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Reconsidering azobenzene as a component of small-molecule hypoxia-mediated cancer drugs: A theranostic case study

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ABSTRACT

An azobenzene scaffold serves as both a fluorescence quencher and nitrogen mustard deactivator in a mitochondrial targeting unit bearing theranostic drug delivery system (DDS). The DDS exhibited a tissue selectivity for tumors with aggressive phenotypes, and the efficient *in vitro* and *in vivo* azoreduction under hypoxia conditions resulted in bright fluorescence at the tumor site as well as the *in situ* activation of the prodrug. *In vivo* therapeutic experiments demonstrated a significant reduction in tumor growth versus number of controls and *ex vivo* tissue analysis confirmed tissue normalization with strongly reduced angiogenic markers and suppressed cell proliferation. Mechanistic insight of the DDS's mode of action was gained by gene and protein expression experiments, aided by a proteomic analysis, revealing the circumvention of cellular drug resistance pathways as well as the normalization of Slit-Robo signaling, and the involvement of granzyme-triggered mitochondria-mediated apoptosis. Overall, the combined high sensitivity and synthetic ease as well as excellent therapeutic response suggests a revival of the azobenzene class of hypoxia activated drugs, especially applied to theranostics, is warranted.

1. Introduction

As a consequence of rapid proliferation and erratic, highly irregular tumor neovasculature, both structural and temporal hypoxia are key hallmarks of solid tumors [1,2]. Cellular responses to hypoxia include decreased cellular proliferation, suppressed apoptosis, increased mutation rates and drug resistance [2]. Poorly oxygenated tumors exhibit increased tumor angiogenesis [3] and an aggressive phenotype [4] and metastatic profile [5], as such hypoxia is an indicator for poor prognosis and therapeutic outcome [1,2]. As the vast majority of non-targeted and non-activated anti-cancer drugs rely on rapid proliferation to ensure differential toxicity to cancerous and healthy tissues, hypoxia results in dramatically reduced toxicity profiles. On a more fundamental level, the requirement of oxygen for the success of radiation therapy [1] and photodynamic therapy [6] also provides a protective mechanism for hypoxic cells. The selective elimination of hypoxic cells, a key component of solid tumors, is despite their relatively minor constitution of the utmost importance.

Seemingly contradictory, tumor hypoxia also represents a remarkably exploitable target for cancer therapy as the low levels of oxygen encountered in solid tumors are not observed elsewhere in healthy tissues. A variety of drugs have been developed, which are activated by intracellular 1-electron reductases in the absence of oxygen. Nitrobenzene and nitroimidazole based triggers are common motifs of hypoxia activated trigger-drug conjugate type prodrugs, however they suffer from drawbacks, often exhibiting suboptimal redox chemistry [7] and a lengthy synthetic procedure [8], respectively.

In recent years, sensitive diazobenzene based fluorescent hypoxia indicators have been reported to be readily activated under

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mild hypoxia [9–14]. Initially the fluorescence is quenched as the excited state energy is rapidly dissipated due to conformational changes around the azo-bond [10,15]. Upon reduction of this bond, the de-excitation pathway is eliminated, thus restoring the fluorescence. Azobenzene containing, reductively activated small molecule cancer prodrugs have enjoyed brief interest many decades ago [7], to date this motive has not been explored in the context of theranostic DDS, yet as we will show in this work, it holds great promise.

Nitrogen mustards, a commonly used class of alkylative drugs, induce toxicity primarily as a result of persistent DNA damage due to intra- and inter-strand linkage of nuclear DNA at the guanine *N*-7 position [16], although DNA-protein cross-linking has been implicated as well [17]. Whereas the initial response to this type of drugs is encouraging, most patients however develop drug resistance due to efficient DNA repair mechanisms [16], and as a result of the mutagenic effect of these drugs, the risk of secondary drug-induced malignancies is non-negligible, especially after prolonged treatment [18].

Rerouting these drugs to the mitochondria was found to be greatly beneficial, presenting a more sensitive target while lowering the mutagenic potential of drugs and bypassing several cellular drug resistance pathways [19–21]. However, the lack of an *in situ* activation method would likely give rise to off-target activity of the drug *in vivo*, especially in the liver, as PET probes sharing a similar mitochondria targeting motif have been found to be targeted to this organ as well [22].

In the current work, we report on a hypoxia activated mitochondria targeted DNA alkylating agent (**1**), combining both a tumor and mitochondria targeted delivery mechanism with a tissue selective activation to maximize the therapeutic window. Under hypoxia conditions, the azo-bond is reduced, resulting in the release of *N*,*N*-bis(2-chloroethyl)-1,4-benzenediamine, previously reported to be a mildly potent (nuclear) DNA alkylating agent [23], with the concomitant release of a fluorescent rhodamine 123/B analogue attesting to the activation of the drug. Mitochondrially localized DNA cross-coupling and alkylative damage subsequently results in cell death, as outlined in Scheme 1.

2. Materials and procedures

2.1. Materials and instrumentation

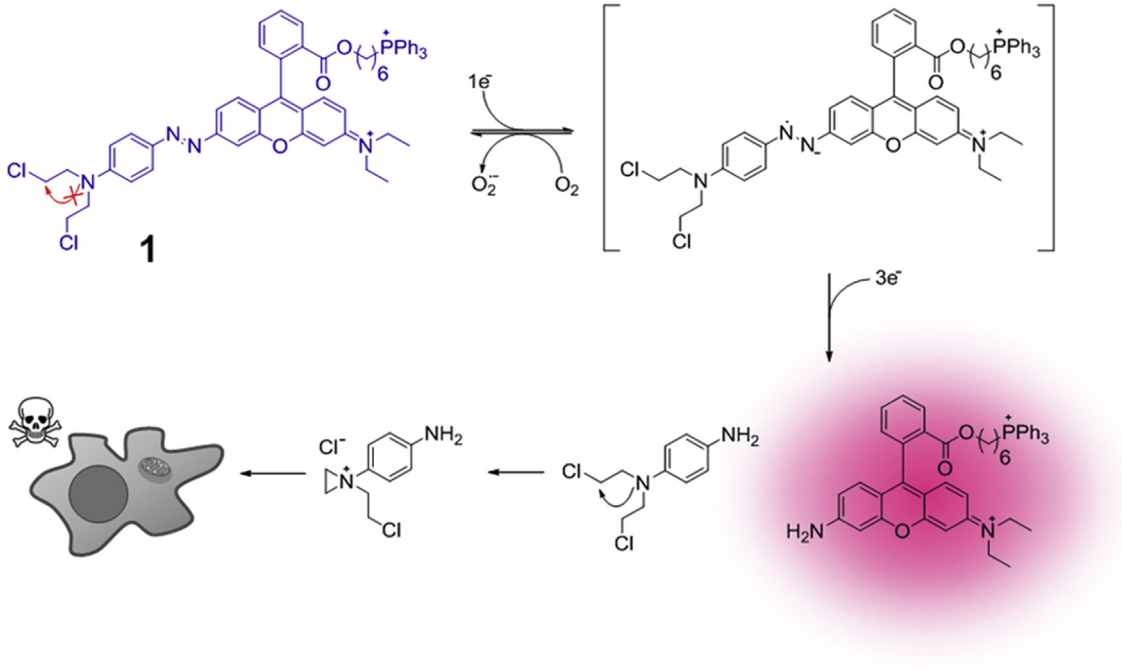
Reagents and solvents have been obtained from commercial sources (Sigma-Aldrich, Alfa Aesar, Samchung, TCI) and have been used as received. Silica gel 60 (Merck) was used for column chromatography and ^{1}H and ^{13}C NMR spectra were recorded on a Varian instrument (300 MHz and 400 MHz). Mass spectra (electron spray injection) were obtained on a Shimadzu LC/MS-2020 Series instrument.

2.2. UV/Vis and fluorescence spectroscopic methods

Spectroscopic results have been obtained on a Scinco S-3100 spectrometer and fluorescence spectra were obtained using a Shimadzu RF-5301PC spectrofluorophotometer. All fluorescence spectra have been recorded at absorbance values lower than 0.1 at the excitation wavelength, the instrument settings and solvents have been provided in the image captions (samples contain maximum 1% DMSO). The quantum yield of the fluorophore (**7**) was determined in MeOH or PBS (10 mM, pH = 7.4) relative to Rhodamine 6G in EtOH (Φ = 0.95%) [24]. The sodium dithionite (2 mM) mediated azoreductuction of **1** (1 μM) was performed in PBS (10 mM, pH = 7.4, 1% DMSO) at 37 °C, time course spectra were recorded at 557 nm upon excitation at 524 nm and the time dependent fluorescence spectra upon excitation at 514 nm (slit ex/em 5/5).

2.3. Cell culture

Four human carcinoma cell lines: prostate cancer cells (DU145), breast cancer cells (MDA-MB-231), lung cancer cells (A549), hepatic carcinoma cells (Huh7), were purchased from the Korean Cell Line Bank (Seoul, Republic of Korea). A normal human fibroblast cell (BJ),



Scheme 1. Proposed mode of action of prodrug **1**.