BIOCHEMICAL PHARMACOLOGY 71 (2006) 1683–1694

available at www.sciencedirect.com

SCIENCE DIRECT®

journal homepage: www.elsevier.com/locate/biochempharm

Radiolytic and cellular reduction of a novel hypoxia-activated cobalt(III) prodrug of a chloromethylbenzindoline DNA minor groove alkylator

G-One Ahn, K. Jane Botting, Adam V. Patterson, David C. Ware, Moana Tercel, William R. Wilson *

Auckland Cancer Society Research Centre, The University of Auckland, Private Bag 92019, Auckland, New Zealand

ARTICLE INFO

Article history:
Received 19 January 2006
Accepted 9 March 2006

Keywords:
Tumour hypoxia
Prodrug
Ionising radiation
Co(III) complex
CBI
DNA minor groove alkylator

ABSTRACT

Metabolic reduction can be used to activate prodrugs in hypoxic regions of tumours, but reduction by ionising radiation is also theoretically attractive. Previously, we showed that a cobalt(III) complex containing 8-hydroxyquinoline (8-HQ) and cyclen ligands releases 8-HQ efficiently on irradiation in hypoxic solutions [Ahn G-O, Ware DC, Denny WA, Wilson WR. Optimization of the auxiliary ligand shell of cobalt(III)(8-hydroxyquinoline) complexes as model hypoxia-selective radiation-activated prodrugs. Radiat Res 2004;162:315–25]. Here we investigate an analogous Co(III) complex containing the potent DNA minor groove alkylator azachloromethylbenzindoline (azaCBI, 1) to determine whether it releases 1 on radiolytic and/or enzymatic reduction under hypoxia. Monitoring by HPLC, the azaCBI ligand in the Co(III)(cyclen)(azaCBI) complex (2) slowly hydrolysed in aqueous solution, in contrast to the free ligand 1 which readily converted to its reactive cyclopropyl form. Irradiation of 2 (30–50 μM) in hypoxic solutions released 1 with yields of 0.57 μmol/J in formate buffer and 0.13 μmol/J in human plasma. Using bioassay methods, cytotoxic activation by irradiation of 2 at 1 μM in hypoxic plasma was readily detectable at clinically relevant doses (≥1 Gy), with a estimated yield of 1 of 0.075 μmol/J. Release of 1 from 2 was also observed in hypoxic HT29 cultures without radiation, with subsequent conversion of 1 to its *O*-glucuronide. Surprisingly, overexpression of human cytochrome P450 reductase in A549 cells did not increase the rate of metabolic reduction of 2, suggesting that other reductases and/or non-enzymatic reductants are responsible. Thus the cobalt(III) complex 2 is a promising prodrug capable of being activated to release a very potent cytotoxin when reduced by either ionising radiation or cells under hypoxic conditions.

1. Introduction

There is ongoing interest in the development of therapeutic agents capable of selectively killing hypoxic cells in tumours, including prodrugs that are activated by enzymatic reduction (bioreductive drugs) in the absence of oxygen [1–5]. This approach is attractive because hypoxic cells limit the response of tumours to radiation therapy in at least some sites [6–10]. In addition, because hypoxia is more severe in tumours than in normal tissues [11] it represents a potentially exploitable feature for selective cancer therapy, especially if prodrug activation results in the formation of relatively stable

* *Corresponding author*. Tel.: +64 9 373 7599x86883; fax: +64 9 373 7571.
E-mail address: wr.wilson@auckland.ac.nz (W.R. Wilson).
0006-2952/$ – see front matter
doi:10.1016/j.bcp.2006.03.007

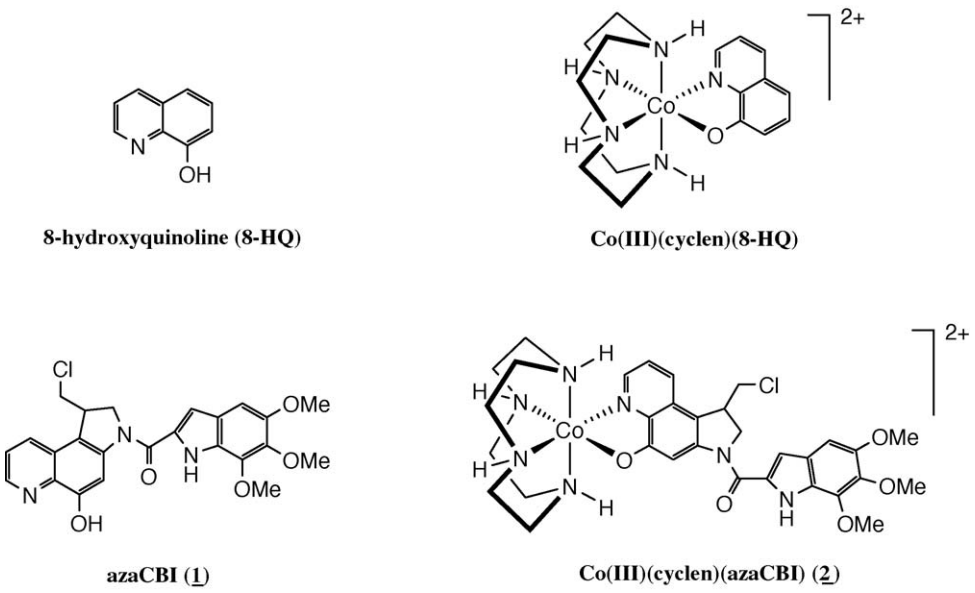

Fig. 1 – Structures of compounds investigated.

metabolites that can diffuse out of hypoxic zones to also kill tumour cells at higher oxygen concentrations. To date most attention has focused on quinones [12,13], nitro compounds [14–16] and *N*-oxides [17,18] as substrates for one-electron reduction in hypoxic cells, but some cobalt(III) complexes can also be activated under hypoxia by reduction [19–21]. Since Co(II) complexes are typically orders of magnitude more kinetically labile than Co(III) complexes [22], reduction of the latter can potentially be used to release cytotoxic ligands such as nitrogen mustards [23–25] or DNA intercalators [26,27] under hypoxic conditions. A similar mechanism has been proposed for Cu(II) complexes, containing nitrogen mustard ligands, as bioreductive drugs [28]. Thus, although relatively unexplored, transition metal complexes containing ligands that are cytotoxic when released warrant consideration as hypoxia-selective bioreductive prodrugs.

We [29,30] and others [31–33] have suggested an alternate approach to reductive activation of prodrugs in hypoxic tissue in which ionising radiation, rather than enzymes and their reducing cofactors, provides the reducing species. This is theoretically possible because absorption of radiation by water generates the strongly reducing aquated electron (e_{aq}^-) which is relatively long-lived in the absence of oxygen. Radiation-activated prodrugs (RAPs) would offer a number of theoretical advantages over enzyme-activated hypoxia prodrugs. If RAPs could be designed to be activated only by radiation, not by reductases, it would be possible to use the spatial targeting ability of radiotherapy to confine activation to the tumour, thereby avoiding toxicity in moderately (physiologically) hypoxic normal tissues such as the retina [34]. In addition, activation would be independent of reductase expression in tumours, which is currently difficult to assess on an individual basis. Further, metabolically stable prodrugs would overcome the problem of excessively rapid consumption as the prodrugs diffuse into hypoxic regions, which is an important limitation in the development of enzyme-activated bioreductive drugs such as tirapazamine [35,36]. If they released stable metabolites able to diffuse appreciable distances, RAPs would also be able to exploit necrotic regions which are hypoxic but lack reductases.

Recently we demonstrated that Co(III) complexes containing 8-hydroxyquinoline (8-HQ; Fig. 1) and a polyazamacrocyclic auxiliary ligand release 8-HQ when reduced by ionising radiation under hypoxic conditions [37]. 8-HQ release was most efficient (showing approximately one-electron stoichiometry) when the auxiliary ligand was a tetradentate macrocycle such as 1,4,7,10-tetraazacyclododecane (cyclen). We used 8-HQ as a model cytotoxic ligand because it contains the same metal chelation site as the DNA alkylator azachloromethylbenzindoline (**1**; azaCBI; 1-(chloromethyl)-3-[(5,6,7-trimethoxy-1*H*-indol-2-yl)carbonyl]-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinolin-5-ol; Fig. 1). This compound is a ring-opened or *seco* analogue of the very potent antitumour antibiotics CC-1065 and the duocarmycins [38,39]. Such ring-opened forms readily cyclise to cyclopropyl intermediates (see for example the formation of **4** from **1** in Fig. 2), and the latter are known to alkylate DNA at N3 of adenine in a sequence selective manner [40]. A very close analogue of **1** (substituted in the pyridine ring with a methoxycarbonyl group) has been shown to exhibit cytotoxicity in the pM range against the L1210 cell line [41]. We anticipated that **1** would also exhibit the high cytotoxicity required for radiation-activation, to compensate for the low molar yield of e_{aq}^- during radiotherapy (typically 0.5 μmol/kg for each radiation fraction, or ca. 30 μmol/kg over a course of fractionated radiotherapy).

In the present study we evaluate whether the novel cobalt complex Co(III)(cyclen)(azaCBI) (**2**; SN 27892; Fig. 1) has appropriate properties for development as a hypoxia-selective radiation-activated or enzyme-activated prodrug. We show that the complex is relatively stable in culture medium, and is much less cytotoxic than the free azaCBI ligand **1**. It is efficiently reduced by radiation to release **1** in hypoxic solutions, including human plasma, and this provides

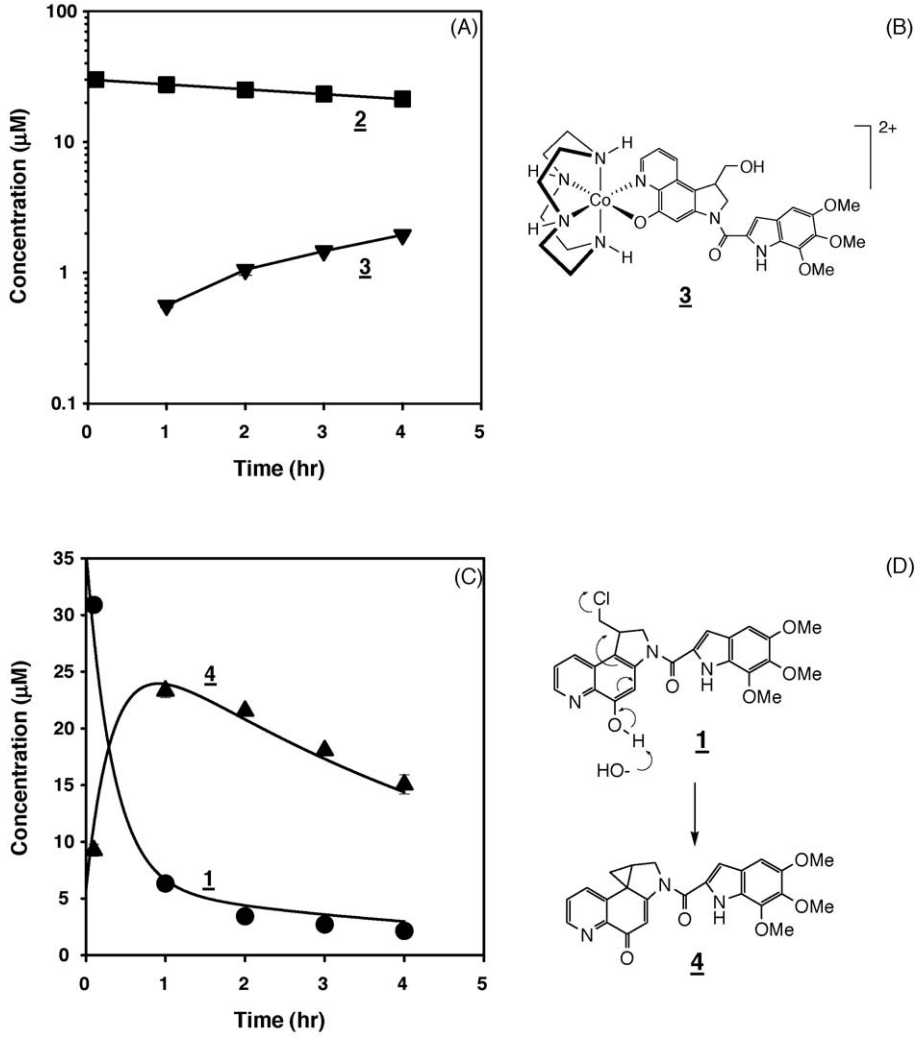

Fig. 2 – Stability of 1 and 2 in culture medium (αMEM with 5% FCS) at 37 °C. (A) Loss of 2 and formation of its hydrolysis product 3; (B) structure of 3; (C) loss of 1 and formation of the cyclopropyl form 4. Lines are fits to the kinetic model described in the text. (D) Scheme for base-induced conversion of 1 to 4.

increased bioactivity (cytotoxicity) even at doses of ionising radiation as low as 1 Gy. The cobalt complex 2 is also significantly activated by metabolic reduction in hypoxic cell suspensions without irradiation.

2. Materials and methods

2.1. Compounds

The azachloromethylbenzindoline (azaCBI) 1, and the Co(III)(-cyclen)(azaCBI) complex 2, were synthesized in this laboratory [42]. Structures of both were confirmed by nuclear magnetic resonance spectroscopy and high-resolution mass spectrometry. 1 was a pale yellow powder and 2, purified as the perchlorate salt, was a purple solid. 1 had a purity of >98% by HPLC, based on absorbance at 320 nm, with a trace of the corresponding cyclopropyl form 4 (see below). An authentic sample of 4 was prepared by addition of NaOH (5 mM final concentration) to a 30 μM solution of 1 in DMSO. 2 was a mixture of two isomers by HPLC; based on absorbance at 320 nm the major and minor isomers comprised 93% and 6% of the total, respectively. Both compounds were stored at −20 °C.

2.2. Radiolytic reduction: HPLC analysis

The cobalt complex 2 was dissolved at 30–50 μM in 5 mM phosphate buffer (pH 7.0) containing 0.1 M sodium formate. Solutions (5 ml) in amber vials were deoxygenated for 2 min using a vacuum pump and irradiated using a ^{60}Co-source (dose rate 21 Gy/min determined by NaCl-modified Fricke dosimetry [43]). Immediately after irradiation, an equal volume of isopropanol was added to each vial to solubilize any released 1. The mixture was then frozen in a cryogenic vial in a dry ice/ethanol bath and stored at −80 °C until analysis by HPLC.

The cobalt complex was also irradiated in human plasma using a method described previously [37]. Briefly, frozen human blood plasma (containing citrate phosphate dextrose solution as anticoagulant) was thawed and centrifuged at 1500 × *g* for 10 min. Plasma was made anoxic by stirring in a

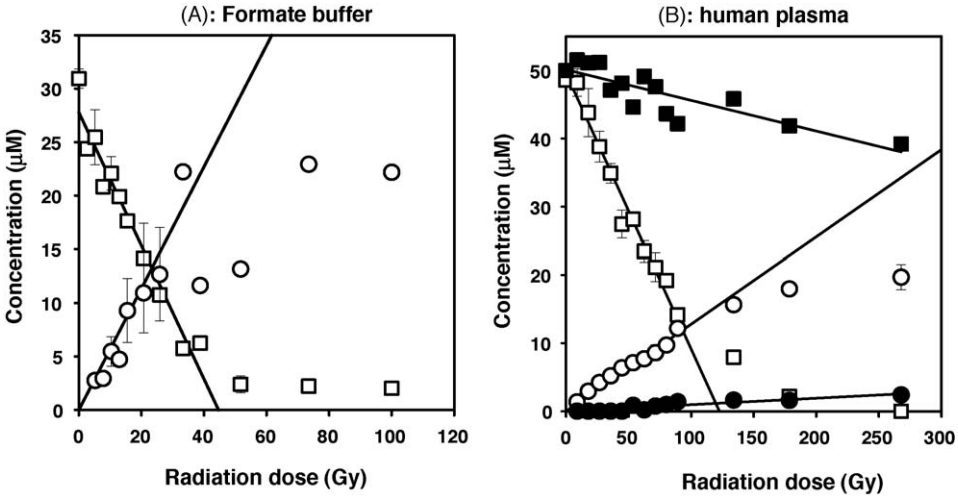

Fig. 3 – Radiolytic reduction of 2 in anoxic formate buffer (A) and oxic (filled symbols) or anoxic (open symbols) human plasma (B), determined by HPLC after gamma irradiation. Squares: loss of 2. Circles: formation of azaCBI (sum of 1 + 4). Errors where shown are ranges for duplicate determinations; other values are single measurements. Lines are linear regression fitted through the initial data points, used to determine *G* values.

glass Petri dish for 3 h in a Pd-catalyst anaerobic chamber (Sheldon Manufacturing Inc., Portland, OR). 2 was dissolved in anoxic dimethylsulfoxide (DMSO) in the chamber and added to 1 ml aliquots of anoxic plasma in 4 ml amber vials (Sun international, Wilmington, NC) to give 50 μM 2 and <1% DMSO. Vials were sealed and removed from the anaerobic chamber for irradiation as above. Oxic plasma containing 2 was irradiated under 20% O_2. The irradiated plasma was deproteinized using 5 vol of ice-cold methanol and spiked with acridan-9-one (100 μM) as internal standard. After freezing at −20 °C for at least 2 h, samples were centrifuged (12,000 × *g* for 10 min at 4 °C). Supernatants were concentrated to a volume of ca. 10 μl using a centrifugal evaporator (Savant Instruments, Farmingdale, NY), diluted to ca. 50 μl with 0.2 M ammonium formate buffer, pH 4.5, and 20–30 μl analysed by HPLC.

2.3. *Radiolytic reduction: bioassay*

Human plasma was made anoxic, and the cobalt complex 2 was added to make a final concentration of 1 μM, otherwise as above. Vials were irradiated using a ^{60}Co source (1 Gy/min; Eldorado G teletherapy unit Nordion Ltd., Ottawa, Ontario). The irradiated plasma was diluted at least 10-fold with culture medium (αMEM containing 5% fetal calf serum (FCS), penicillin (100 IU/ml) and streptomycin (100 μg/ml)) and was bioassayed against log-phase UV4 cells using an in vitro cytotoxicity assay [37]. Briefly, UV4 cells were seeded at 300 cells/50 μl in 96 well plates. The diluted irradiated or non-irradiated human plasma was added the following day, and the plates were incubated (5% CO_2, 37 °C) for 4 h before washing with fresh medium. The cells were grown for further 4 days, fixed with 40% trichloroacetic acid, stained with 0.4% sulforhodamine B, and the absorbance was determined at 490 nm with a reference at 450 nm. The IC_{50} was defined as the apparent drug concentration giving 50% inhibition relative to controls on the same plate.

The equivalent concentration of released cytotoxic effector, [*E*], was calculated according to Eq. (1) which assumes additive effects of the prodrug (2) and effector (1) in mixtures of both species [44] and no significant toxicity for any other products from the reaction:

$$[E] = \frac{((IC_{50},E/IC_{50},M)\times[P]_0) - ((IC_{50},E/IC_{50},P)\times[P]_0)}{1-(1/Y)(IC_{50},E/IC_{50},P)} \quad (1)$$

where $[P]_0$ is the initial prodrug concentration, and IC_{50}, *E* and IC_{50}, *P* are the IC_{50} values of the pure effector (1) and prodrug (2), respectively, and IC_{50}, *M* is the IC_{50} of the mixture of prodrug and effector after irradiation expressed as the apparent IC_{50} of the prodrug. *Y* is the number of moles of 1 formed per mole of 2 consumed; the value of *Y* was taken to be 0.325 based on the stoichiometry of 1 released from 2 in Fig. 3(B). (The estimated *G* value for release of 1, i.e. [*E*]/dose, is relatively insensitive to the assumed value of *Y*, decreasing by only 3% if *Y* is unity).

2.4. *HPLC and LC/MS*

The HPLC system was an Agilent 1100 with diode array detector and/or single quadrupole mass spectrometer (LC/MSD, model D, Agilent Technologies, Walbronn, Germany), using a reverse phase 150 mm × 3.2 mm Altima C8 column (Alltech Associates Inc., Chicago, IL) and a flow rate of 0.5 ml/min. The mobile phase was a gradient of 80% acetonitrile/water (A) and 0.2 M (for HPLC) or 0.1 M (for LC/MSD) ammonium formate buffer in water, pH 4.5, with 5% of A initially, increasing linearly to 95% at 10 min, maintaining at 95% for further 5 min, and decreasing to 5% at 20 min. Absorbance detection was 320 nm (bandwidth 4 nm), with quantitation by integration of peak areas (Chemstation software) with reference to standard curves prepared with authentic compounds in the same medium. The LC/MSD ionization mode was positive electrospray with a

fragmentation voltage of zero, N_2 drying gas flow of 13 l/min and nebulising pressure of 30 psi.

2.5. Metabolism and cytotoxicity in cell suspensions

HT29 human colon carcinoma cells were grown as multicellular spheroids by seeding 10^5 cells into 100 mm diameter bacteriological Petri dishes and transferring to a spinner flask (Bellco Glass Inc., NJ) 7 days later. They were grown for a further 8 days with daily medium replacement after 3 days. The culture medium was αMEM containing 10% FCS with antibiotics as above. Single cell suspensions at 1.5×10^6 cells/ml were prepared by enzymatic dissociation of spheroids in 0.07% trypsin (Difco Laboratories, Detroit, MI) in saline containing trisodium citrate (14 mM, pH 7.6) for 10 min, followed by DNAse I (1 mg/ml) in culture medium for 10 min at 37 °C. A549 human non-small cell lung cancer cells (obtained from ATCC) and A549–P450^{puro} cells (see below) were grown as monolayers in medium with 5% FCS, the latter with 3 μM puromycin, and late log phase cultures were trypsinised to prepare single cell suspensions.

Aliquots (10 ml) of cell suspensions, or culture media without cells, were transferred to glass universal bottles with a screw cap equipped with gas inlet and outlet ports. Vials were incubated in a 37 °C water bath with magnetic stirring while gassing with 5% CO_2 in O_2 or N_2 as detailed previously [35]. After equilibration for 60 and 90 min under oxic and hypoxic conditions, respectively, compounds were added from N_2-purged stock solutions in DMSO. Oxygen concentrations in the hypoxic suspensions under these conditions were <0.1 μM as determined using an Oxylite fibre optic probe (Oxford Optronics, UK). Samples (1 ml) were removed at intervals; 40 μl was stained with trypan blue (GibcoBRL, NY) to assess cell viability (>80% in all cases) and the balance used to assess clonogenic cell survival or metabolism of the complex. For clonogenic assay, cells were washed with fresh medium by centrifugation, enumerated with an electronic particle counter (Coulter Electronics Ltd., Luton, England), diluted and plated in 60 mm Petri dishes which were incubated for 14 days before staining with methylene blue. Colonies with >50 cells were scored to determine plating efficiency.

Metabolism of the complexes was assessed by centrifuging the cell suspension at 12,000 × *g* for 3 min. The pellets were briefly centrifuged again and remaining medium was aspirated. The cell pellets, and extracellular medium from the first spin, were stored at −80 °C for subsequent analysis by HPLC. Thawed samples of extracellular medium (50 μl) containing 5% FCS were injected into the HPLC without any further work-up. The cell pellet was lysed with milli Q water (90 μl). Ten volumes of ice-cold methanol were added followed by addition of acridan-9-one to 100 μM as internal standard. The dried supernatant was reconstituted in 60 μl of DMSO and 30 μl was analysed by HPLC. The intracellular water content of 1.23 μl/10^6 HT29 cells, determined previously using the spin-through-oil technique [35], was used to calculate intracellular concentrations.

2.6. A549–P450R^{puro} cells

A549 cells were used to generate a stable transfectant (A549–P450R^{puro}) overexpressing human cytochrome P450 reductase (P450R; E.C.1.6.2.4). In brief P450R cDNA was introduced as a 2.3 kb EcoRI-Not I restriction fragment into the bi-cistronic plasmid F373, which has been shown to provide high-level stable gene expression in vitro [45], to create pEF–P450R^{puro}. Restriction mapping and partial sequence analysis confirmed the product and endotoxin-free DNA was prepared (Qiagen Inc, CA) [46,47]. Exponentially growing A549 cells were harvested by trypsinisation and seeded at 2×10^5 cells/ml with overnight recovery. One microgram of the pEF–P450R^{puro} plasmid was mixed with 2 μg of Fugene6 (Roche Diagnostics, CH) in 97 μl αMEM and allowed to stand (1 h) at room temperature. The DNA:liposome mix was added dropwise onto cells in αMEM (containing 5% FCS) and the following day cells were recovered as a single-cell suspension, counted and plated at various densities onto 10 cm diameter tissue culture plates. Forty-eight hours later 1 mM puromycin was added to each plate and at 72 h intervals thereafter. Once no further cell death was observed, the puromycin concentration was escalated to 3 μM. After 18–20 days large colonies had formed which were isolated using cloning rings, replated into 5 μM puromycin, and sub-cloned. Individual colonies were expanded and cryopreserved. A sample of each population was grown on in the absence of puromycin and S9 fractions were prepared and tested for P450R enzyme activity as previously described [48].

2.7. Hypoxic-selective cytotoxicity

Cytotoxicity of **1** and **2** was determined by clonogenic assay under oxic and hypoxic conditions, using HT29 single cells obtained as for the metabolism study. The gassed and stirred single cell suspensions were sampled at intervals and were washed with fresh culture medium by dilution and centrifugation. The washed cells were resuspended in fresh culture medium (5% FCS plus antibiotics) and the cell density was measured by the electronic particle counter. The cells were then plated at densities of up to 2×10^4 cells/ml in 60 mm diameter tissue culture dishes (Becton Dickinson, NJ) and grown for 14 days before staining with methylene blue (GibcoBRL, NY). Cytotoxicity as determined as the drug concentration required to reduce cell survival to 10% of controls, and the ratio of these values were used to assess hypoxic selectivity.

3. Results

3.1. Stability of compounds in culture medium

The Co(III) complex **2** showed good stability in culture medium (at 37 °C; Fig. 2(A)) with a half-life of approximately 20 h. Its slow disappearance was accompanied by formation of a new species **3**, with a shorter HPLC retention time but almost identical absorbance spectrum (data not shown). The positive mode electrospray mass spectrum of **2** showed a base peak (*m*/*z* 348.5) corresponding to the expected M^{2+} molecular ion and also showed the expected ^{13}C and ^{37}Cl nuclide peaks. The mass spectrum of **3** showed a base peak at *m*/*z* 339.7, with no ^{37}Cl peak, consistent with its assignment as the M^{2+} ion of the hydrolysis product of **2** (Fig. 2(B)).

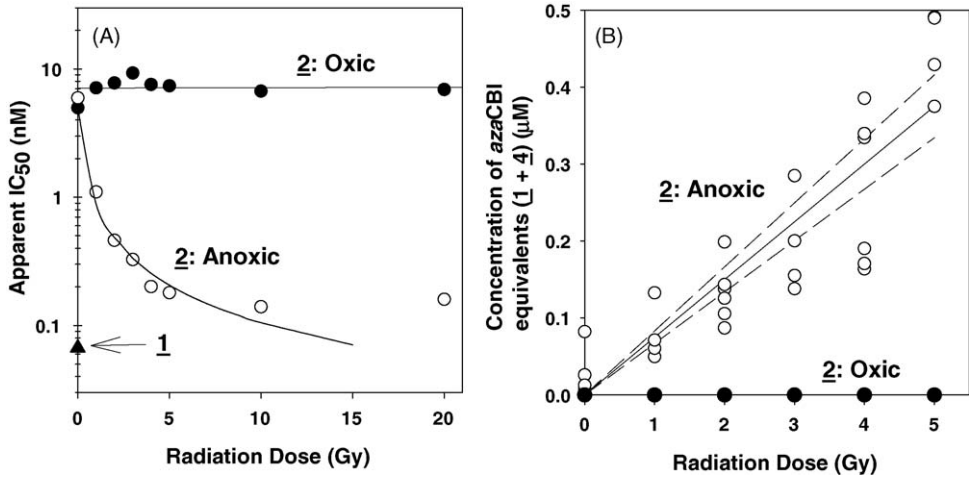

Fig. 4 – Radiolytic reduction of 2 at low concentration (1 μM) in oxic (filled symbols) and anoxic (open symbols) human plasma, determined by bioassay against UV4 cells. (A) changes in apparent IC_{50} of 2 with irradiation under oxic (95% O_2; ●) and anoxic (○) conditions in a single representative experiment. The IC_{50} of 1 was determined in the same experiment by freshly dissolving the azaCBI in DMSO and diluting into culture medium (filled triangle). The line through the anoxic data is the fit to Eq. (1). (B) The estimated release of azaCBI equivalents (1 + 4) from 2 calculated from Eq. (1), for five experiments. The line is the linear regression through the origin, with 95% confidence limits as dashed lines.

Under the same conditions, 1 was less stable with rapid conversion to 4 (Fig. 2(C)), which eluted earlier than 1 and had a distinct absorption spectrum (absorbance maximum 360 nm versus 310 nm for 1). The mass spectrum of 1 showed a base peak at *m/z* 467.9, corresponding to the expected $[M + H]^+$ ion, while 4 showed a base peak at *m/z* of 432.0 with loss of the ^{37}Cl peak. This is consistent with the $[M + H]^+$ ion of the cyclopropyl product of 1. The base-induced intramolecular cyclisation of 1 to 4 is shown in Fig. 2(D). Spirocyclisation of 1 to 4 could be described by zero order kinetics with subsequent first order loss of 4, leading to loss of total azaCBI (1 + 4) with a half-life of about 3 h.

3.2. *Radiolytic reduction of 2*

Whether the cobalt complex releases 1 on reduction was investigated using ionising radiation to generate free radical reductants with known stoichiometry. Irradiation of water generates $OH^•$, $H^•$ and the aquated electron (e_{aq}^-) as the primary radical species, but the oxidizing $OH^•$ and $H^•$ radicals can be converted to the reducing $CO_2^{•-}$ radical by abstraction of $H^•$ from HCO_2^- (formate) to form a cleanly reducing system [49]. As shown in Fig. 3(A), irradiation of 2 in deoxygenated neutral sodium formate buffer resulted in loss of the cobalt complex and formation of azaCBI (quantified as 1 + 4). The *G* value (radiation chemical yields) for loss of 2 ($G(-2)$ = 0.68 μmol/J) and formation of azaCBI ($G(1 + 4)$ = 0.57 μmol/kg) were close to that for total radiolytic reductants in this system ($G(e_{aq}^- + CO_2^{•-})$ = 0.68 μmol/J; [50]) indicating a one-electron stoichiometry of reduction with very efficient release of the cytotoxic effector.

We next tested radiolytic activation in a more physiological milieu, without addition of artificial radical scavengers, by irradiating 2 in anoxic human plasma (Fig. 3(B)). Loss of 2 was almost as efficient as in formate buffer, with a $G(-2)$ of 0.40 μmol/J. Release of azaCBI (1 + 4) was again observed, but with somewhat lowered efficiency ($G(1 + 4)$ = 0.13 μmol/J). This radiolytic consumption of 2 and release of azaCBI was strongly inhibited by oxygen, which lowered $G(-2)$ to 0.05 μmol/J and $G(1 + 4)$ to 0.01 μmol/J.

The oxygen-inhibitable radiolytic reduction of the cobalt complex to release the very potent azaCBI ligand suggested that irradiation of anoxic plasma containing cobalt complex 2 should provide efficient cytotoxic activation of the prodrug. To test this, we irradiated anoxic plasma containing 1 μM 2, diluted the irradiated plasma at least 10-fold into culture medium, and bioassayed against UV4 cells using a proliferation assay as illustrated for a representative experiment in Fig. 4(A). Irradiated plasma lacking 2 did not show any cytotoxicity under these conditions (data not shown), but the apparent IC_{50} of 2 in irradiated anoxic plasma showed a marked decrease with radiation dose to reach a value almost as low as authentic 1 (which showed an IC_{50} of 0.067 ± 0.007 nM over six such experiments). In the experiment in Fig. 4(A), a radiation dose of only 2 Gy was sufficient to lower the apparent IC_{50} of 2 by 11-fold (from 4.94 ± 0.77 nM to 0.43 ± 0.03 nM). In contrast, no change in the IC_{50} of 2 was observed on irradiation in oxygenated plasma. The apparent IC_{50} of the irradiated solutions was used to estimate the yield of azaCBI under anoxic plasma using Eq. (1); Fig. 4(B) shows these estimates combined for all six experiments. This indicated a *G* value for free azaCBI equivalents (1 + 4) of 0.075 μmol/J (95% confidence limits 0.067–0.083 μmol/J), which is 58% of the value in plasma using a 50-fold higher starting concentration of 2 as determined by HPLC above (Fig. 3(B)).

3.3. *Biotransformation of 2 in HT29 cells*

Whether 2 is subject to biotransformation in cells was investigated using stirred suspensions of aerobic and hypoxic HT29 cells at 1.5×10^6 cells/ml, using HPLC to assess the parent prodrug and its products in both the extracellular

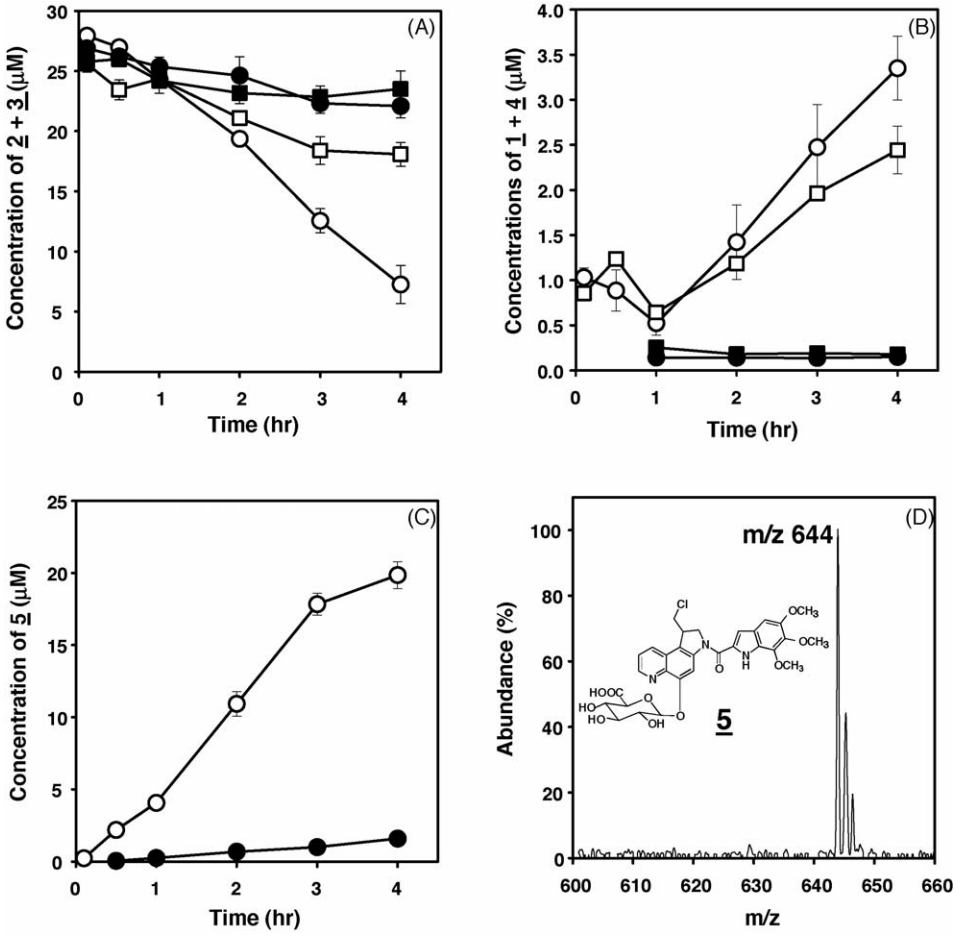

Fig. 5 – Cellular metabolism of 2 in oxic (filled symbols) or anoxic (open symbols) HT29 cell suspensions (1.5×10^6 cells/ml) as detected in the extracellular medium (circles) or in culture medium without cells (squares). Symbols are the mean ± S.E.M. for triplicate determinations. (A) Loss of 2 + 3; (B) formation of azaCBI (1 + 4); (C) formation of the metabolite 5; (D) Positive mode electrospray mass spectrum and deduced structure of 5, the glucuronide metabolite of 1.

medium (Fig. 5) and the intracellular compartment (Fig. 6). Fig. 5(A) shows that concentrations of 2 showed little change with time in the extracellular medium of aerobic cultures, or in aerobic culture medium, with the exception of slow hydrolysis to 3 as noted above. The complex was less stable under hypoxic conditions, showing progressive loss of the parent in the extracellular medium with some loss also seen in hypoxic medium without cells (Fig. 5(A)). In both extracellular medium and cell-free medium the loss of 2 under hypoxia was accompanied by formation of azaCBI (1 and its cyclopropyl form 4; Fig. 5(B)). A new metabolite (5) was observed in the medium of hypoxic HT29 cells, with much lower levels in aerobic cultures (Fig. 5(C)); this species was not detectable in cell-free medium. 5 eluted earlier than 1 and 4 in the HPLC chromatogram but showed a similar absorbance spectrum to 1 (data not shown) so was quantified assuming the same molar extinction coefficient as 1. The electrospray mass spectrum of 5 showed a base peak at *m/z* of 644, consistent with the $[M + H]^+$ ion of the O-glucuronide derivative of 1 and the observed retention of the ^{37}Cl peak at *m/z* of 646 (Fig. 5(D)). This assignment was supported by separate experiments showing that incubation of 1 with aerobic HT29 cells gives a metabolite identical to 5 in retention time, absorbance spectrum and mass spectrum (data not shown).

The intracellular concentrations were also measured in cell pellets, although the methanol deproteinisation/evaporative concentration method used provided variable and low (ca. 10%) recovery of 2 from spiked cell lysates with ca. 10% conversion to 1 + 4 during the concentration step. Estimates were in the range 100–500 μM 2 in cells; the much higher concentrations than in the extracellular medium (Fig. 5(A)) may reflect sequestration in cells because of non-covalent binding of the azaCBI unit in the minor groove of DNA. Higher levels of azaCBI were demonstrated in hypoxic than oxic cells, but concentrations of 1 + 4 in oxic cells were largely accounted for by conversion during workup and reliable quantitation of these species (1, 2 and 4) was therefore not achieved. The O-glucuronide 5 was also detected in cells, at higher concentrations under hypoxia, as was a new product 6 (Fig. 6(A)) which had a mass spectrum (Fig. 6(B)) identical to 1, similar absorbance spectrum, different (longer) retention time and greater stability. On this basis 6 was identified as the ring-expanded analog of 1 arising from nucleophilic attack by Cl^- on the tertiary carbon of the cyclopropyl ring of 4 (Fig. 6(B))

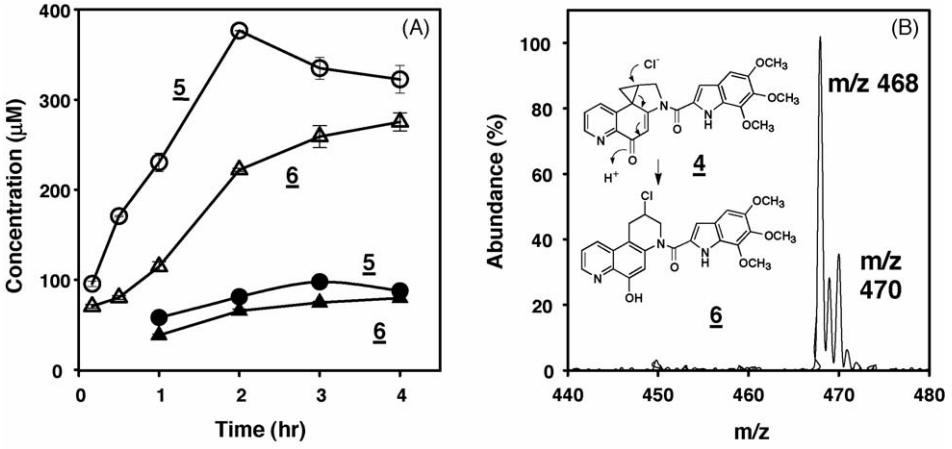

Fig. 6 – Intracellular metabolites of 2 in HT29 cells from the same experiments as Fig. 5. (A) Concentration of the *O*-glucuronide 5 and the ring-expanded metabolite 6 under oxic (filled symbols) or hypoxic (open symbols) conditions. (B) Positive mode electrospray mass spectrum of 6 and its proposed mechanism of formation from the cyclopropyl intermediate 4.

rather than at the secondary carbon which generates 1. 6 was quantified in Fig. 6(A) assuming the same molar extinction coefficient as 1.

The overall mass balance for biotransformation of 2 was evaluated by summing the species in the intracellular and extracellular compartments and expressing as a volume-averaged concentration (Fig. 7). Although not accurately determined as noted above, intracellular concentrations of 1, 2 and 4 were included but these make little contribution to overall mass balance at these cell densities since only 0.2% of the culture volume is intracellular. The mass balance for all species was almost constant over time indicating that the major species are accounted for. In summary, under anoxic conditions the cobalt complex 2 is extensively converted by HT29 cells to the free azaCBI ligand (1) and its derivatives 4, 5, and 6 with the *O*-glucuronide 5 as the major stable end product.

3.4. *Biotransformation of 2 in A549 cells*

The formation of azaCBI and related products in hypoxic HT29 cells suggests enzymatic one-electron reduction of the cobalt complex 2. Because cytochrome P450 reductase (P450R) is considered to be the major one-electron reductase in mammalian cells [51], we overexpressed human P450R in A549 cells and compared rates of reduction of 2 in the wild type and stably transfected cell lines under the same conditions as used for HT29 cells above. The rate of loss of 2 in hypoxic A549–WT cell suspensions was faster than in HT29, with extensive formation of azaCBI species (1, 4 and 6).

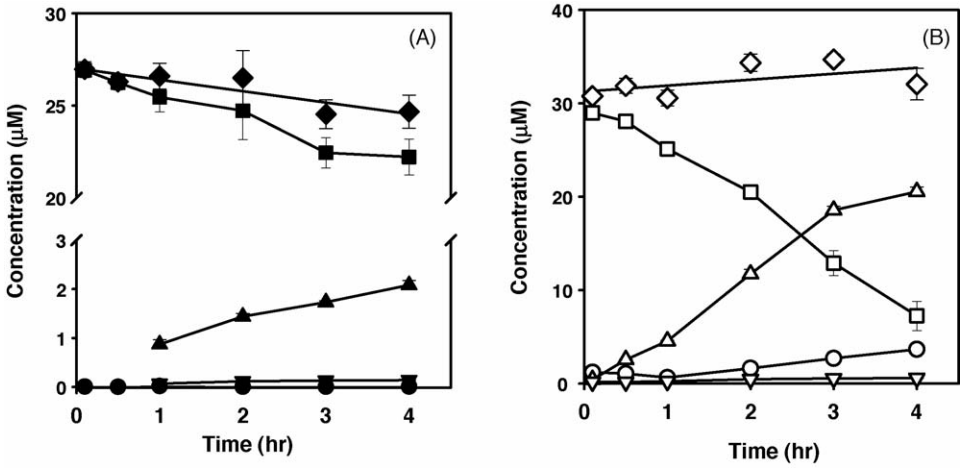

Fig. 7 – Mass balance for the reduction of 2 in HT29 cell suspensions (1.5×10^6 cells/ml) determined by summing the parent complex and products in the intracellular and extracellular compartments and averaging over the whole culture volume. (A) Oxic conditions; (B) anoxic conditions. Squares: cobalt complexes (2 plus its hydrolysis product 3); circles: azaCBI (1 + 4); triangles: the azaCBI glucuronide (5); inverted triangles: 6; diamonds: sum of all species. Values are mean ± S.E.M. for triplicate cultures.

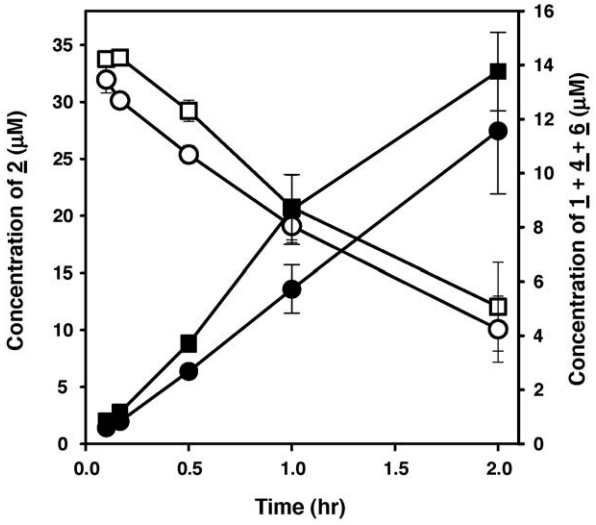

Fig. 8 – Reduction of 2 in hypoxic suspensions of A549–WT (squares) and A549–P450R^{puro} (circles) cells (1.5 × 10^6 cells/ml) as monitored by HPLC of the extracellular medium. Open symbols show loss of the parent complex (2) and filled symbols show the summed concentration of all species derived from uncomplexed azaCBI (1 + 4 + 6). The glucuronide 5 was not formed in these cell lines. Values are the mean ± S.E.M. for triplicate determinations.

Again, the level of 6 was much lower than that of 1 + 4 in the extracellular medium. A conspicuous difference from HT29 cells was the absence of the glucuronide 5. Surprisingly, the kinetics of reduction of 2 was essentially the same in A549–P450R^{puro} cell suspensions (Fig. 8), despite a nine-fold higher activity of P450R in the latter cells (15.0 and 135 nmol cytochrome c reduced/min/mg S9 protein for A549–WT and A549–P450R^{puro}, respectively). This suggests that the major route of reduction of 2 is not via P450R.

3.5. *Hypoxia-selective cytotoxicity of 2 in HT29 cells*

The observation that reduction of 2 releases the cytotoxic azaCBI ligand in hypoxic tumour cells suggests that it might act as a hypoxia-activated prodrug (without any requirement for radiolytic activation). To test this, the clonogenic survival of HT29 cells was examined after incubating with 1 and 2 under aerobic or hypoxic conditions. The free ligand 1 was potently cytotoxic (Fig. 9(A)), with concentrations of ca. 0.05 μM providing one log kill and no difference between aerobic or hypoxic conditions. 2 showed approximately 90-fold lower cytotoxic potency than 1 under aerobic conditions at 1 h, decreasing to 50-fold lower by 4 h (Fig. 9(B)). Importantly, 2 was selectively toxic under hypoxic conditions with a hypoxic cytotoxicity ratio (aerobic/hypoxic concentration for one-log cell kill) of 20 at both 1 and 4 h (Fig. 9(B)).

4. Discussion

This study demonstrates that the novel cobalt complex Co(III)(cyclen)(azaCB) (2) can be reduced efficiently under hypoxic conditions, both by ionising radiation and by tumour cells, to release the highly potent azaCBI cytotoxin 1. The latter is a new compound, but is closely related in structure to a previously-reported azaCBI derivative with the same hydroxyquinoline core structure and trimethoxyindole sidechain [41]. The natural (+) enantiomer of the latter compound, in its open chain (*seco*) form, had a reported IC_{50} of 21 pM for continuous exposure of L1210 cells. The potent cytotoxicity of azaCBI (1) in the present study was demonstrated in clonogenic assays with HT29 cells (one-log kill after 1 h exposure at ca. 0.05 μM; Fig. 9), and in antiproliferative assays with 4 h exposure of UV4 cells (IC_{50} value 0.067 ± 0.007 nM; e.g. Fig. 4(A)). This potency is high relative to the yield of primary reducing radicals during a course of radiotherapy (typically 30–40 μmol/kg), making 2 a candidate for use in cancer therapy as a RAP. Only one other system has been reported in which such

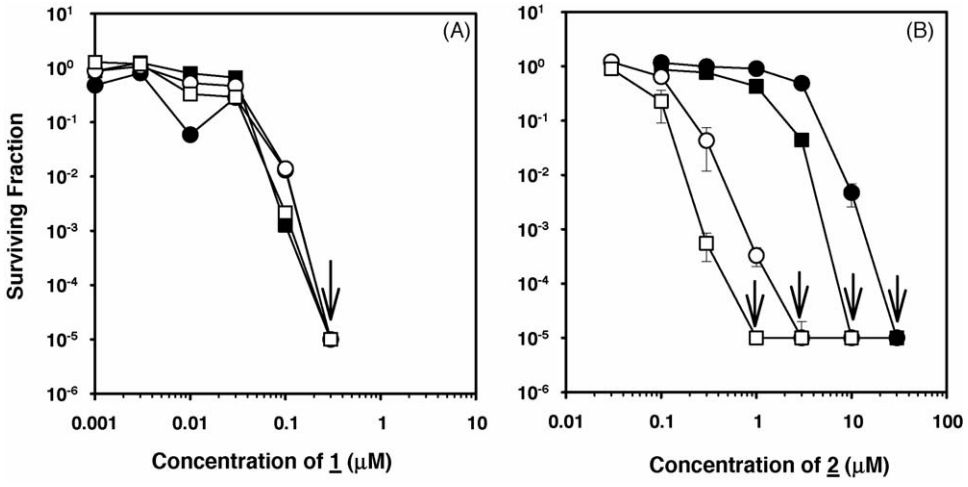

Fig. 9 – Cytotoxicity of free azaCBI, 1 (A) and its cobalt complex 2 (B) in HT29 cells (1.5 × 10^6 cells/ml) under oxic (filled symbols) and hypoxic (open symbols) conditions at 1 h (circles) and 4 h (squares) exposure time, determined by clonogenicity. Symbols in B are the mean ± range for two independent experiments. Arrows indicate that cell survival was below the sensitivity limit of the assay.

potent cytotoxins can be generated by radiolytic reduction [52]; in the latter case the cytotoxic effector is also a CBI derivative but is released by reduction of a nitroheterocyclic quaternary ammonium salt.

For 2 to be useful as a RAP for treatment of hypoxic tumours, it would need to satisfy several additional requirements. The complex (prodrug) must be much less cytotoxic than the free ligand (effector); this is the case for 2, which shows a 40–90-fold prodrug/effector differential in aerobic HT29 cultures (Fig. 9) and a differential of ca. 100-fold in UV4 IC_{50} assays (Fig. 4(A)). The prodrug must also be reduced efficiently by ionising radiation under hypoxic conditions, including in complex biological milieu. The design of 2 was based on a simpler model compound, Co(III)(cyclen)(8-HQ) which we have shown to release 8-HQ at close to the theoretical value for a one-electron process on radiolytic reduction under hypoxia [37]. The azaCBI complex 2 shows very similar features, with essentially stoichiometric release of azaCBI (1) on reduction in formate buffer (Fig. 3). As previously noted with Co(III)(cyclen)(8-HQ) [37], radiolytic loss of 2 also occurs efficiently in anoxic human plasma although the yield of 1 is lower than the loss of 2. This presumably reflects competing consumption of 2, without reduction of the Co(III) centre, by other radiation-induced radicals such as the hydroxyl radical.

A critical test of 2 as a RAP was to determine whether it could be activated significantly in hypoxic human plasma using clinically relevant radiation doses. We show in Fig. 4(A) that doses as low as 1 Gy provide a significant increase in the cytotoxicity of 2 when the plasma is bioassayed against UV4 cells following irradiation. UV4 cells are defective in nucleotide excision repair, but this makes only a small difference in sensitivity to CBI drugs [53,54]. If the *G* value for release of 1 in hypoxic regions of tumours were as high as in hypoxic plasma (estimated as 0.075 μmol/J by fitting the apparent IC_{50} versus radiation dose data to Eq. (1); Fig. 4), then a 2 Gy dose of radiation (as used typically each day during a course of radiotherapy) would be expected to provide ca. 0.15 μM of 1. This is in the range giving substantial tumour cell killing in vitro (Fig. 9(A)). The actual outcome will depend on the intratumour pharmacokinetics of 2 and 1, and also on the *G* value in tissue which might be lower because of competing electron acceptors. It is also theoretically possible that reduction of 2 would generate free Co^{2+} ions, and that these could exert additional biological effects via stabilization of HIF-1α. However, we have previously shown that the analogous Co(cyclen)(8-HQ) complex does not give rise to free Co^{2+} on reduction because the latter remains complexed to cyclen [37]. Given that 8-HQ provides an identical chelating environment to azaCBI, the same can be expected with 2. Biological effects of Co^{2+} are also unlikely at the very low concentrations which would be generated, even in the absence of such complexation.

A complicating factor, although not necessarily disadvantageous, is that 2 is also activated by reduction in hypoxic tumour cell cultures (without radiation) as shown in Figs. 5–8. This was unexpected since the 8-HQ analogue was stable in HT29 cultures under the same conditions [37]. The products of reduction of 2 by hypoxic tumour cells include the free ligand 1 and a rearrangement product (6) which was seen only in the intracellular fraction and can plausibly be accounted for as arising from chloride ion attack on the cyclopropyl intermediate 4 (Fig. 6(B)). In addition, in HT29 cells but not A549 cells, a metabolite (5) with mass spectrum consistent with the *O*-glucuronide of 1 was observed. This assignment is supported by formation of 5 on addition of 1 to aerobic HT29 cells, and by the known high activity of uridine diphosphate glucuronosyl transferase in HT29 cells [55]. It is also consistent with the known facile glucuronidation of 8-HQ in rodents [56]. All of these azaCBI products are present at higher concentrations under hypoxic conditions, but presumably 1 (and its cyclopropyl form 4 which was not reliably distinguished from 1 because of interconversion during sample preparation) is the main cytotoxic metabolite. The *O*-glucuronide 5, by analogy with the 5-*O*-glycosidic CBI prodrugs reported by Tietze et al. [57] is expected to be strongly deactivated with respect to DNA reactivity. In contrast, 6 may retain some cytotoxicity by analogy with duocarmycin C1 [58].

Given that essentially all known bioreductive drugs (with the exception of obligate 2-electron acceptors like the tertiary amine di-*N*-oxide AQ4N) are metabolized by cytochrome P450 reductase by virtue of the low reduction potential of its flavosemiquinone/dihydroflavin couples [59], it was a surprise to find that overexpression of this enzyme in A549 cells did not increase the rate of reductive metabolism of 2. This finding is consistent with recent cytotoxicity studies showing that under anoxic conditions the same A549–P450R^{puro} cell line described here is hypersensitive, relative to parental A549 cells, to tirapazamine, RSU-1069, CB 1954 and SN 23862 but not to 2 or the Co(III)-nitrogen mustard complex SN 24771 (Wilson et al., in preparation). It will be important to establish whether other enzymes are responsible for reduction of these Co(III) complexes, or whether the reaction might be mediated by reductants such as ascorbate, thiols or NAD(P)H. The observation that 2 undergoes slow reduction in hypoxic culture medium is consistent with a role for non-enzymatic pathways. A further possibility is that these lipophilic cations concentrate in mitochondria and are reduced by the mitochondrial electron transport chain. Whatever the identity of the key cytotoxin, or activating enzyme/reductant(s), it is clear that cellular reduction under hypoxia provides significant cytotoxicity, giving a hypoxic cytotoxicity ratio of ca. 20-fold in HT29 cell cultures.

The present study thus demonstrates that 2 can be reduced, resulting in release of its highly potent azaCBI cytotoxin, by both radiolytic and non-radiolytic pathways under hypoxic conditions. It is not yet clear which of these broad strategies is likely to be more useful for exploiting tumour hypoxia with the new Co(III)(azaCBI) prodrug class. Based on the findings of this study, the pharmacokinetics of 2 in mice, and its therapeutic activity against hypoxic cells in human tumour xenografts, is currently being examined in the context of both radiolytic and non-radiolytic activation. In addition, analogues with higher hypoxic selectivity in cell culture are under investigation.

Acknowledgements

We thank Dr. Angela G. Kriste for her assistance in conducting the bioassays. This study was supported by grants from the

Foundation of Research, Science and Technology of New Zealand (UOAX0211) and a Laing Foundation Fellowship from the Cancer Society of New Zealand (to G-OA).

REFERENCES

[1] Kennedy KA, Teicher BA, Rockwell S, Sartorelli AC. The hypoxic tumor cell: a target for selective cancer chemotherapy. Biochem Pharmacol 1980;29:1–8.
[2] Workman P, Stratford IJ. The experimental development of bioreductive drugs and their role in cancer therapy. Cancer Metast Rev 1993;12:73–82.
[3] Denny WA, Wilson WR, Hay MP. Recent developments in the design of bioreductive drugs. Br J Cancer 1996;74(Suppl. XXVII):S32–8.
[4] Brown JM. The hypoxic cell: a target for selective cancer therapy—eighteenth Bruce F. Cain Memorial Award lecture. Cancer Res 1999;59:5863–70.
[5] Brown JM, Wilson WR. Exploiting tumor hypoxia in cancer treatment. Nat Rev Cancer 2004;4:437–47.
[6] Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 1996;41:31–9.
[7] Brizel DM, Dodge RK, Clough RW, Dewhirst MW. Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol 1999;53:113–7.
[8] Fyles AW, Milosevic M, Wong R, Kavanagh MC, Pintilie M, Sun A, et al. Oxygenation predicts radiation response and survival in patients with cervix cancer. Radiother Oncol 1998;48:149–56.
[9] Subarsky P, Hill RP. The hypoxic tumour microenvironment and metastatic progression. Clin Exp Metast 2003;20:237–50.
[10] Wouters BG, Weppler SA, Koritzinsky M, Landuyt W, Nuyts S, Theys J, et al. Hypoxia as a target for combined modality treatments. Eur J Cancer 2002;38:240–57.
[11] Vaupel P. Tumor microenvironmental physiology and its implications for radiation oncology. Semin Radiat Oncol 2004;14:198–206.
[12] Rockwell S, Sartorelli AC, Tomasz M, Kennedy KA. Cellular pharmacology of quinone bioreductive alkylating agents. Cancer Metast Rev 1993;12:165–76.
[13] Jaffar M, Naylor MA, Robertson N, Stratford IJ. Targeting hypoxia with a new generation of indolequinones. Anticancer Drug Des 1998;13:593–609.
[14] Denny WA, Wilson WR. Considerations for the design of nitrophenyl mustards as agents with selective toxicity for hypoxic tumor cells. J Med Chem 1986;29:879–87.
[15] Cole S, Stratford IJ, Fielden EM, Adams GE, Leopold W, Elliott W, et al. Dual function nitroimidazoles less toxic than RSU 1069: selection of candidate drugs for clinical trial (RB 6145 and/or PD 130908. Int J Radiat Oncol Biol Phys 1992;22:545–8.
[16] Papadopoulou MV, Bloomer WD. NLCQ-1 (NSC 709257): exploiting hypoxia with a weak DNA-intercalating bioreductive drug. Clin Cancer Res 2003;9:5714–20.
[17] Denny WA, Wilson WR. Tirapazamine: a bioreductive anticancer drug that exploits tumour hypoxia. Expert Opin Invest Drugs 2000;9:2889–901.
[18] Patterson LH. Bioreductively activated antitumor N-oxides: the case of AQ4N, a unique approach to hypoxia-activated cancer chemotherapy. Drug Metab Rev 2002;34:581–92.
[19] Ware DC, Palmer BD, Wilson WR, Denny WA. Hypoxia-selective antitumor agents. 7. Metal complexes of aliphatic mustards as a new class of hypoxia-selective cytotoxins. Synthesis and evaluation of cobalt(III) complexes of bidentate mustards. J Med Chem 1993;36:1839–46.
[20] Wilson WR, Moselen JW, Cliffe S, Denny WA, Ware DC. Exploiting tumor hypoxia through bioreductive release of diffusible cytotoxins: the cobalt(III)–nitrogen mustard complex SN 24771. Int J Radiat Oncol Biol Phys 1994;29: 323–7.
[21] Anderson RF, Denny WA, Ware DC, Wilson WR. Pulse radiolysis studies on the hypoxia-selective toxicity of a cobalt–mustard complex. Br J Cancer 1996;(Suppl. 27): S48–51.
[22] Simic M, Lilie J. Kinetics of ammonia detachment from reduced cobalt(II) complexes based on conductometric pulse radiolysis. J Am Chem Soc 1973;96:291–2.
[23] Ware DC, Palmer HR, Pruijn FB, Anderson RF, Brothers PJ, Denny WA, et al. Bis(dialkyl)dithiocarbamato cobalt(III) complexes of bidentate nitrogen mustards: synthesis, reduction chemistry and biological evaluation as hypoxia-selective cytotoxins. Anticancer Drug Des 1998;13:81–103.
[24] Ware DC, Brothers PJ, Clark GR, Denny WA, Palmer BD, Wilson WR. Synthesis, structures and hypoxia-selective cytotoxicity of cobalt(III) complexes containing tridentate amine and nitrogen mustard ligands. J Chem Soc Dalton Trans 2000;925–32.
[25] Craig PR, Brothers PJ, Clarke GR, Wilson WR, Denny WA, Ware DC. Anionic carbonato and oxalato cobalt(III) nitrogen mustard complexes. J Chem Soc Dalton Trans 2004;611–8.
[26] Perrin LC, Wilson WR, Denny WA, McFadyen WD. The design of cobalt(III) complexes of phenazine-1-carboxamides as prointercalators and potential hypoxia-selective cytotoxins. Anticancer Drug Des 1999;14:231–41.
[27] Craig PR. Cobalt(III) and chromium(III) complexes as anticancer drugs. PhD Thesis, The University of Auckland, 1998.
[28] Parker LL, Lacy SM, Farrugia LJ, Evans C, Robins DJ, O'Hare CC, et al. A novel design strategy for stable metal complexes of nitrogen mustards as bioreductive prodrugs. J Med Chem 2004;47:5683–9.
[29] Wilson WR, Tercel M, Anderson RF, Denny WA. Radiation-activated prodrugs as hypoxia-selective cytotoxins: model studies with nitroarylmethyl quaternary salts. Anticancer Drug Des 1998;13:663–85.
[30] Kriste AG, Tercel M, Anderson RF, Ferry DM, Wilson WR. Pathways of reductive fragmentation of heterocyclic nitroarylmethyl quaternary ammonium prodrugs of mechlorethamine. Radiat Res 2002;158:753–62.
[31] Nishimoto SI, Hatta H, Ureshima H, Kagiya T. 1-(5′-fluoro-6′-hydroxy-5′,6′-dihydrouracil-5′-yl)-5-fluorouracil, a novel N(1)-C(5)-linked dimer that releases 5-fluorouracil by radiation activation under hypoxic conditions. J Med Chem 1992;35:2711–2.
[32] Shibamoto Y, Tachi Y, Tanabe K, Hatta H, Nishimoto SI. In vitro and in vivo evaluation of novel antitumor prodrugs of 5-fluoro-2′-deoxyuridine activated by hypoxic irradiation. Int J Radiat Oncol Biol Phys 2004;58:397–402.
[33] Tanabe K, Makimura Y, Imagawa-Sato A, Nishimoto S. Hypoxia-selective activation of 5-fluorodeoxyuridine prodrug possessing indolequinone structure: radiolytic reduction and cytotoxicity characteristics. Bioorg Med Chem Lett 2005;15:2321–4.
[34] Lee AE, Wilson WR. Hypoxia-dependent retinal toxicity of bioreductive anticancer prodrugs in mice. Toxicol Appl Pharmacol 2000;163:50–9.
[35] Hicks KO, Pruijn FB, Sturman JR, Denny WA, Wilson WR. Multicellular resistance to tirapazamine is due to restricted extravascular transport: a pharmacokinetic/pharmacodynamic study in HT29 multicellular layer cultures. Cancer Res 2003;63:5970–7.

[36] Pruijn FB, Sturman JR, Liyanage HDS, Hicks KO, Hay MP, Wilson WR. Extravascular transport of drugs in tumor tissue: effect of lipophilicity on diffusion of tirapazamine analogs in multicellular layer cultures. J Med Chem 2005;48:1079–87.
[37] Ahn G-O, Ware DC, Denny WA, Wilson WR. Optimization of the auxiliary ligand shell of Cobalt(III)(8-hydroxyquinoline) complexes as model hypoxia-selective radiation-activated prodrugs. Radiat Res 2004;162:315–25.
[38] Hanka LJ, Dietz A, Gerpheide SA, Kuentzel SL, Martin DG. CC-1065 (NSC-298223), a new antitumor antibiotic. Production, in vitro biological activity, microbiological assays and taxonomy of the producing microorganism. J Antibiot 1978;31:1211–7.
[39] Ichimura M, Ogawa T, Takahashi K, Kobayashi E, Kawamoto I, Yasuzawa T, et al. Duocarmycin SA, a new antitumor antibiotic from *Streptomyces* sp.. J Antibiot 1990;43:1037–8.
[40] Boger DL, Johnson DS. CC-1065 and the duocarmycins: understanding their biological function through mechanistic studies. Angew Chem Int Ed Engl 1996;35:1438–74.
[41] Boger DL, Boyce CW. Selective metal cation activation of a DNA alkylating agent: synthesis and evaluation of methyl 1,2,9,9a-tetrahydrocycloprop[c]pyrido[3,2-*e*]indol-4-one-7-carboxylate (CPyI). J Org Chem 2000;65:4088–100.
[42] Denny WA, Wilson WR, Ware DC, Atwell GJ, Mibank JBJ, Stevenson RJ. Anti-cancer 2,3-dihydro-1H-pyrrolo[3,2-*f*]quinoline complexes of cobalt and chromium. PCT Patent Application WO02059122A1, 2002.
[43] Fricke H, Hart EJ. Chemical dosimetry. In: Attix FH, Roesch WC, editors. Radiation dosimetry, vol. 2. New York: Academic Press; 1966. p. 167–239.
[44] Sykes BM, Atwell GJ, Hogg A, Wilson WR, O'Connor CJ, Denny WA. *N*-Substituted 2-(2,6-dinitrophenylamino)propanamides: novel prodrugs that release a primary amine via nitroreduction and intramolecular cyclization. J Med Chem 1999;42:346–55.
[45] Hobbs S, Jitrapakdee S, Wallace JC. Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. Biochem Biophys Res Commun 1998;252:368–72.
[46] Patterson AV, Saunders MP, Chinje EC, Talbot DC, Harris AL, Stratford IJ. Overexpression of human NADPH:cytochrome c (P450) reductase confers enhanced sensitivity to both tirapazamine (SR 4233) and RSU. Br J Cancer 1997;76:1338–47.
[47] Cowen RL, Patterson AV, Telfer BA, Airley R, Hobbs S, Phillips RM, et al. Viral delivery of P450 reductase recapitulates the ability of constitutive overexpression of reductase enzymes to potentiate the activity of Mitomycin C in human breast cancer xenografts. Mol Cancer Ther 2003;2:901–9.
[48] Patterson AV, Barham HM, Chinje EC, Adams GE, Harris AL, Stratford IJ. Importance of P450 reductase activity in determining sensitivity of breast tumour cells to the bioreductive drug, tirapazamine (SR 4233). Br J Cancer 1995;72:1144–50.
[49] Buxton GV. Radiation chemistry of the liquid state: (1) water and homogeneous aqueous solutions. In: Farhataziz, Rodgers MAJ, editors. Radiation chemistry: principles and applications. New York: VCH Publishers; 1987. p. 321–49.
[50] Mulazzani QG, D'Angelantonio M, Venturi M, Hoffman MZ, Rodgers MAJ. Interaction of formate and ozalate ions with radiation-generated radicals in aqueous solution. Methylviologen as a mechanistic probe. J Phys Chem 1986;90:5347–52.
[51] Gutierrez A, Grunau A, Paine M, Munro AW, Wolf CR, Roberts GC, et al. Electron transfer in human cytochrome P450 reductase. Biochem Soc Trans 2003;31:497–501.
[52] Kriste AG, Tercel M, Stribbling SM, Botting KJ, Wilson WR. Radiation-activated prodrugs: hypoxia-selective radiolytic release of potent chloromethylbenzindoline (CBI) cytotoxins from novel nitroarylmethyl quaternary (NMQ) prodrugs. Br J Cancer 2002;86(Suppl. 1):S30–1.
[53] Atwell GJ, Tercel M, Boyd M, Wilson WR, Denny WA. Synthesis and cytotoxicity of 5-amino-1-(chloromethyl)-3-[(5,6,7-trimethoxyindol-2-yl)carbonyl]-1,2-dihydro-3*H*-benz[e]indole (amino-*seco*-CBI-TMI) and related 5-alkylamino analogues: new DNA minor groove alkylating agents. J Org Chem 1998;63:9414–20.
[54] Atwell GJ, Milbank JJ, Wilson WR, Hogg A, Denny WA. 5-Amino-1-(chloromethyl)-1,2-dihydro-3*H*-benz[e]indoles: relationships between structure and cytotoxicity for analogues bearing different DNA minor groove binding subunits. J Med Chem 1999;42:3400–11.
[55] Franklin TJ, Jacobs VN, Jones G, Ple P. Human corelectal carcinoma cells in vitro as a means to assess the metabolism of analogs of mycophenolic acid. Drug Metab Dispos 1997;25:367–70.
[56] Monson TP, Henle KJ, Nagle WA, Mansouri A. Tumor-targeted delivery of 8-hydroxyquinoline. Int J Radiat Oncol Biol Phys 1991;20:1263–71.
[57] Tietze LF, Lieb M, Herzig T, Haunert F, Schuberth I. A strategy for tumor-selective chemotherapy by enzymatic liberation of *seco*-duocarmycin SA-derivatives from nontoxic prodrugs. Bioorg Med Chem 2001;9: 1929–39.
[58] Ichimura M, Ogawa T, Takahashi K, Takahashi I, Nakano H. Interconversion and stability of duocarmycins, a new family of antitumor antibiotics: correlation to their cytotoxic and antimicrobial activities in vitro. Oncol Res 1993;5:165–71.
[59] Wardman P. Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia. Curr Med Chem 2001;8:739–61.