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Probing cytochrome P450 bioactivation and fluorescent properties with morpholinyl-tethered anthraquinones

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ABSTRACT

Structural features from the anticancer prodrug nemorubicin (MMDX) and the DNA-binding molecule DRAQ5™ were used to prepare anthraquinone-based compounds, which were assessed for their potential to interrogate cytochrome P450 (CYP) functional activity and localisation. 1,4-disubstituted anthraquinone **8** was shown to be 5-fold more potent in EJ138 bladder cancer cells after CYP1A2 bioactivation. In contrast, 1,5-*bis*((2-morpholinoethyl)amino) substituted anthraquinone **10** was not CYP-bioactivated but was shown to be fluorescent and subsequently photo-activated by a light pulse (at a bandwidth 532–587 nm), resulting in punctuated foci accumulation in the cytoplasm. It also showed low toxicity in human osteosarcoma cells. These combined properties provide an interesting prospective approach for opto-tagging single or a sub-population of cells and seeking their location without the need for continuous monitoring.

The cytochrome P450 (CYP) enzymes are responsible for the oxidation of a diverse range of xenobiotic and endogenous compounds. There are 57 transcriptionally active genes encoding CYPs, which are classified into families and subfamilies according to their nucleotide sequence.[1] CYPs function mainly to detoxify xenobiotics and endogenous molecules, but some evidence also indicates a link to signaling events.[2] Many probes based on various chemical scaffolds have been explored in order to identify chromophores that can be used to explore CYP activity.[3] Generally, it has proven difficult to develop CYP isoform-selective fluorophores, in particular because CYPs are encoded by large gene families, and their functions cannot be predicted from their gene sequence.[4] However, it is possible to achieve CYP-selectivity as we have shown with duocarmycin bioprecursors reengineered to target CYP1A1 and CYP2W1 for tumour-selective bioactivation.[5–7] However, the duocarmycin scaffold[8] is not suited as a fluorophore for monitoring CYP functional activity as the pharmacophore is poorly fluorescent.

Nemorubicin (3′-deamino-3′-[2-(*S*)-methoxy-4-morpholi-nyl]-doxorubicin; MMDX, Fig. 1), a doxorubicin derivative bearing a methoxymorpholinyl group on the carbohydrate moiety, has

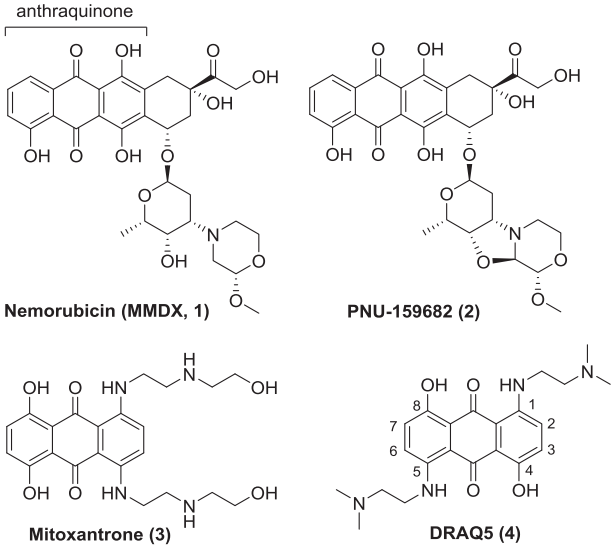

Fig. 1. Anthraquinone-based bioactive compounds.

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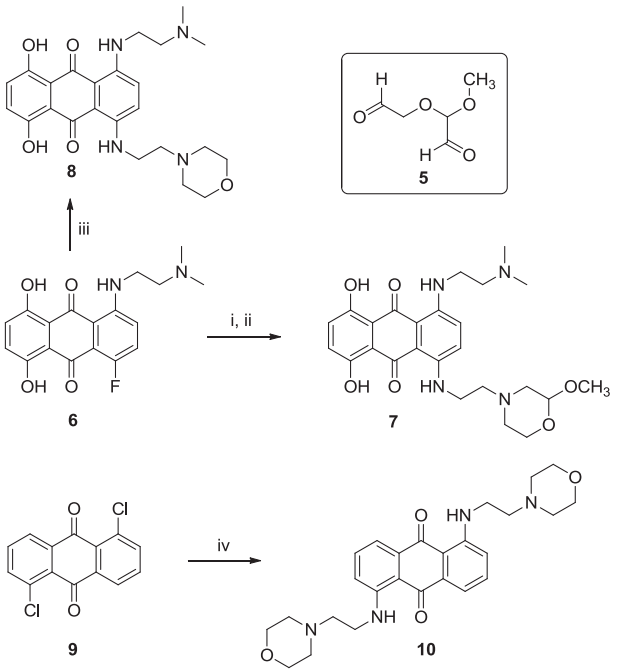

Scheme 1. Synthesis of target compounds **7**, **8** and **10**. (i) $H_2NCH_2CH_2NH_2$ (neat), 42%, (ii) **5**, $NaBH_3CN$, glacial acetic acid, 32%, (iii) amine (neat), 78%, (iv) excess amine (neat), 50 °C, 1–3 h, 60–85%.

undergone clinical evaluation.[9] MMDX is at least 80-fold more potent *in vivo* compared with doxorubicin, which is attributed to generation of metabolite PNU-159682 (**2**) via CYP3A bioactivation that can covalently adduct DNA.[10,11] The latter is likely to follow DNA intercalation via the planar anthraquinone pharmacophore, which we have explored to discover libraries of dual-targeting DNA-affinic covalent binding agents.[12–18] The anthraquinone also plays a key part in the anticancer drug mitoxantrone (**3**) and DRAQ5 (**4**), a far-red DNA label used to detect nuclei and quantify DNA content in live or fixed cells.[19,20] The anthraquinone possesses fluorescent properties that can be exploited in measuring nuclear or cytoplasmic events.[21] Here, we speculated whether combining features of MMDX and DRAQ5™ could be used to discover new molecules, which could simultaneously be used to assess CYP functional activity across a heterogeneous cell population.

Synthesis of target anthraquinone probes **7**, **8** and **10** were prepared from either 1-monosubstituted building block **6** or commercially available **9** (Scheme 1). Compound **6** was reacted with neat ethylenediamine and purified by column chromatography before the resulting 1,4-disubsituted aminoanthraquinone was reacted with 2-methoxy-2-(2-oxoethoxy)acetaldehyde **5** under reductive amination conditions to produce target molecule **7**.[22] Intermediate **5** was generated following the published procedure.[23] Anthraquinone probes **8**[24] and **10**[25,26] were obtained by stirring **6** or **9** respectively in neat 2-morpholinoethylamine followed by removal of excess amine and purification by flash column chromatography.

To determine if compounds **7** and **8** were substrates for CYP bioactivation. We evaluated these in the EJ138 bladder cancer cell line (identical to the T24 cell line[27]) due to its very low levels of CYPs.[6] Using previously published methodology,[28] metabolites were generated via incubation of compounds **7** and **8** with 20 pmol CYP bactosomes in 50 mM Tris-HCl buffer (2 mM NADPH, 1 mM $MgCl_2$, pH 7.4). Generally, none of the extracted metabolite incubates were shown to exert increased anti-proliferative activity over their parental compounds **7** and **8** using the MTT assay (Table 1). The only exception was that CYP1A2-mediated metabolites from incubation with compound **8** possessed approximately a 5-fold potentiation in activity. Evaluation was also performed in SW480-mock and 2 W1-transfected colon carcinoma cell lines,[7] but no bioactivation was observed for compounds **7** (IC_{50} = 1.27 μM in mock cells and 1.53 μM in CYP2W1 cells) and **8** (IC_{50} = 0.97 μM in mock cells and 1.25 μM in CYP2W1 cells).

Both the morpholinyl and 2-methoxymorpholinyl moieties contain motifs that could be hydroxylated by CYPs in the 3-position with loss of H_2O and generation of activated imine or hydroxylation at the 3-methoxy group with loss of formaldehyde, ring opening and generation of an aldehyde intermediate. Such CYP-bioactivated metabolites would have the propensity to form covalent adducts with DNA and hence increased potency would be expected. However, the lack of any significant bioactivation by CYP3A4 and 3A5 indicates that the carbohydrate moiety of **1** in the proximity of the 2-methoxymorpholino group, subsequent to CYP3A hydroxylation, is vital for intramolecular formation of a potent cytotoxin and emphasizes the importance of the MMDX structural configuration.[10]

To investigate the fluorescent properties of the compounds we decided to use human non-small cell lung (A549) and osteosarcoma (U2-OS) cancer cell lines, which we routinely use as models for assessing compounds with fluorescent properties[20,29]; these cell lines are also relevant for studying CYP1A2 as the human protein atlas shows moderate expression levels of its isoform in both cell types, with U-2 OS cancer cells showing 2-fold RNA levels above A549 cancer cells.[30]

The absorbance spectrum of **7** alone (Fig. 2A) gave two maxima at wavelengths 610 and 650 nm; with an emission profile peak at 690 nm and hence the compound can be best detected at a bandwidth of 650–790 nm. The molecule was readily taken up by living A549 cells and was shown to be localised in the *peri*-nuclear vesicular region of cells (Fig. 2D). The lack of DNA binding by compound **7** is evidenced by a nuclear-to-cytoplasmic (n/c) ratio of fluorescence intensity of 0.3 ± 0.06 (n = 9). In contrast, the benchmark molecule DRAQ5™ gave a similar far-red spectral performance, but a n/c ratio of 7.7 ± 1.8 (n = 6). Unlike compounds **7** and **10** DRAQ5™ has two highly DNA affinic moieties in the 1,5 position of the anthraquinone chromophore, giving an equilibrium of nuclear compartment localisation and hence the molecule binds DNA in a stoichiometric fashion. Next we interrogated molecule **10** without the 3-methoxy functionality to assess the effect of the unsubstituted morpholinyl moiety on fluorescent signal while maintaining the 1,5-disubstituted anthraquinone symmetry. This probe showed a blue-shifted absorbance profile to a single peak of 520 nm with an emission profile maxima at 635 nm (Fig. 2B). However, continuous exposure to 20 μM of **10** again in A549 cells resulted in a distinct nuclear labelling and a perinuclear compartment which also contained bright punctate labelling (Fig 2E) and a n/c ratio of 4.1 ± 0.7 (n = 17). The lower ratio compared to DRAQ5™

Table 1
Growth inhibition of parental anthraquinone and CYP bactosomes generated metabolite fractions against EJ138 cell line.

ID	EJ138	CYP1A1	PF	CYP1A2	PF	CYP1B1	PF	CYP2D6	PF	CYP3A4	PF	CYP3A5	PF
7	0.32 ± 0.02	0.28 ± 0.06	1.1	0.25 ± 0.04	1.3	0.34 ± 0.04	0.9	0.31 ± 0.05	1.0	0.29 ± 0.09	1.1	0.25 ± 0.02	1.3
8	0.34 ± 0.04	0.30 ± 0.08	1.1	0.07 ± 0.01	4.6	0.28 ± 0.09	1.2	0.25 ± 0.06	1.3	0.33 ± 0.06	1.0	0.36 ± 0.02	0.9

[a] IC_{50} (μM) values are the mean ± SD of at least three independent assays; PF = potentiation factor.