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Antagonism in effectiveness of evofosfamide and doxorubicin through intermolecular electron transfer

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ABSTRACT

Hypoxic cells pose a problem in anticancer chemotherapy, in which often drugs require oxygen as an electron acceptor to bring about the death of actively cycling cells. Bioreductive anticancer drugs, which are selectively activated in the hypoxic regions of tumours through enzymatic one-electron reduction, are being developed for combination with chemotherapy-, radiotherapy- and immunotherapy-containing regimens to kill treatment-resistant hypoxic cells. The most clinically-advanced bioreductive drug, evofosfamide (TH-302), which acts by releasing a DNA-crosslinking mustard, failed to extend overall survival in combination with doxorubicin, a topoisomerase II inhibitor, for advanced soft tissue sarcoma in a pivotal clinical trial. However, the reasons for the lack of additive efficacy with this combination are unknown. Here, we show that the radical anion of evofosfamide undergoes electron transfer to doxorubicin in kinetic competition to fragmentation of the radical anion, thus suppressing the release the cytotoxic mustard. This electron transfer process may account, at least in part, for the lack of overall survival improvement in the recent clinical trial. This study underlines the need to consider both redox and electron transfer chemistry when combining bioreductive prodrugs with other redox-active drugs in cancer treatment.

1. Introduction

Severe hypoxia, which frequently arises in neoplasms due to the dysfunction of tumour vasculature and imbalances between oxygen supply and consumption [1], has been investigated as a pharmacological target as a result of its contributions to malignant progression and resistance to therapy. Hypoxia arises early in the course of tumour development and selects for the outgrowth of clones that are resistant to apoptosis [2], genetically unstable [3] and refractory to some widely-used chemotherapeutic agents [4]. Moreover, hypoxia enhances tumour angiogenesis [5], epithelial-to-mesenchymal transition [6] and invasive and metastatic potential [7]. As a result, tumour hypoxia has been associated with poor prognosis across multiple primary tumour sites [8]. Hypoxic cells are also notably resistant to radiotherapy due to the requirement for fixation by molecular oxygen of initial ionising radiation-induced DNA free radicals to generate cytotoxic strand breaks [9,10], leading to poor clinical outcomes [11]. Hypoxia may also serve as a reservoir for tumour regrowth by stimulating vasculogenic responses after irradiation [12]. Accordingly, compelling clinical data indicates that extensive tumour hypoxia predicts for poor radiotherapy outcomes, most notably in squamous cell carcinomas of the head and neck [13], a setting in which preliminary evidence supports the view that hypoxia-modifying therapy may improve the efficacy of radiation [14,15]. More recently, a key role for tumour hypoxia in the establishment of an immune-suppressive microenvironment has been elucidated, leading to interest in the use of hypoxia-targeting agents to sensitise tumours to immune checkpoint blockade [16].

Reflecting such considerations, bioreductive drugs are being developed to exploit hypoxic regions of tumours in which they act as prodrugs by being initially one-electron reduced by cellular reductases to release cytotoxins, at the one or multi-electron reduction level, to specifically kill such treatment-resistant cells [17,18]. Normoxic cells are protected to a major extent by the obligate intermediate radical anion being back-oxidised by oxygen, a process competing with the

Abbreviations: $E^{0'}$, one-electron reduction potential at pH 7; Gy, gray(J.kg^{-1}); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CI, combination indices; IC50, the drug concentration inhibiting cell growth by 50% relative to untreated controls; ED50, ED75, ED90, the drug concentration providing 50%, 75% or 90% of maximal cell growth inhibition, f_a, the fraction of cell growth inhibition, where 0 is nil inhibition and 1.0 is maximal inhibition

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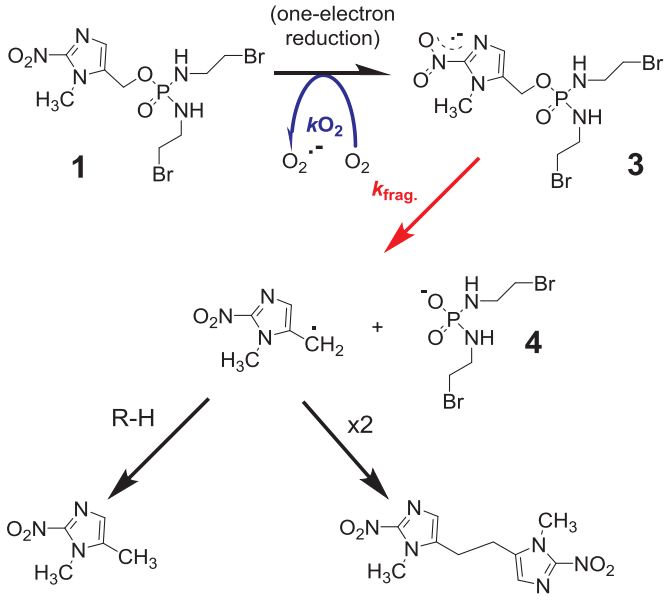

Fig. 1. Radical reactions upon one-electron reduction of evofosfamide, **1**. One-electron reduction, either by proteins or radiolytic methods, form radical anion **3**. Back oxidation of **3** to reform **1** is by reaction with O_2 with a 2nd-order rate constant kO_2 in competition to fragmentation of **3** with a 1st-order rate constant $k_{frag.}$ to release the cytotoxic mustard compound **4**.

release of cytotoxins from the bioreductive prodrugs. Hence, there is considerable interest in combining standard-of-care chemotherapy and radiotherapy treatments aimed at in-cycle normoxic cancer cells with bioreductive drugs to kill hypoxic cancer cells, so as to improve therapeutic outcomes for patients. Evofosfamide (also known as TH-302), a 2-nitroimidazole prodrug of the potent DNA cross-linking bromo-*iso*-phosphoramide mustard [19], is the most clinically advanced bioreductive prodrug. Evofosfamide shows excellent anti-tumour activity in a range of preclinical models [20] and has been the subject of 27 clinical studies, most notably in combination Phase II trials with gemcitabine for pancreatic adenocarcinoma [21] and with doxorubicin for soft-tissue sarcoma [22].

One-electron reduction of evofosfamide (**1**) forms its radical anion (**3**) which undergoes spontaneous fragmentation of the methyl-ether bond to release bromo-*iso*-phosphoramide mustard (**4**) and a radical which abstracts an H-atom or dimerises to form products [23], Fig. 1. Fragmentation of the radical anion is in kinetic competition with its back-oxidation under oxic conditions, as seen for a wide range of nitro- and *N*-oxide- aromatic compounds [24,25]. The recent Phase III clinical trial, TH-CR-406/SARC021 (NCT01440088), combining evofosfamide (**1**) with doxorubicin (**2**) for the first-line treatment of locally-advanced, unresectable or metastatic soft tissue sarcoma, failed to show overall survival benefit over doxorubicin alone [26]. The reasons for this failure, in light of manageable toxicity interactions [27] and evidence of enhanced growth delay [28] and favourable pharmacodynamics interactions [29] in tumour models, remains largely unknown. The outcome is unlikely to reflect intrinsic resistance to evofosfamide in soft-tissue sarcoma, given that ifosfamide (a close analogue of bromo-*iso*-phosphoramide) is a standard agent for this indication [30]. Here we examine both the radical chemistry and *in vitro* cytotoxicity response when combining both drugs in anticancer treatment.

2. Materials and methods

2.1. Materials

All solutions were prepared in sodium phosphate buffer (5 mM, pH 7) unless otherwise stated. Doxorubicin was purchased from L & G Chemicals and used as supplied. Evofosfamide was a gift from Threshold Pharmaceuticals, CA. All other chemicals were purchased from Aldrich Chemical Company and were of Analar grade. Water was purified by a MilliQ system.

2.2. Cell Culture

The lingual squamous carcinoma cell line UT-SCC-74B was acquired from Professor Bradly Wouters (Princess Margaret Cancer Centre, Toronto). UT-SCC-74B was cultured in humidified CO_2 incubators in Minimal Essential Medium supplemented with 10% fetal calf serum, 4.5 mg/mL D-GLUCOSE, 1.9 mg/mL sodium bicarbonate, 1 mM sodium pyruvate and 20 mM HEPES. Cells were routinely tested for *Mycoplasma* infection using the PlasmoTest kit (InvivoGen) and authenticated against short tandem repeat profiles generated in-house.

2.3. Cytotoxicity assays

UT-SCC-74B cells in logarithmic-phase growth were seeded into 96-well plates at a density of 800 cells per well and allowed to adhere over 2 h under aerobic (21% gas-phase O_2), hypoxic (0.1% O_2) or anoxic (< 10 ppm O_2) conditions. Drugs were then added over two-fold serial dilution series and exposed for 4 h before wash-out. The cultures were then regrown under ambient oxygen for 5 days and cell density assessed colourimetrically using sulphorhodamine B staining. Four-parameter logistic regressions were fitted to the data and IC50 values defined, by interpolation, as drug concentrations reducing staining to 50% of untreated control cultures on the same plate. Hypoxic and anoxic incubations were performed in a Don Whitley H45 Hypoxystation using media and consumables equilibrated for ≥3 days. Combination drug exposures were established by diluting evofosfamide and doxorubicin sequentially along two orthogonal dimensions of a 96-well plate, such that wells in the diagonal dimension contained a dilution series of both drugs in a constant equipotent ratio (i.e. at equivalent multiples of their single-agent IC50). The interaction of the two drugs relative to their monotherapy activity on the same plate was then analysed with CalcuSyn software (Biosoft) using the Chou-Talalay method [31] and standard computations, where combination indices (CI) > 1 indicated antagonism. Within each experiment, the CI was computed separately for the ED50, ED75 and ED90 (i.e. at 50%, 75% and 90% growth inhibition relative to untreated controls). The mean of these three measures was taken as the overall CI for that experiment. The combination index (CI) equation is based on the multiple drug-effect equation of Chou-Talalay,

$$CI = (D)_1/(D_x)_1 + (D)_2/(D_x)_2$$

where $(D_x)_1$ and $(D_x)_2$ are the concentrations of drug 1 and drug 2, respectively, which inhibit cell growth by x% as single agents, whereas D_1 and D_2 are the concentrations of drug 1 and drug 2 that provide x% of growth inhibition in combination.

2.4. Pulse radiolysis

Time-resolved optical absorption and kinetic studies were carried out at 22 °C using the pulse radiolysis instrumentation at the University of Auckland's Free Radical Research Facility, which utilizes a 4-MeV linear accelerator to deliver 200 ns electron pulses with doses of 2–20 Gy to a 2 cm path-length optical cell. The optical detection system and dosimetry method have been described previously [32].

One-electron reduction of compounds **1** and **2** to their radical anions, **3** and **5**, were carried out in pulse irradiated N_2O-saturated solutions containing either formate ions (0.1 M) or propan-2-ol (0.2 M) and phosphate buffer (5 mM, pH 7) to form the carbon dioxide radical anion, $CO_2^{\bullet-}$ and the α-hydroxy-2-propanyl radical, $(CH_3)_2C^{\bullet}OH$, respectively.