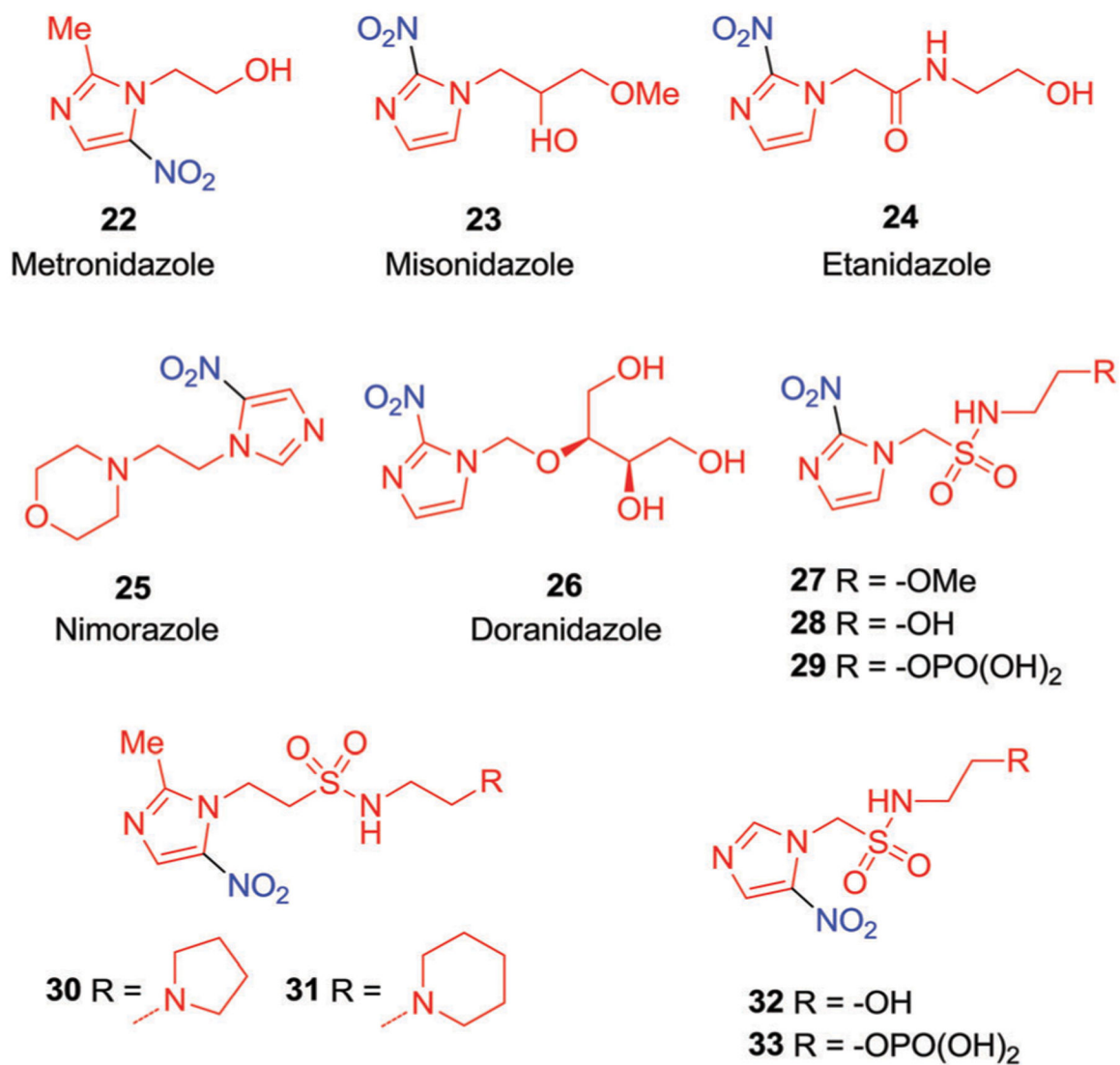


Fig. 9. Chemical structures of oxygen-mimetic radiosensitizers and novel nitroimidazole alkyl sulfonamide radiosensitizers that have been investigated clinically.

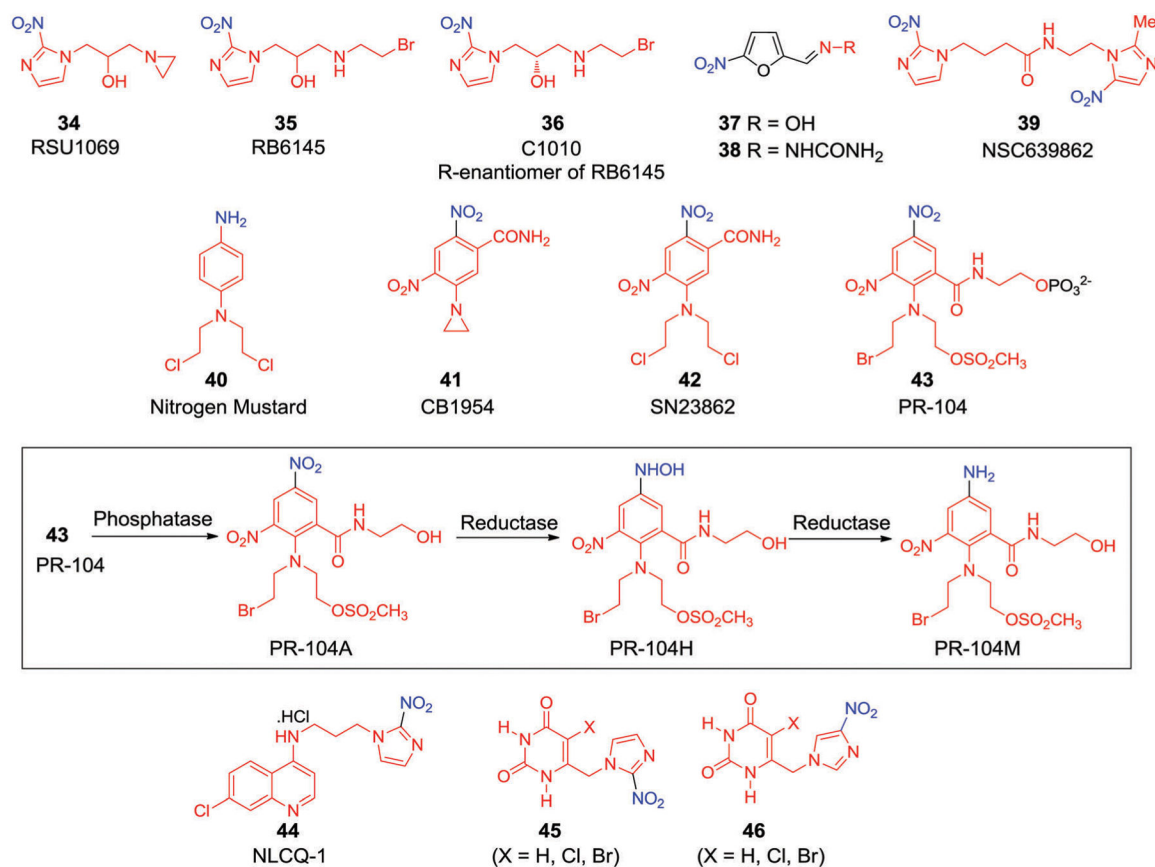


Fig. 10.
Structures of some hypoxia-selective cytotoxins.

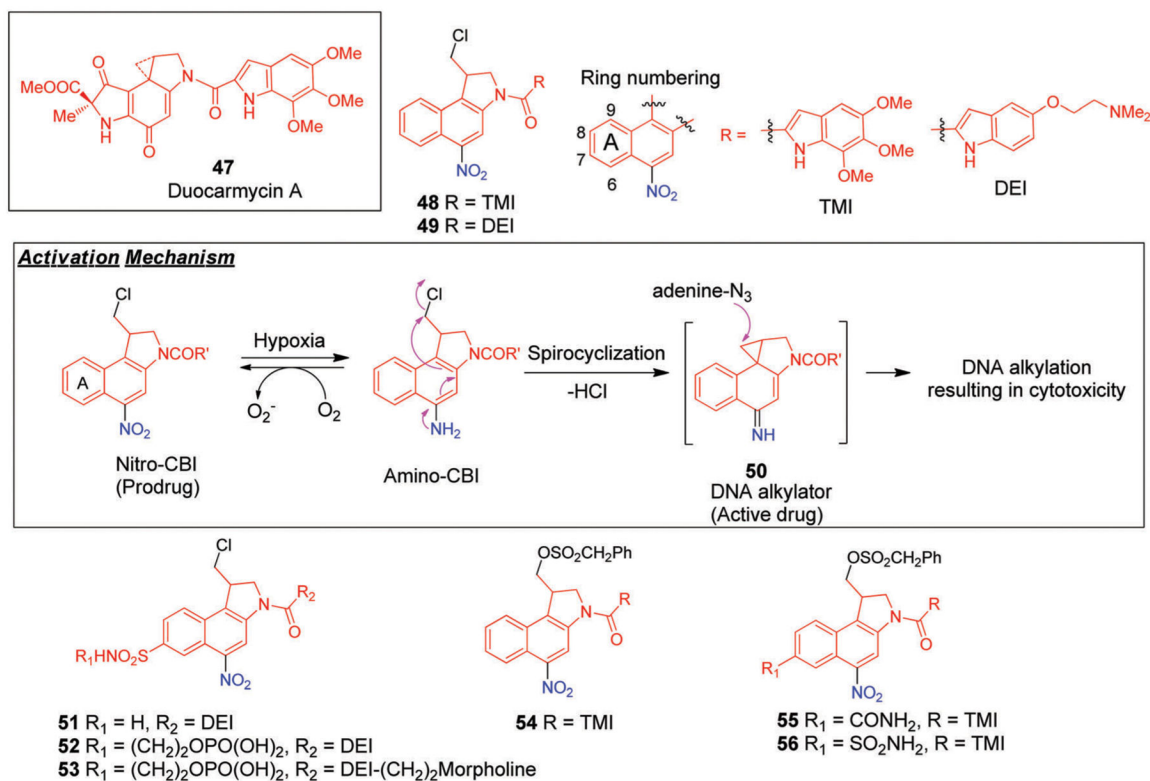


Fig. 11. Structures of 7-substituted hypoxia-activated prodrugs of nitro-chloromethylbenzindolines (nitro-CBIs) bearing 5,6,7-trimethoxyindole (TMI) and 5-[(dimethylamino)ethoxy]indole (DEI) side chains.

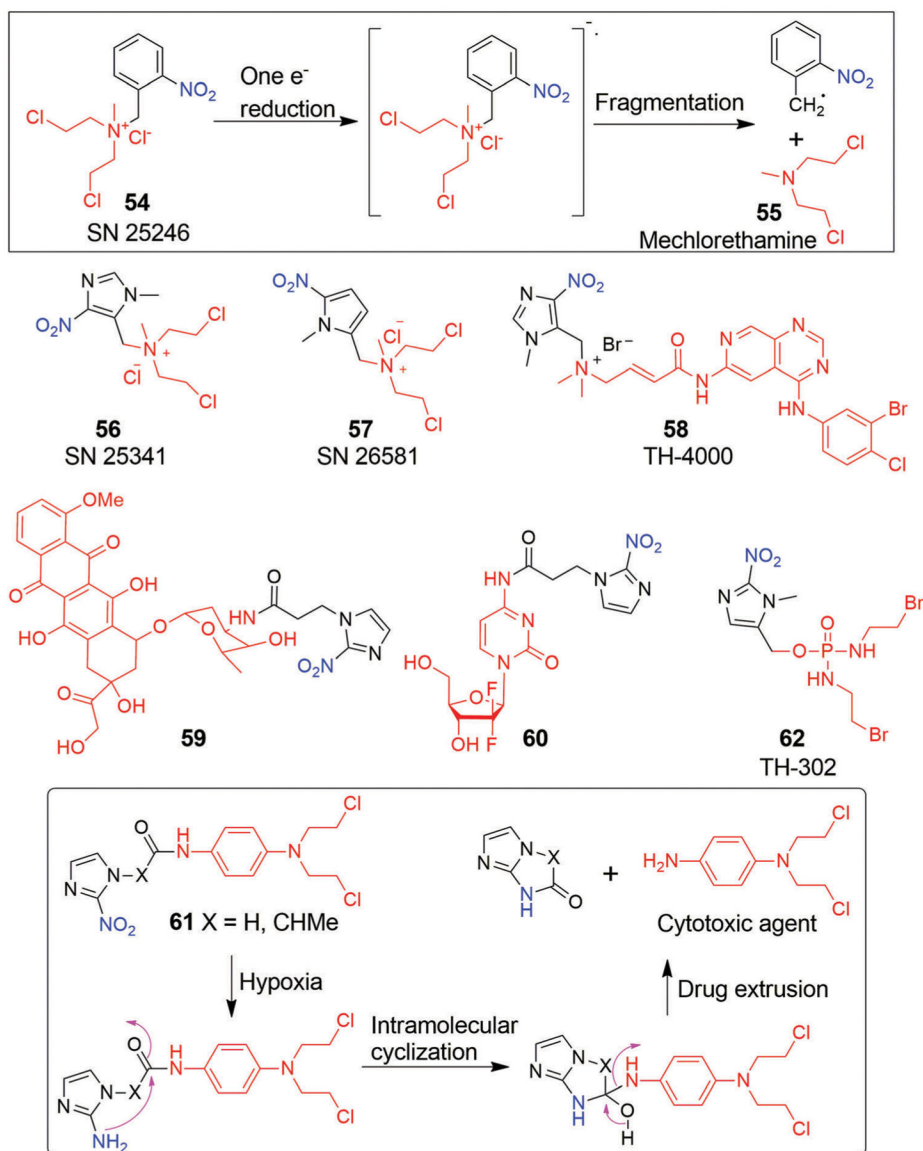


Fig. 12. Hypoxia-activated prodrugs based upon nitrogen mustard and their proposed activation mechanism.

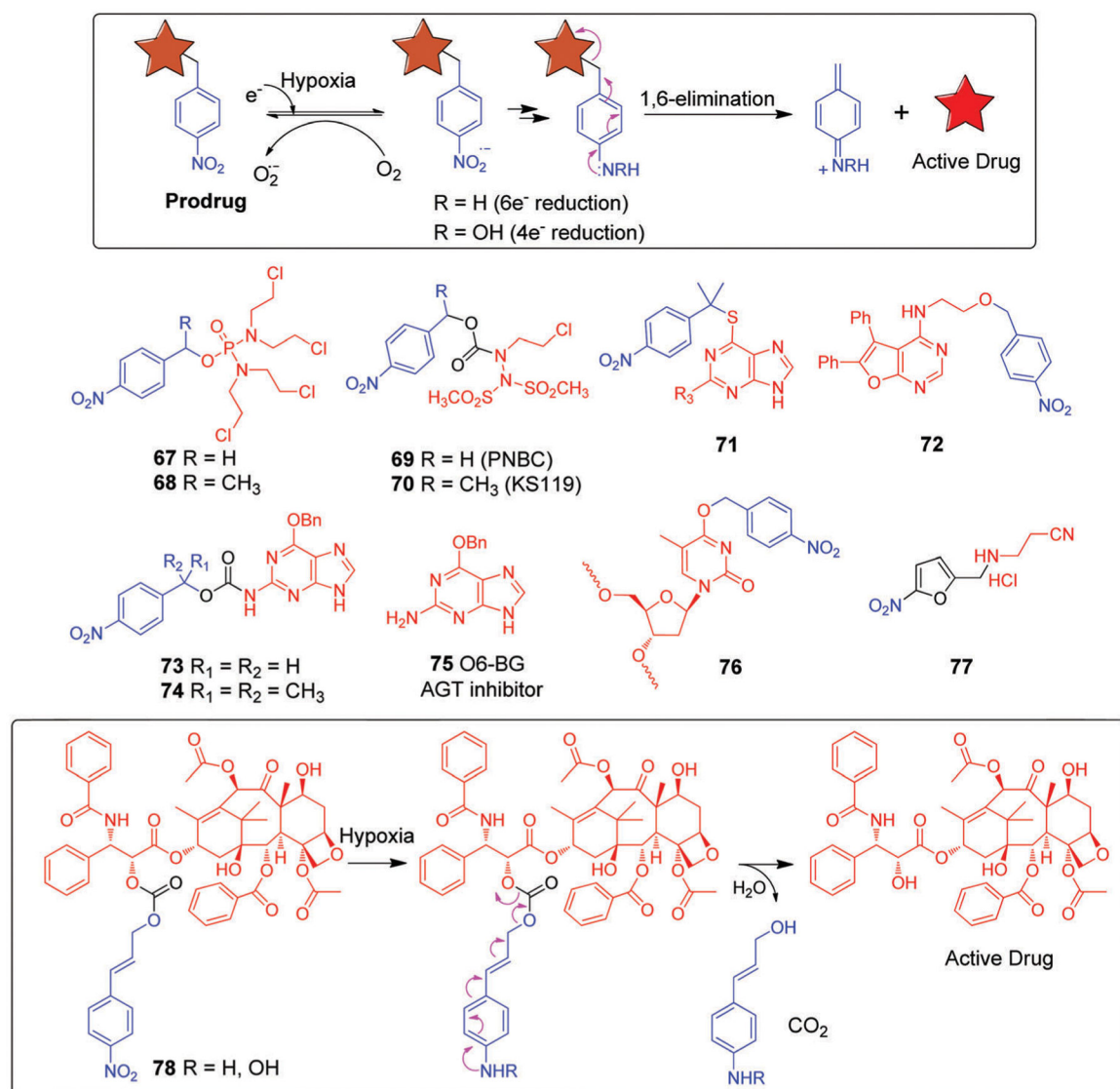


Fig. 13. Nitrobenzyl-based hypoxia-activated prodrugs. Also shown is their proposed mechanism of drug release, which involves 1,6-elimination under hypoxic conditions.

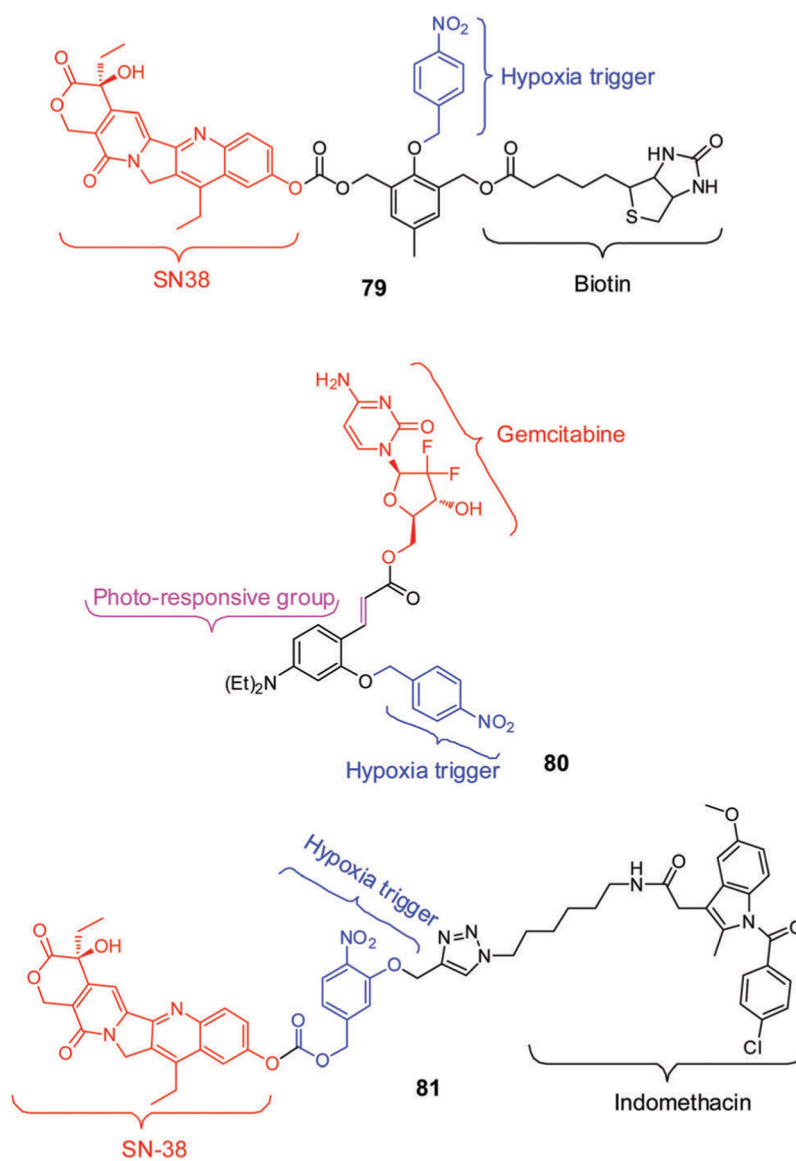


Fig. 14. Structures of hypoxia-activated theranostics **79**, **80**, and **81**.

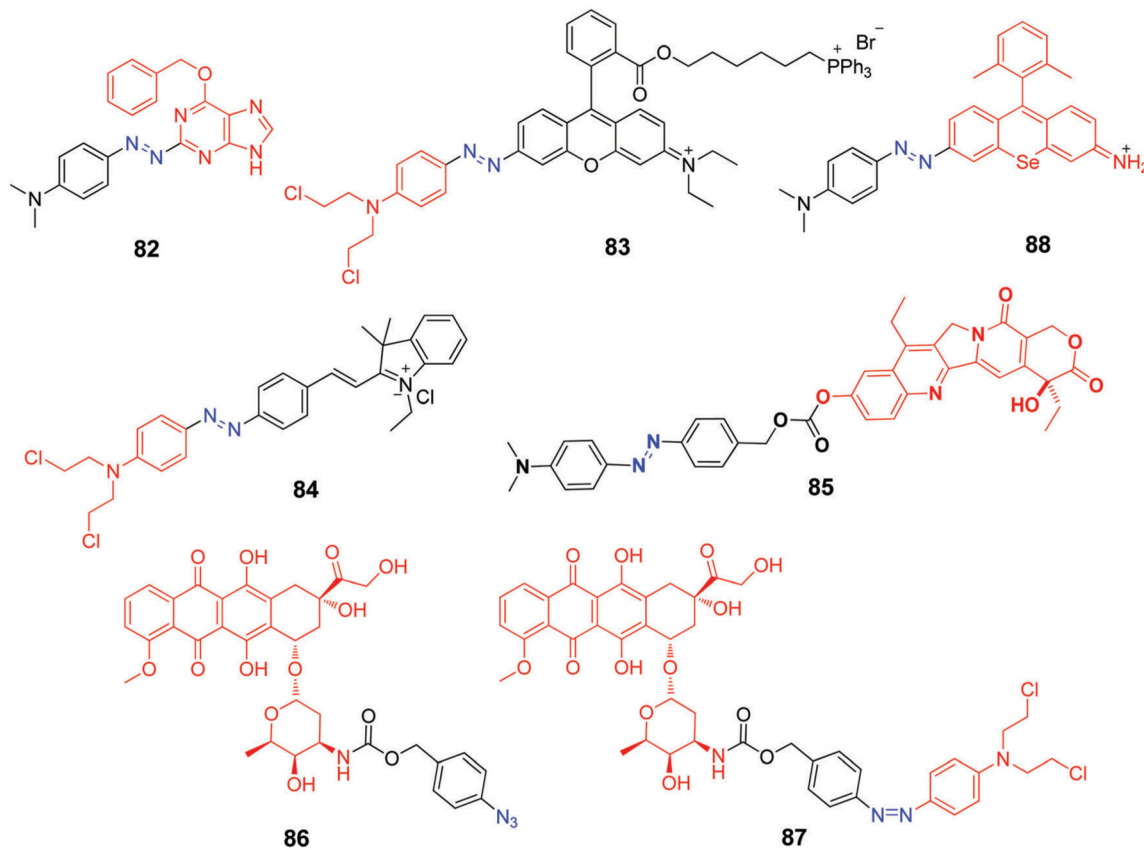
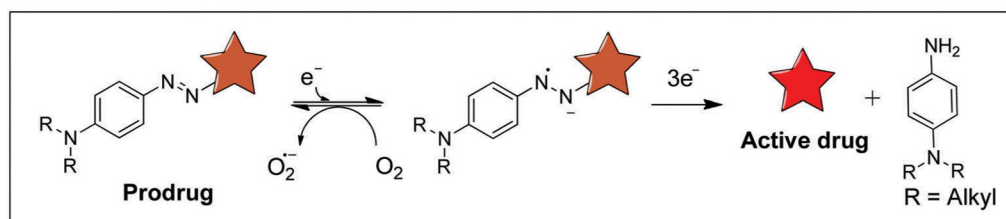


Fig. 15. Mechanism of azo-based prodrug activation and structures of azo-based drug delivery systems.

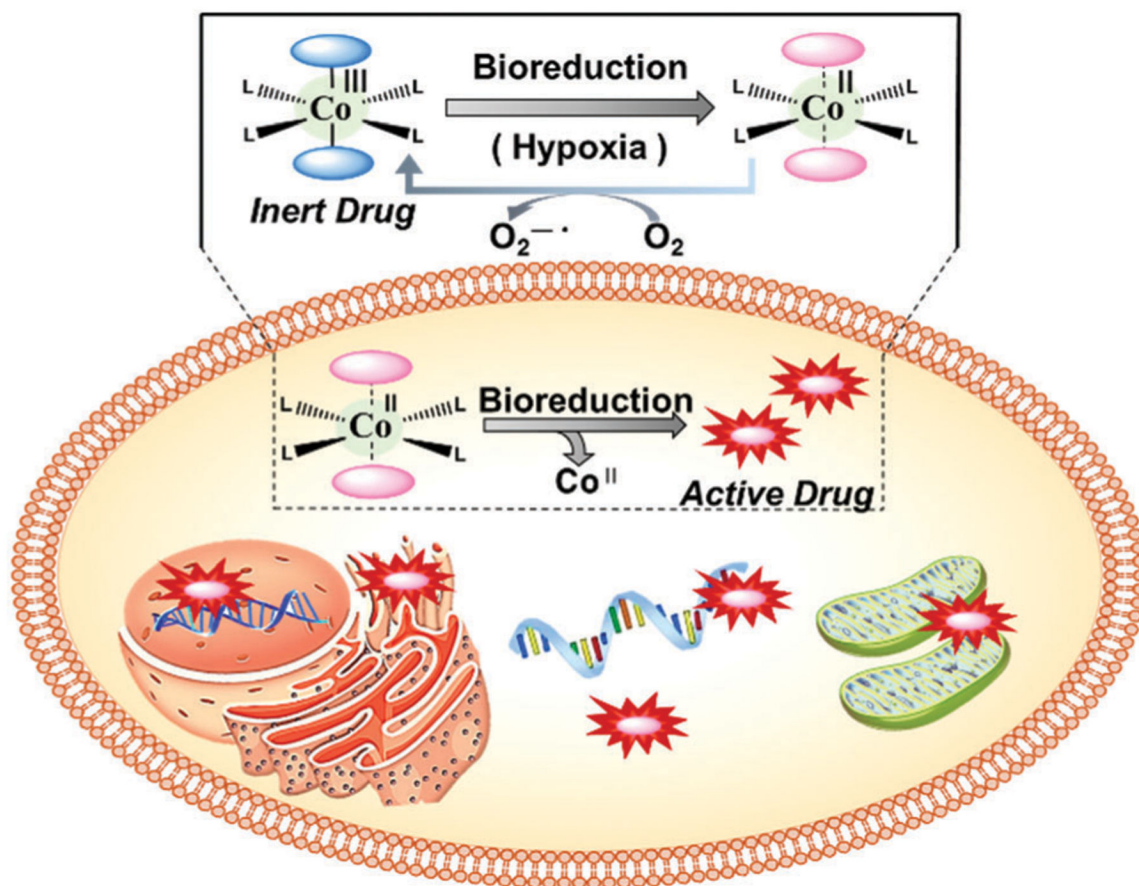


Fig. 16. Proposed mechanism of reductive activation of Co(III) prodrugs.

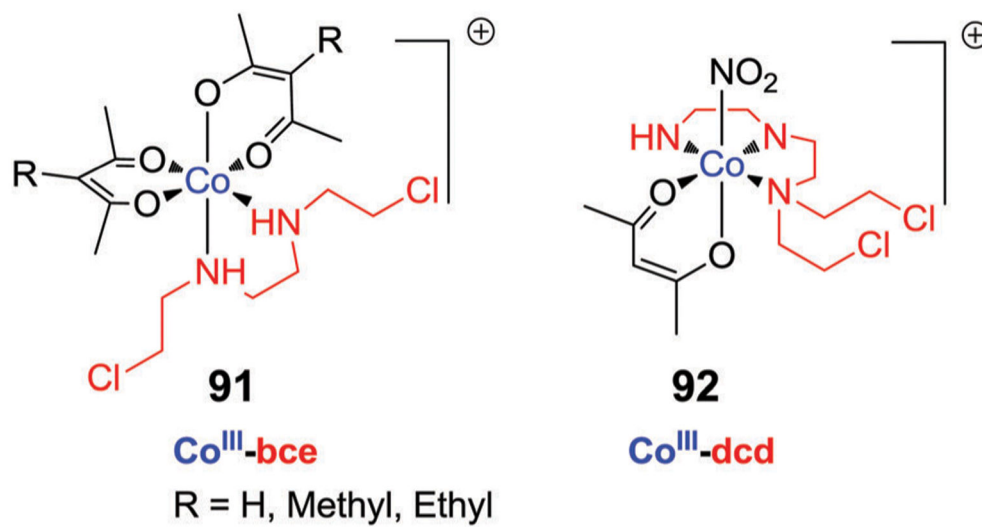
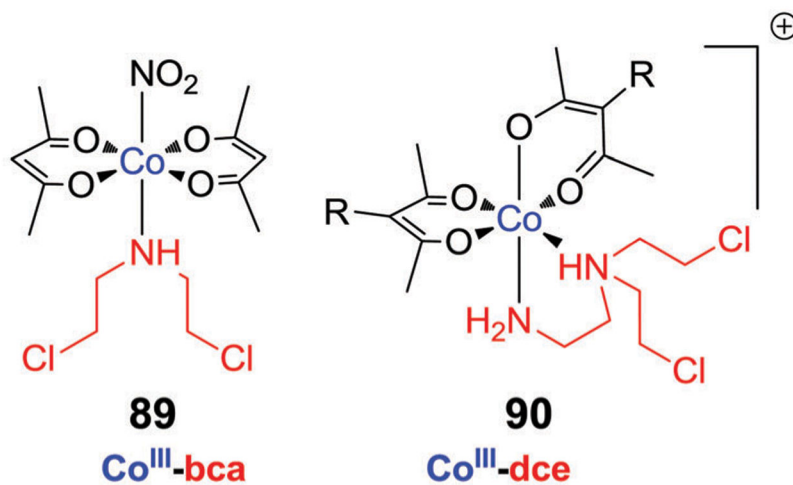


Fig. 17.
Chemical structures of hypoxia-activated cobalt(III) complexes incorporating various ligands.

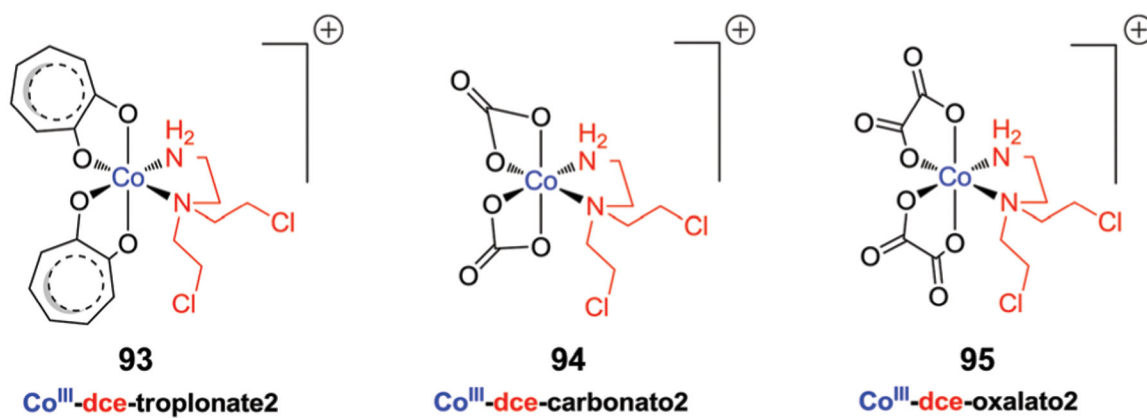


Fig. 18.
Chemical structures of hypoxia activated cobalt(III) complexes.

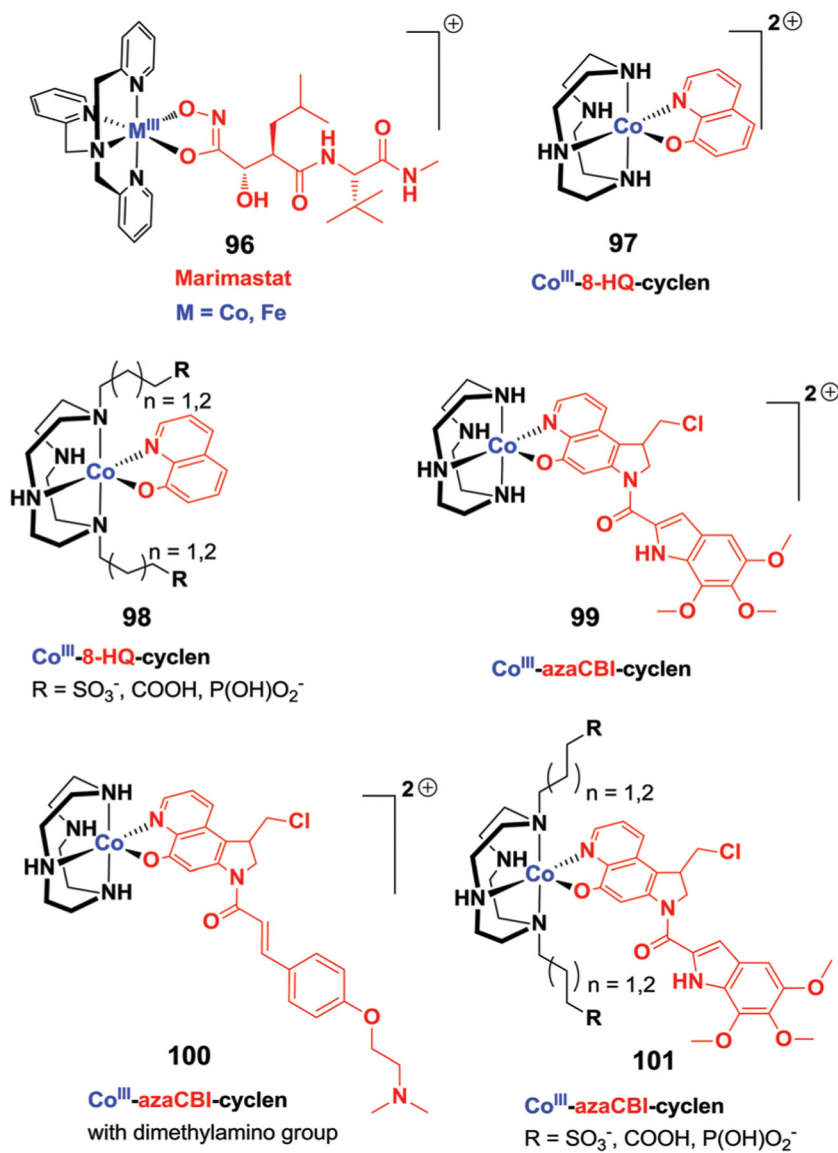


Fig. 19. Chemical structures of $\text{Co}(\text{III})$ complexes bearing tridentate and tetradentate ligands.

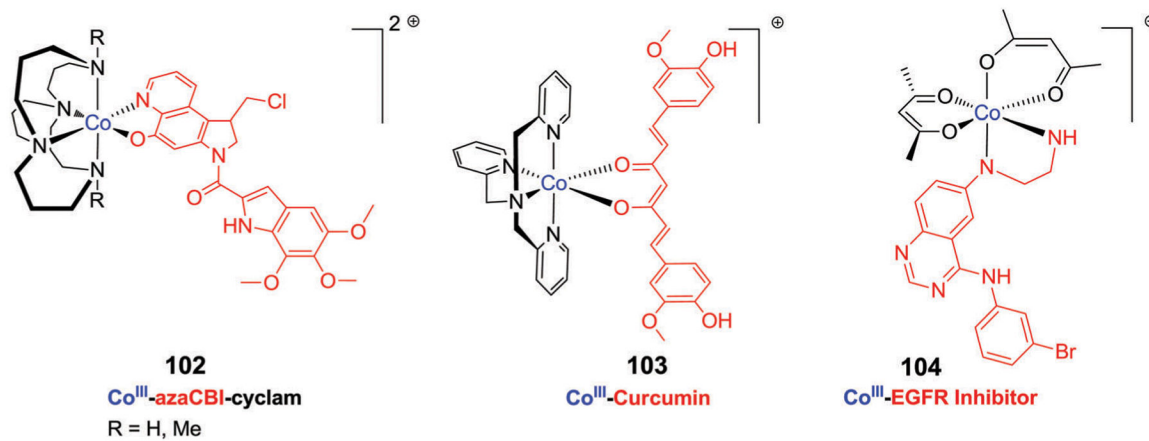


Fig. 20. Hypoxia activated Co(III) cytotoxin complexes bearing various tethered ligands.

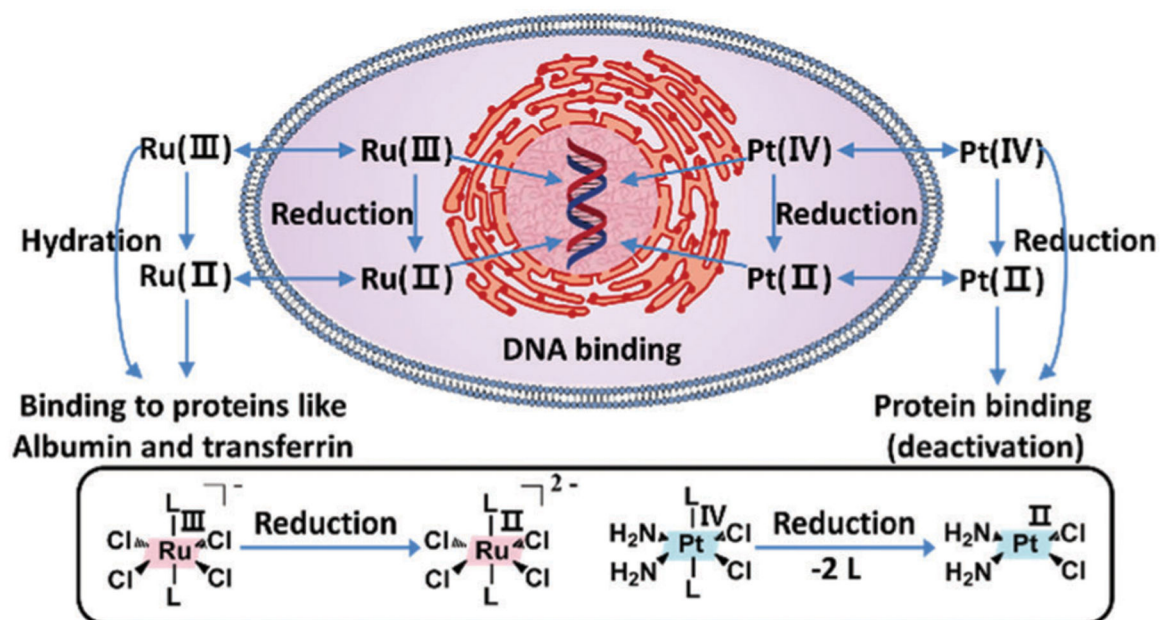


Fig. 21.
Proposed reduction mechanism for Ru(III) and Pt(IV) prodrugs inside and outside the cell.

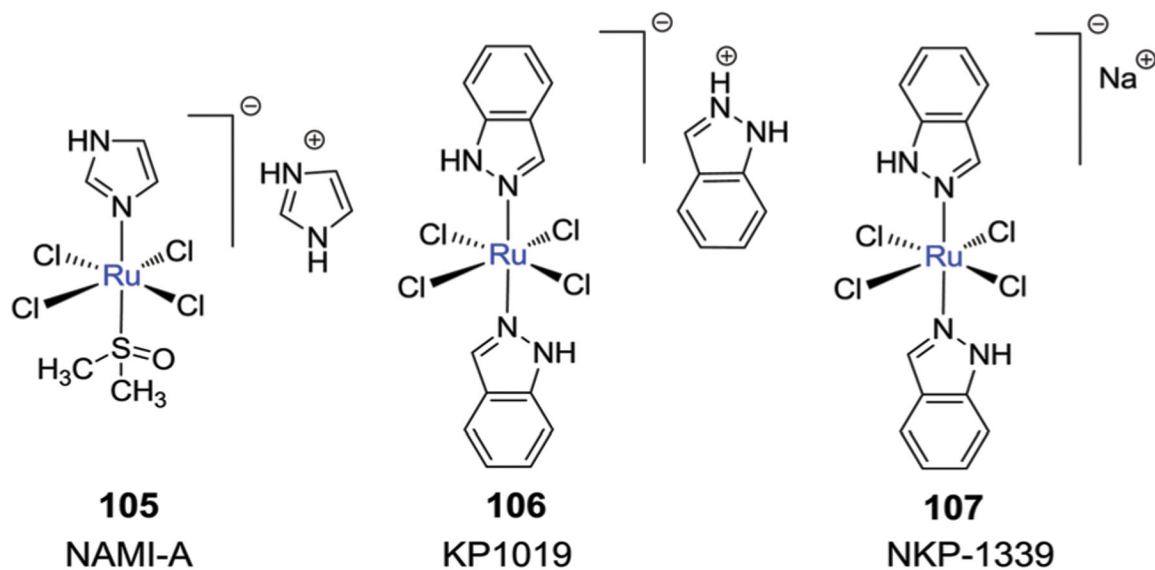


Fig. 22.
Chemical structures of ruthenium complexes of interest as potential anticancer agents.
Currently, **NAMI-A** and **NKP-1339** are under clinical evaluation.

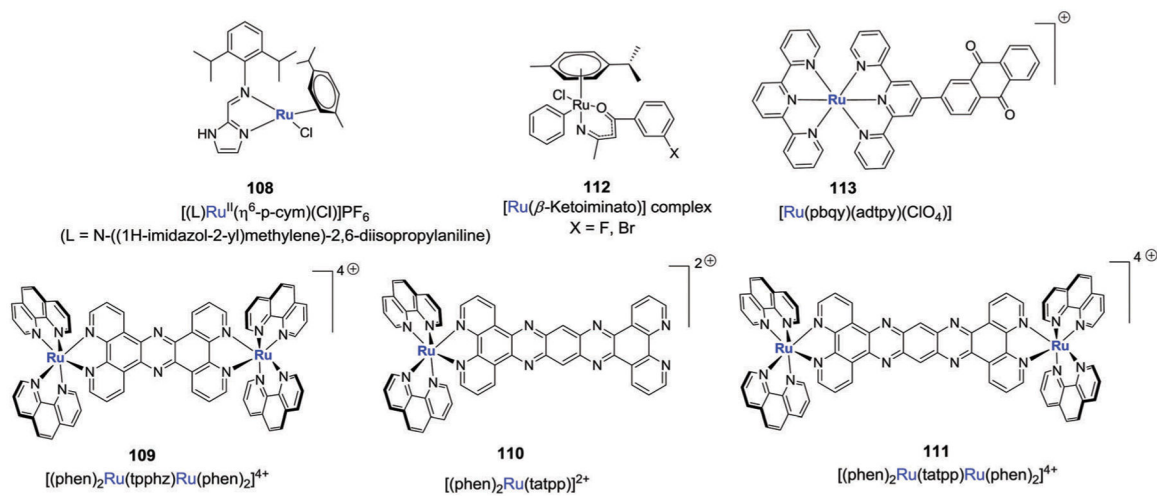


Fig. 23.
Chemical structures of ruthenium complexes (**102–107**).

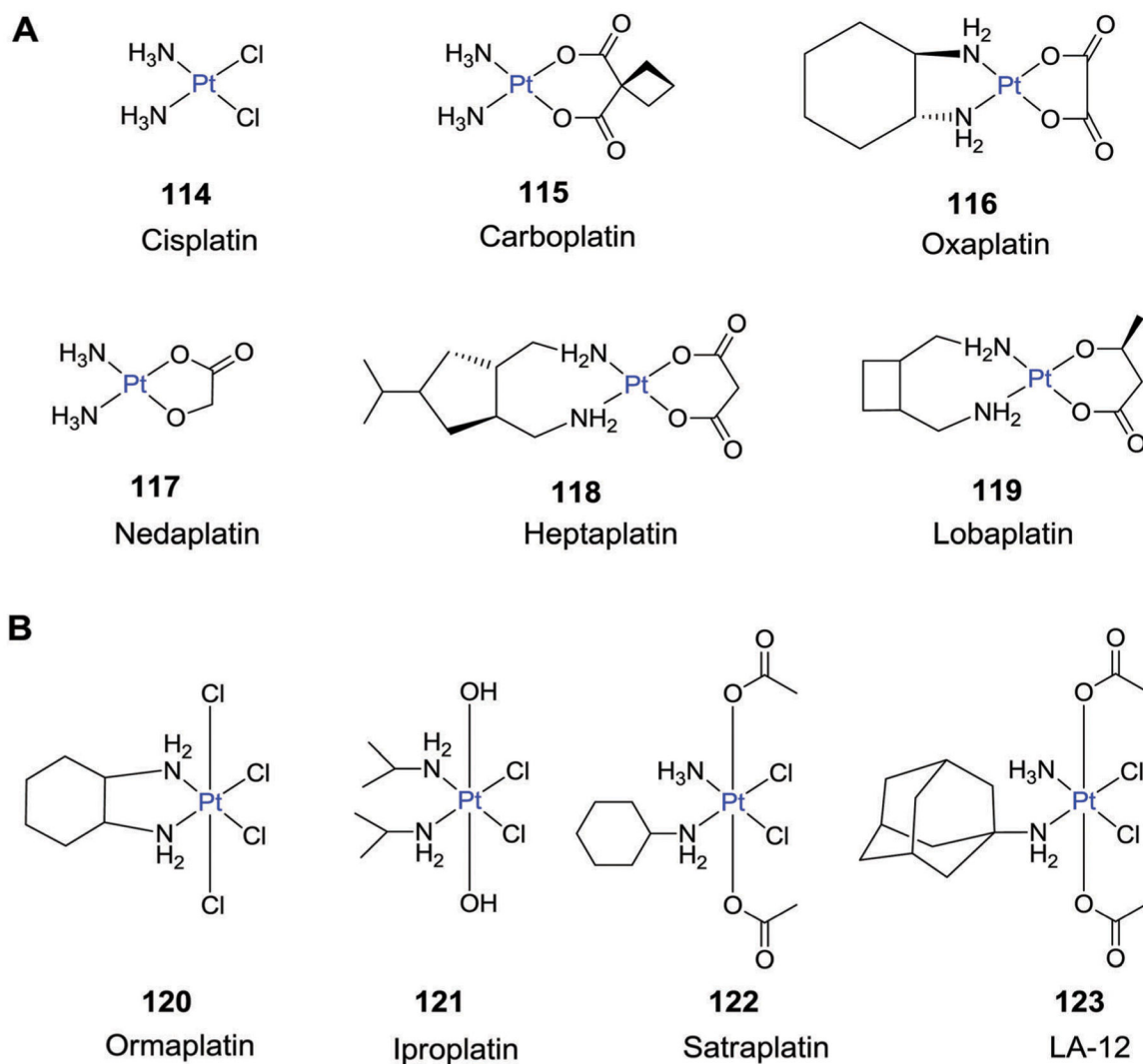


Fig. 24. Chemical structures of (A) clinically approved/marketed platinum(II) complexes and (B) platinum(IV) complexes under clinical investigations.

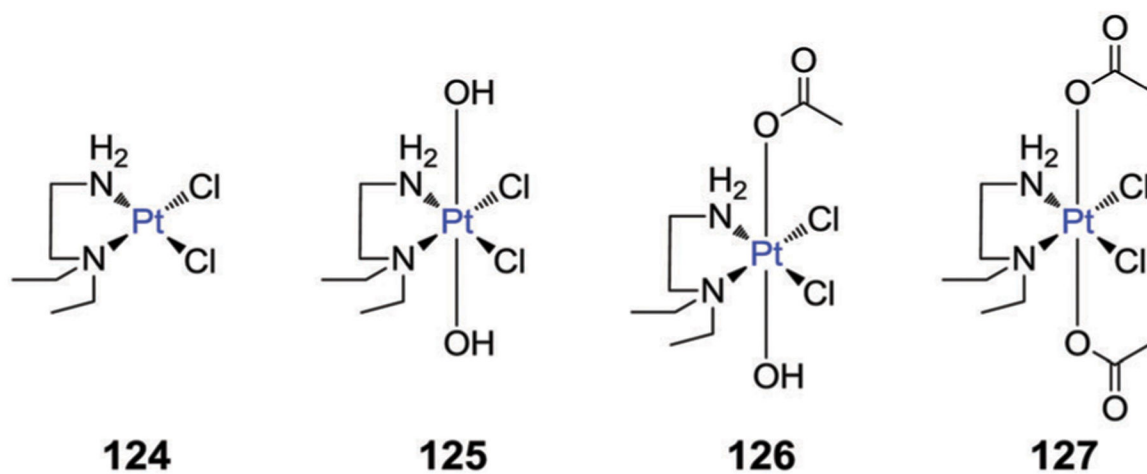


Fig. 25.
Chemical structures of hypoxia-responsive Pt(IV) complexes.

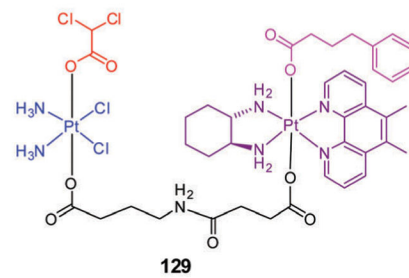
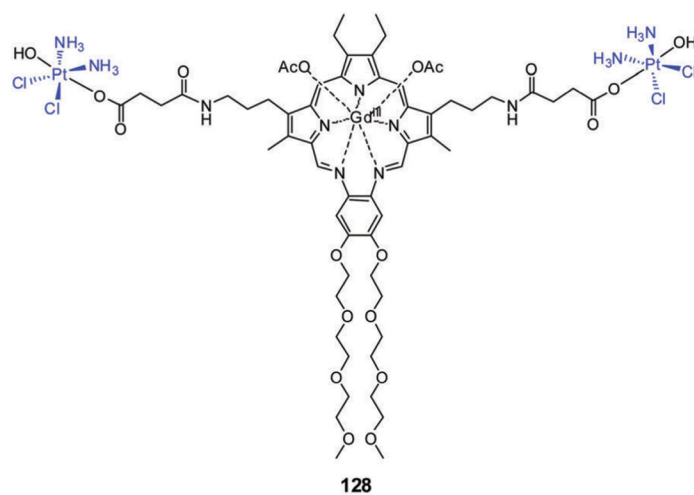


Fig. 26.
Chemical structures of platinum(IV) complexes.

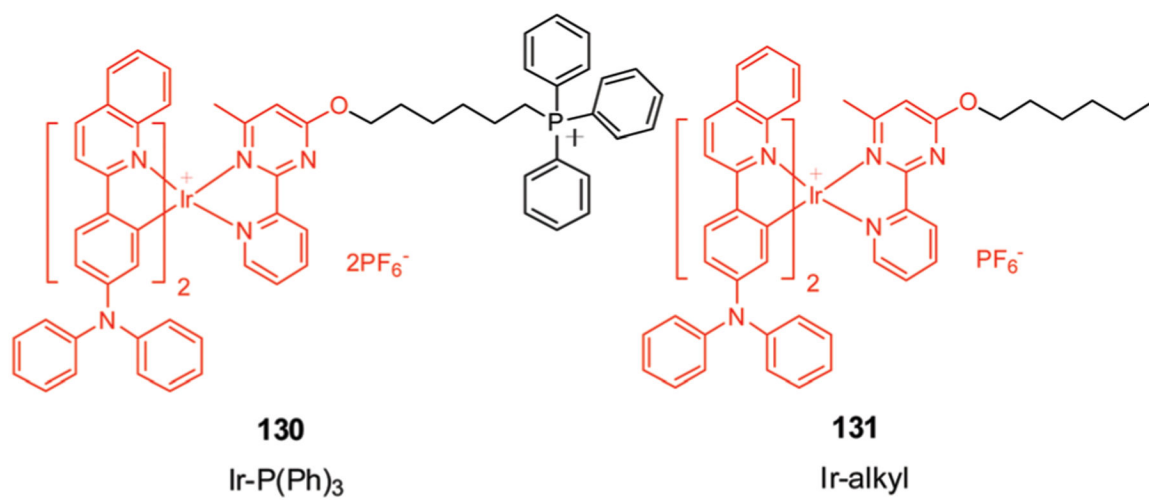


Fig. 27.
Chemical structures of (a) Ir-P(Ph)₃ **124** and (b) Ir-alkyl **125**.

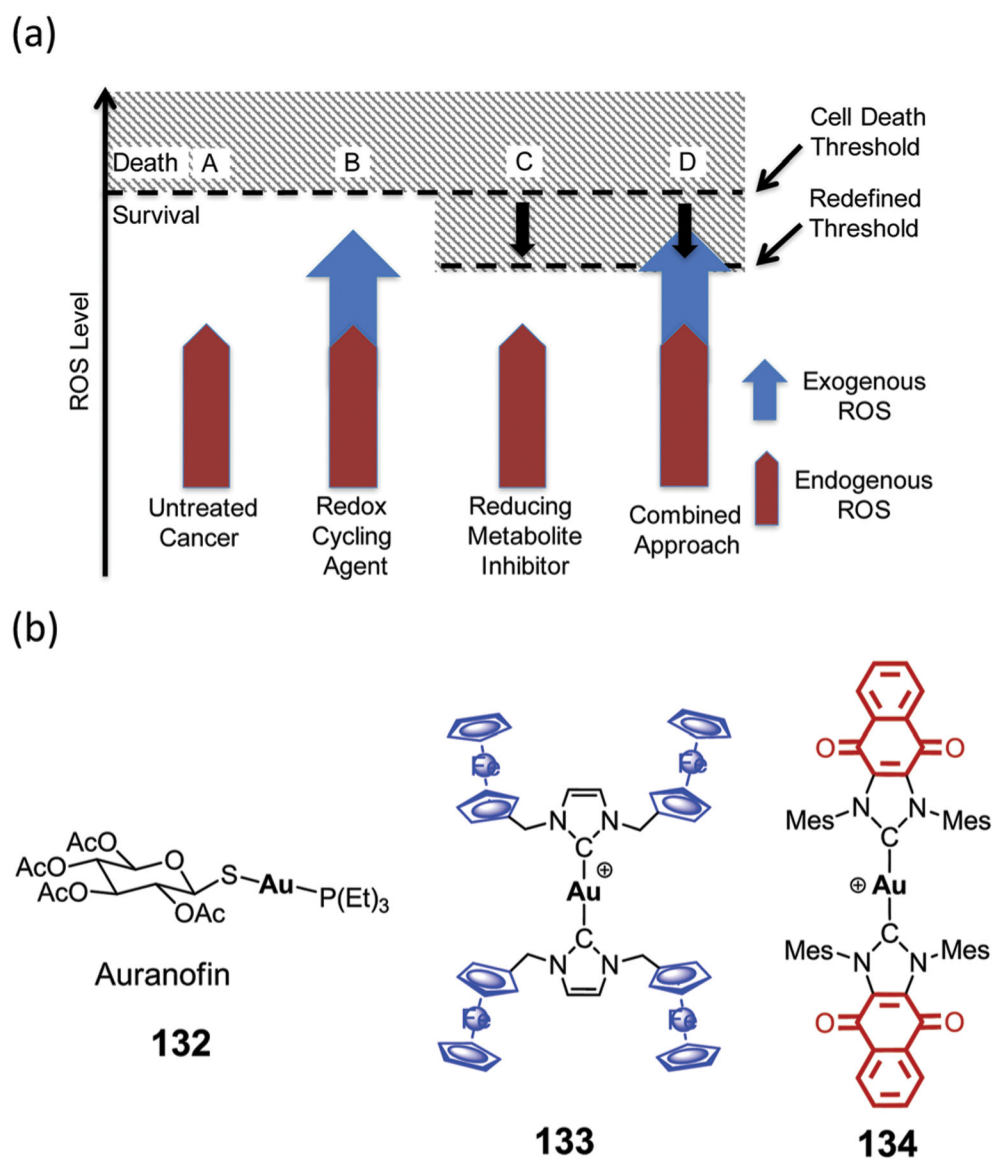


Fig. 28.

(a) Mechanism-based rationale for a dual targeting approach to drug design. (A) Elevated endogenous ROS can be tolerated by cancer cells. (B) Accentuating exogenous ROS by a single mechanism may not reach the cell death threshold. (C) Antioxidant inhibitors reduce the concentrations of reducing metabolites, thus lowering the cell death threshold. (D) The dual targeting approach involves the use of (1) a so-called redox cycling agent to accentuate exogenous ROS that is combined with (2) a reducing metabolite inhibitor to lower the cell death threshold. This combination is expected to overwhelm the system and drive it towards death. (b) Chemical structures of auranofin **132** and dual targeting redox active Au(I) N-heterocyclic carbene-based complexes **133** and **134**. The portion of this figure appearing in (a) was first published in ref. 407 and reproduced with permission. Copyright Royal Society of Chemistry.

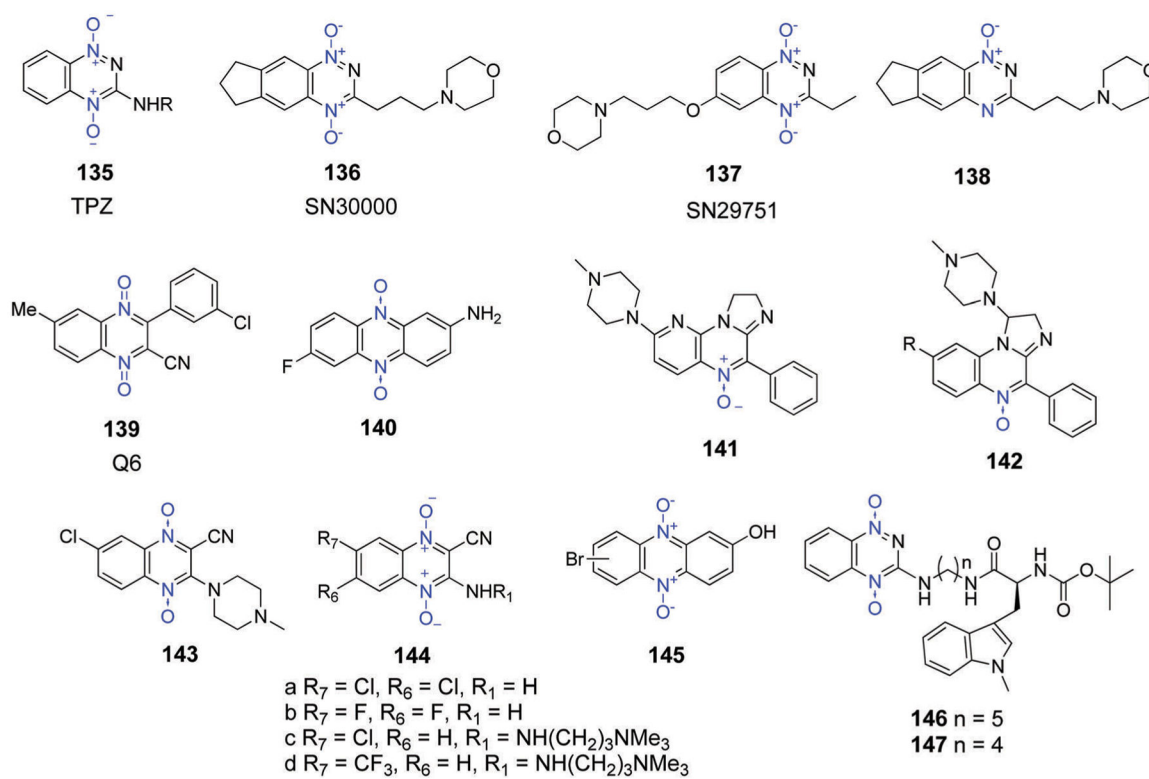


Fig. 29.
Structures of hypoxia-activated aromatic *N*-oxide prodrugs.

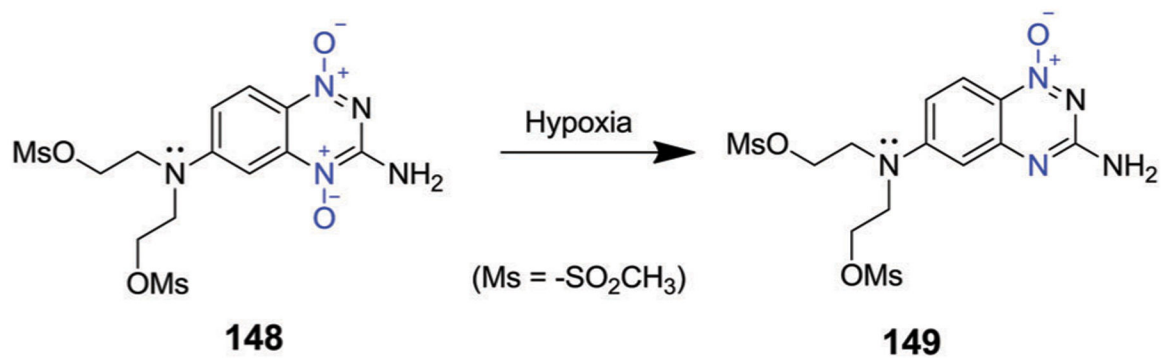


Fig. 30.
Structure and activation of hypoxia-activated aromatic *N*-oxide prodrug **140**.

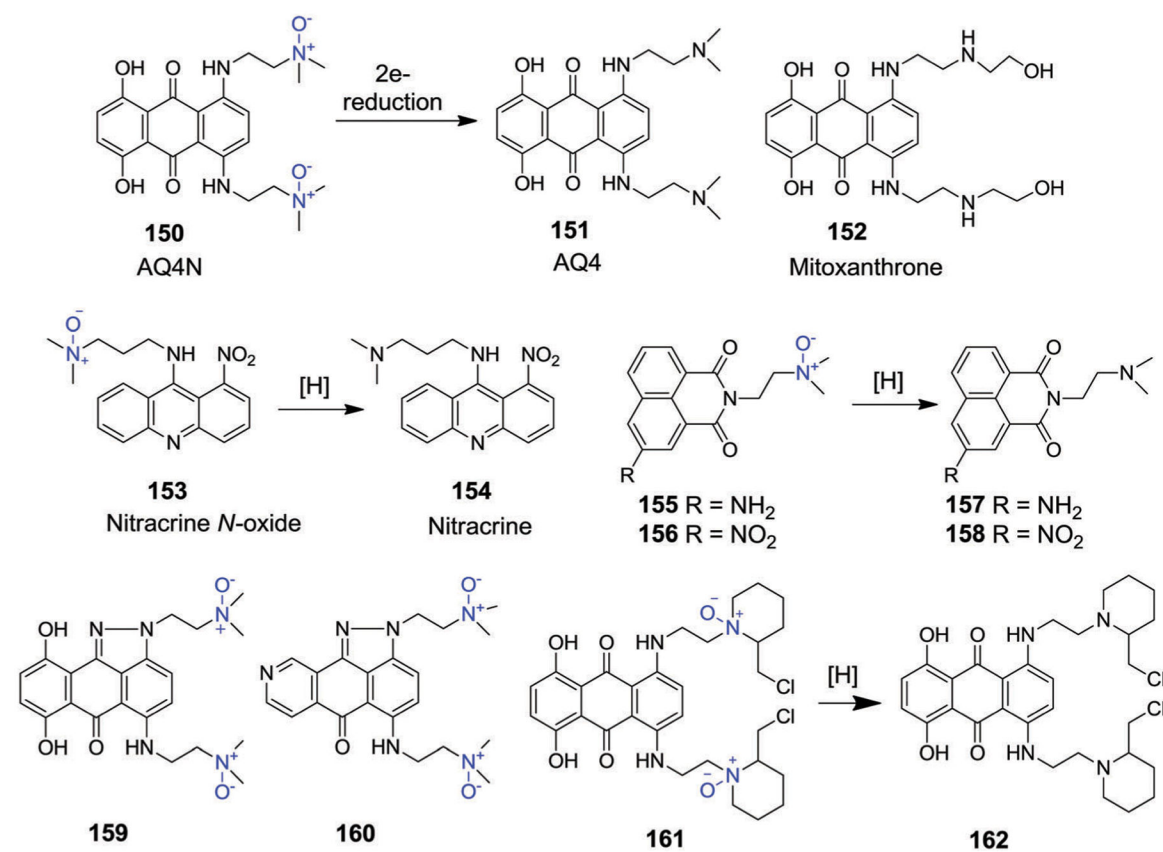


Fig. 31.
Structures of alkyl tertiary amine-based *N*-oxide prodrugs.

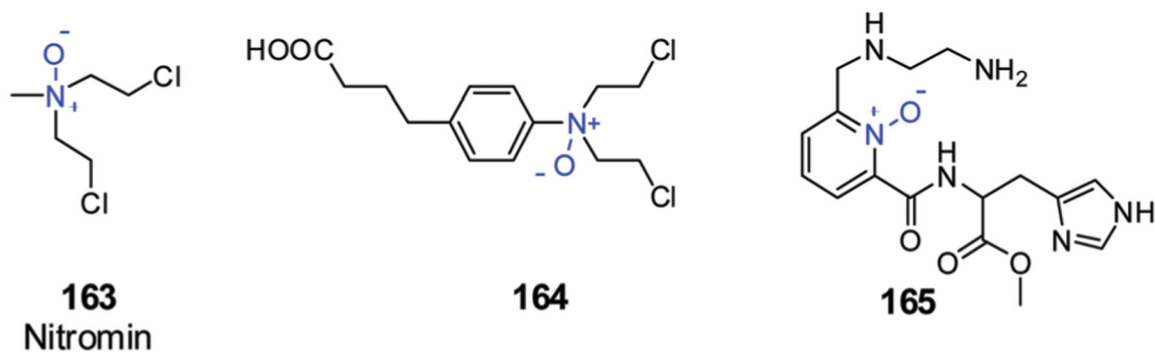


Fig. 32.
Structures of hypoxia-activated prodrugs (HAPs) based on masked cytotoxic agents.