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Bexarotene prodrugs: Targeting through cleavage by NQO1 (DT-diaphorase)

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ABSTRACT

Bexarotene, a retinoid X receptor (RXR) agonist, is being tested as a potential disease modifying treatment for neurodegenerative conditions. To limit the peripheral exposure of bexarotene and release it only in the affected areas of the brain, we designed a prodrug strategy based on the enzyme NAD(P)H/quinone oxidoreductase (NQO1) that is elevated in neurodegenerative diseases. A series of indolequinones (known substrates of NQO1) was synthesized and coupled to bexarotene. Bexarotene-3-(hydroxymethyl)-5-methoxy-1,2-dimethyl-1H-indole-4,7-dione ester **7a** was cleaved best by NQO1. The prodrugs are not cleaved by esterase.

Bexarotene (Fig. 1) is a retinoid X receptor (RXR) agonist approved for T cell lymphoma.[1] In 2012, bexarotene was reported to have neuroprotective properties in rodent models of Alzheimer's Disease (AD)[2] and a year later in Parkinson's Disease (PD).[3] This caused a lot of excitement in the AD field, and clinical phase II trials started early 2013 (ClinicalTrials.gov identifier: NCT01782742). However, systemic exposure leads to peripheral side effects such as hypertriglyceridemia, hypothyroidism and leukopenia.[4] In addition, bexarotene undergoes extensive cytochrome P450 metabolism,[5,6] leading to potential drug–drug interactions if it is co-administered with other CYP450 substrates, inducers or inhibitors. It would therefore be highly desirable to find selective prodrugs for brain targeting of bexarotene to avoid those side effects. In addition, it was recently shown that bexarotene displays neuroprotective activity at low doses, making a prodrug strategy feasible.[3] Herein we disclose the first investigation of prodrugs for pathophysiological release of bexarotene, in an ongoing research project of Pathology Activated Therapies (PATs).

NAD(P)H/quinone oxidoreductase (QR1, NQO1, formerly DT-diaphorase; EC 1.6.99.2) is a two electron transfer obligate oxidoreductase. NQO1 is induced when levels of L-dopa are raised.[7] Physiologically, it keeps L-dopa, dopamine and other easily oxidizable catecholamines in their reduced state.[8,9] When tissues

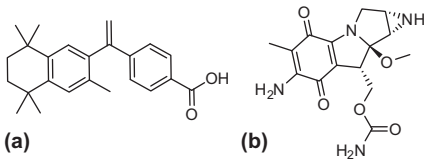

Figure 1. Structural formula of (a) bexarotene and (b) mitomycin C.

are subjected to oxidative stress, as in tumors, inflammation or neurodegenerative diseases, detoxification enzymes, such as NQO1 are induced.[10–12] For instance, NQO1s activity is elevated in several tumor tissues, for example up to 80 fold in lung tumors.[13] Also, it is overexpressed in neurodegenerative diseases like in active multiple sclerosis lesions[12] and in the substantia nigra in Parkinson's Disease (PD).[14,15] In Alzheimer's disease, neurons containing diaphorase are selectively spared from degeneration.[16]

In addition to catecholamines, other quinones can be substrates for NQO1.[17,18] They have been used for tumor targeting; for example diaphorase plays a key role in mitomycin C (see Fig. 1) activation in tumor tissue.[19] To function as a promoiety for disease targeting, indolequinones have a hydroxymethyl substituent in C2 or C3 which can be connected to the active drug (see Fig. 2).[20] In tissues with high diaphorase levels, the quinones are reduced and the drug is released.

With the potential toxicity of the quinones taken into account, the indolequinone structures shown in Figure 2 were chosen

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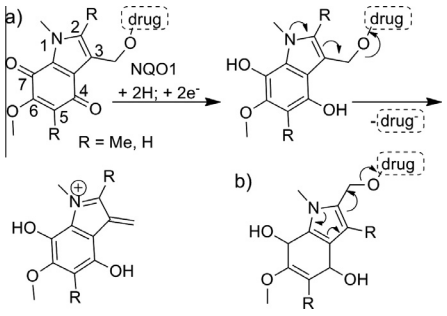
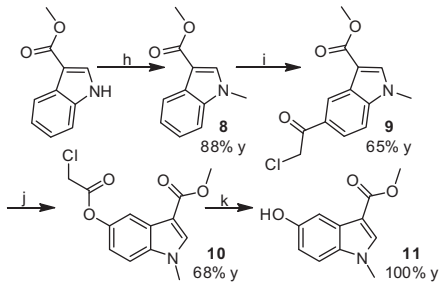


Figure 2. Mechanism of drug release from indolequinones by NQO1: Cleavage from (a) the 3-hydroxymethyl, (b) the 2-hydroxymethyl position.

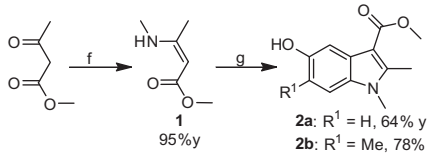


Scheme 2. Reagents and conditions: Precursors of **11**: h: MeI, KOH, acetone; i: 2-chloroacetyl chloride, DCM, $AlCl_3$; j: mCPBA, Na_2HPO_4, $CHCl_3$; k: NaOH, MeOH, rt, 1 h.

because they combine reasonable affinity to NQO1 with reduced toxicity. For example, an aziridine instead of the methoxy group in C6 results in higher affinity, but also much higher toxicity.[21] In this study, both C2- and C3-derivatives were synthesized and coupled to bexarotene and evaluated. The free carboxylic acid is crucial for interaction with RXR, so the esterified prodrug is not expected to show any activity.

The synthesis of the test compounds **7a**, **7b**, **7c** and **18** was accomplished by three different routes (Schemes 1–4). The route for **7a** and **b** started from methyl acetoacetate which was condensed with methylamine to form **1** (Scheme 1).[22] A subsequent Nenitzescu reaction with quinone or methyl quinone formed the indoles **2a** or **2b**.[23] Synthesis of **7c** could not be accomplished this way. We therefore started out from commercial methyl 1*H*-indole-3-carboxylate (Scheme 2), which was methylated to **8** and then subjected to Friedel–Crafts acylation with chloroacetyl chloride to yield **9** (the 6-chloroacetyl isomer which was formed in small amounts was removed by column chromatography).[24] Compound **9** was further oxidized to the ester **10** with mCPBA and hydrolyzed to **11**. Compounds **2a**, **2b** and **11** were methylated to form **3a–c** and then nitrated with acetic acid/HNO_3 to **4a–c** (Scheme 3).[17] Reduction with Sn/HCl yielded amines **5a–c**. A subsequent reduction of the esters using $LiAlH_4$ yielded the corresponding alcohols, evaporated and the resulting residue was immediately oxidized with Fremy's salt to the quinones **6a–c**.[25] The 3-hydroxymethyl bearing indolequinones **6a–c** were coupled to bexarotene under Mitsunobu conditions to form **7a–c**. The synthesis of the 2-hydroxymethyl indolequinones is shown in Scheme 4. Methyl α-ethylacetoacetate, sodium nitrite and *p*-anisidine reacted to **13** in a Fischer indole cyclization via the Japp–Klingmann azo-ester intermediate **12**.[26,27] **13** was nitrated with HNO_3 in DCM to give **14** and methylated to form **15**. It was then reduced with tin/HCl to yield **16**. Reduction and quinone formation was also accomplished with $LiAlH_4$ and Fremy's salt successively to form **17**, before it was coupled to bexarotene to form compound **18**.[28]

To evaluate the compounds as prodrugs, **7a**, **7b**, **7c** and **18** were solved in DMSO and then diluted 100 fold with tris buffer. 5 μM solutions of compounds were incubated at 37 ± 1 °C with or without diaphorase (75 μg protein/mL; 50 U/mL), 0.07% BSA, 300 μM NADH in tris buffer (pH 7.4).[29] Cleavage of the conjugates was monitored with HPLC (RP-18 column, acetonitrile/water gradient, 0.1% TFA). Injections were performed every 60 min. Experiments were carried out in triplicate.



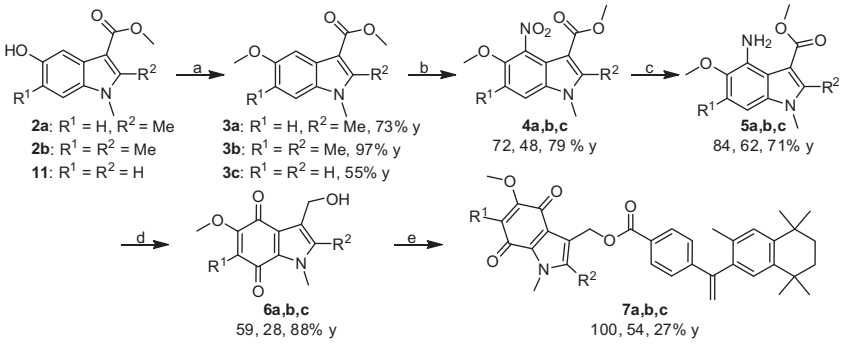
Scheme 1. Reagents and conditions: Precursor of **2a** and **2b**: f: $MeNH_2$, rt, overnight, g: quinone (**2a**) or methylquinone (**2b**), nitromethane, 24 h, rt.

Bexarotene is cleaved from compound **7a**, a 3-hydroxymethyl ester (Fig. 3). 50% bexarotene is released after 120 min, after which no further cleavage could be observed. This might be due to NQO1 inhibitory properties of compound **7a**, as some 3-hydroxymethyl derivatives are inhibitors of NQO1.[25,30] Compound **7b** was prepared to evaluate if the positive inductive effect of the additional methyl group might facilitate the release of bexarotene. For **7b** and **7c**, free bexarotene can also be observed, but cleavage is slower and plateaus at a much lower level (Fig. 4). A reason could be that compound **7b** is too sterically demanding to be cleaved by NQO1 because of the additional methyl group. The behavior of compound **18** is displayed in Figure 3. No release of bexarotene occurred during the observation period of 4 h. Even after 24 h of incubation, no cleavage could be observed (data not shown). This was unexpected, as 2-hydroxymethyl indolequinones were previously reported to be the better substrates for NQO1.[20,21] The reduced peak area of compound **18** might be due to association with the enzyme without cleavage. The behavior of compounds **7a–c** and **18** at 37 °C in 1 M HCl (to mimic gastric acid) was also monitored. There was no release of bexarotene during 4 h.

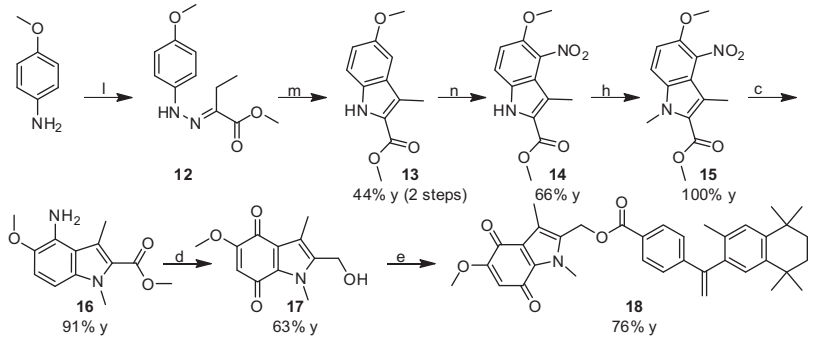
To investigate if the stagnation in the formation of the peak area of bexarotene was due deterioration of NADH over time, additional NADH was added to the test solutions after 30 and 60 min. However, no change in peak area was observed (data not shown). The reasons could be either substrate inhibition or product inhibition. Two model compounds, a 2-hydroxy- and a 3-hydroxyindole conjugated to 3-methyl-4-nitrophenol, were used to elucidate whether the indolequinones inhibit the diaphorase enzyme (see Supplementary information). As the model compounds were cleaved completely within minutes, this seems unlikely. In addition, in a more physiological environment, neither the reaction products or starting materials would accumulate in the way they do in a reaction vial.

Lastly, compound **19** was prepared with an additional methyl group on the 3-hydroxymethyl (Fig. 4, for synthesis see Supplementary information). A compound coupled to the same indolequinone had been released more than 10 times faster than the one without the methyl in a reductive elimination study because of its positive inductive effect on the release position.[17] The conjugate of this indolequinone with bexarotene (**19**) was not cleaved at all (data not shown).

Compounds were also tested for enzymatic stability using porcine liver esterase using a modified method from Bonina,[31] employing a final concentration of 76 μg/mL (1.3 U/mL) esterase in tris buffer pH 7.4. The conjugates (5 μM) showed no cleavage of the ester over 180 min and only 13% free bexarotene after 24 h (see Supplementary information for compound **7a** as example), whereas ethylbenzoate (100 μM) as positive control was cleaved completely after 7 min. Hence, even though cleavage



Scheme 3. Reagents and conditions: a: MeI, KOH, DMSO, rt, 3 h; b: HNO_3, acetic acid, −10 °C, 2 h; c: Sn, HCl, EtOH, 30 min reflux; d: (1) $LiAlH_4$, THF, 0 °C, 30 min, (2) $K_4[ON(SO_3)_2]_2$, acetone, rt, 1 h; e: bexarotene, DEAD, PPh_3, THF, 0 °C → rt, overnight.



Scheme 4. Reagents and conditions: l: (1) $NaNO_2$, HCl, 0 °C; (2) KOH, methyl 2-ethylacetoacetate; m: HCl, reflux; n: HNO_3, DCM, −20 °C; h: MeI, KOH, acetone, rt, time; c: Sn, HCl, EtOH, 30 min reflux; d: (1) $LiAlH_4$, THF, (2) $K_4[ON(SO_3)_2]_2$, acetone; e: bexarotene, DEAD, PPh_3, THF, 0 °C → rt.

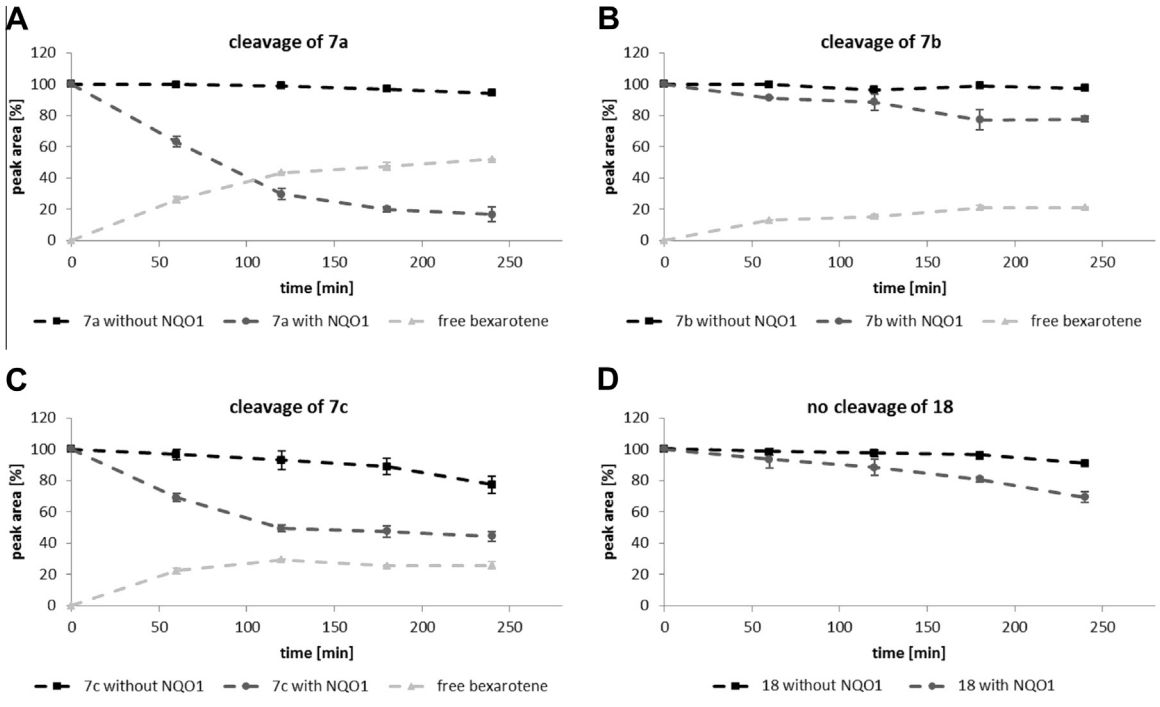


Figure 3. Release of bexarotene from (A) **7a**, (B) **7b** and (C) **7c** by NQO1, (D) no release of bexarotene from **18** could be observed.

by NQO1 appears to be rather slow, there is no competing cleavage by esterase.

2-Hydroxymethyl derivatives had been found to be better substrates for NQO1 and are also preferentially cleaved by it.[20] 3-Hydroxymethyl indolequinones like compounds **7a–c** may even be inhibitors of NQO.[25,30] In summary, the 2-hydroxymethyl indolequinone derivative **18** should have released the drug better than the 3-hydroxymethyl derivatives **7a–c**. Contrary to the literature, the 2-hydroxymethyl indolequinone **18** was not cleaved at all, but **7a** was cleaved best. Others studied radiolytic cleavage of

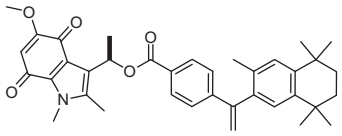


Figure 4. Compound **19** with an additional methyl group (bold) on the release position.

aspirin from 2- and 3-hydroxymethyl indolequinones and also found that only the 3-hydroxymethyl derivatives were cleaved, but no explanation was given.[32] However, these studies were done in the absence of O_2 to mimic hypoxia. O_2 was not excluded in our experiments, but we still found the 3-hydroxymethyl derivatives to be cleaved instead of 2-hydroxymethyl.

Further research is being conducted on other prodrugs for pathology activated therapies in Parkinson's disease.

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Supplementary data

Supplementary data (experimental procedures for all compounds, diagrams for incubation with esterase) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.03.003.

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