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# Design, Synthesis, and Characterization of Brequinar Conjugates as Probes to Study DHODH Inhibition**

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**Abstract:** Brequinar, a potent dihydroorotate dehydrogenase (DHODH) inhibitor, has been evaluated in multiple clinical trials as a potential treatment for cancer. To further understand brequinar-based DHODH inhibition and DHODH's therapeutic relevance in cancer, we have developed novel brequinar-based probes. We disclose a 16-step convergent synthesis of the first brequinar-PROTAC and a four-step approach towards the first mitochondrial-directed brequinar probe. A PROTAC and mitochondria-directed probe of brequinar both possess cytotoxicity that is superior to brequinar in a colony formation assay.

Over 20 years ago, a potent dihydroorotate dehydrogenase (DHODH) inhibitor, brequinar, was evaluated in multiple cancer clinical trials.[1] In preclinical studies, brequinar inhibited rapid cellular growth by inducing pyrimidine depletion and suppressed $>90\%$ tumor growth in murine studies at 20–40 mg $kg^{-1}$ $day^{-1}$.[2] However, its transition from bench to bedside was disappointing as few patients met their objective response despite the fact that analysis of patient-derived samples showed that DHODH was inhibited and uridine depletion had occurred.[3] As newer studies emerged suggesting that DHODH may be an effective target,[4] we designed novel molecular probes to better understand its therapeutic relevance in cancer. In this work, we report our convergent syntheses of novel brequinar probes to induce DHODH degradation and improve brequinar's accumulation in the mitochondria. Our approach was to incorporate late-stage functionalization of the brequinar scaffold with molecular "tags" to generate brequinar-based probes.

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[**] *DHODH = dihydroorotate dehydrogenase*

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To induce intracellular DHODH knockdown, we planned to develop a proteolysis-targeting chimera (PROTAC) of brequinar. A bifunctional PROTAC probe can induce intracellular protein degradation by utilizing the cell's ubiquitin proteasome system.[5] In short, a small molecule with a known target can be attached to an established E3 ligase ligand (tag) and when both moieties are simultaneously bound to their respective target, the E3 ligase may ubiquitinate lysine residues on the target protein.[6] This ubiquinated protein is then signaled for degradation by cellular proteasomes. Many examples of this approach have been published.[5,7] Our second probe was designed to direct subcellular localization to the mitochondria by incorporating a membrane-permeable delocalized lipophilic cation onto a target compound. The electrochemical gradient required for mitochondrial respiration drives permeable cations across the membrane.[8] Our plan was to incorporate a large triphenylphosphonium (TPP) cation, which should hinder water solvation and reduce unfavorable interactions with hydrophobic membranes. As a result, these cations are more membrane permeable, leading to selective accumulation in the mitochondria. Because DHODH is located within the mitochondria, we propose that improving brequinar's exposure to the target may improve potency in cells.

We postulated that a properly designed PROTAC or TPP probe of brequinar would be more potent in cells than brequinar. To evaluate this hypothesis, we designed probes to optimize the effectiveness of our functional tags without significantly decreasing inhibitory activity for DHODH. The design of the PROTAC probe focused on identifying linker attachment sites on brequinar that would allow for an E3 ligase to be recruited. The brequinar-analogue/DHODH co-crystal structure (1D3G) shows the presence of multiple lysine residues near the binding pocket (Figure 1).[9] Using this, we targeted probe **11**, shown in Scheme 1, in which the *para* position of the terminal aromatic ring of brequinar incorporates a linker that should not significantly decrease DHODH potency and permit solvent exposure to the tag. Our second probe, which was aimed at improving mitochondrial accumulation, was designed to ensure an overall positive charge on the probe. Brequinar's carboxylic acid functionality is mostly deprotonated at the pH (7.4) of cellular assays leaving the molecule with an overall negative charge, thus resulting in poor membrane permeability. To successfully transport a charged molecule to the mitochondria, it must possess an overall positive charge and have a high degree of lipophilicity. Toward this end, compound **16**

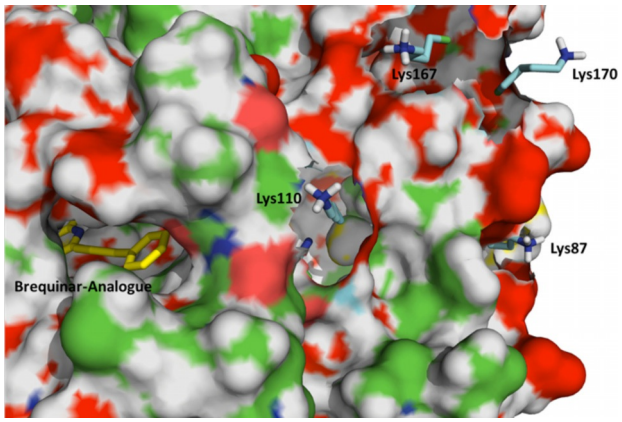



**Figure 1.** Depiction of the brequinar binding pocket in DHODH and lysine residues with solvent exposure near the binding pocket (PDB 1D3G).

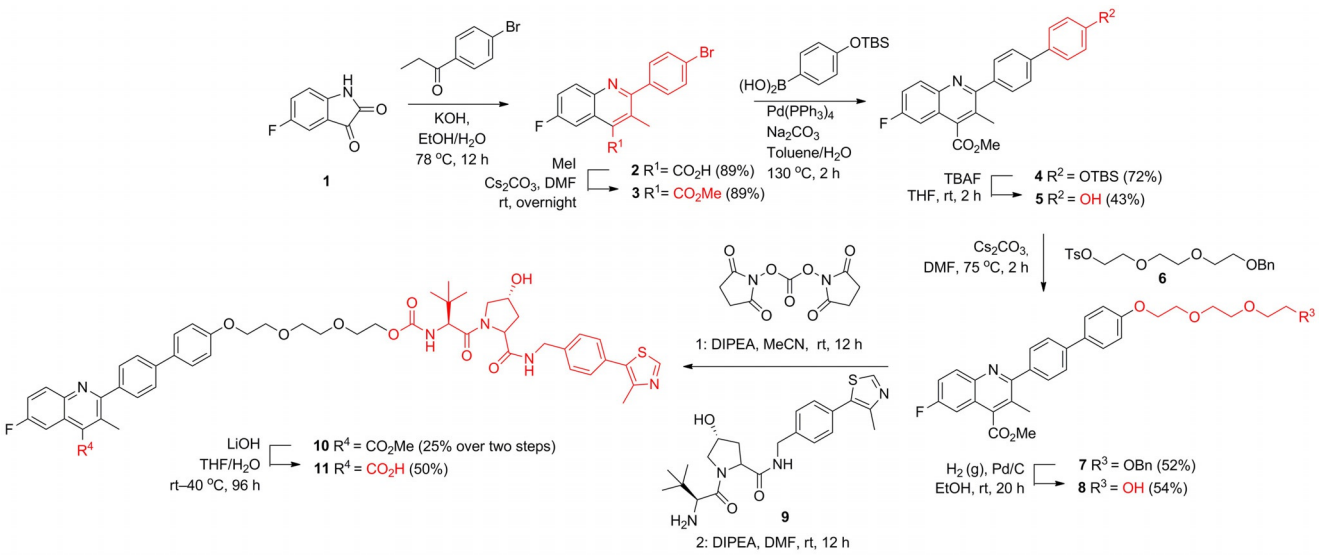
(Scheme 2) with the triphenylphosphonium head group became our target probe.

Our synthetic approach for each probe focused on utilizing a convergent strategy with late-stage incorporation of functional tags. The synthesis of a brequinar-PROTAC probe incorporating an ether attachment at the *para* position of the terminal aromatic ring is outlined in Scheme 1. The highly substituted quinoline ring was constructed using the Pfitzinger reaction to make **2**, which was readily esterified to afford **3** in 89% yield upon generation of the cesium salt and exposure to iodomethane.[10] Suzuki coupling of **3** with a suitable phenylboronic acid gave the TBS (*tert*-butyldimethylsilyl)-protected phenol (**4**) in good yield (72%), which was then treated with TBAF (tetrabutylammonium fluoride) to give **5** in a 25% overall yield from **1**. Condensation of the cesium phenolate salt of **5** with **6**[11] installed a PEG-linker to give **7**, which was positioned for a second-stage attachment of the VHL (von Hippel–Lindau) ligand.

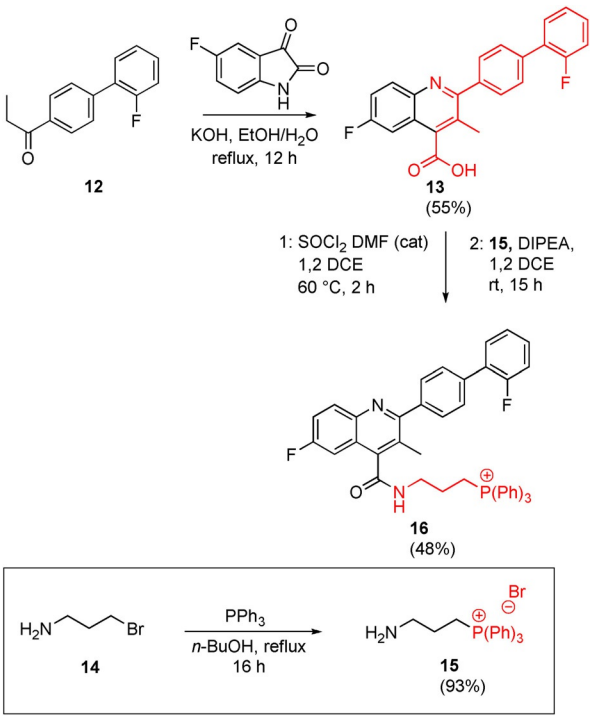
Hydrogenolysis of the benzylated linker of **7** unveiled the terminal alcohol **8** (28% overall yield from **5**), which was then activated with disuccimidyl carbonate. The activated intermediate was reacted under mildly basic conditions with the VHL E3 ligase ligand **9**, which was synthesized following a previously published six-step scheme[6] (see the Supporting Information), to give carbamate **10**. Mild ester hydrolysis of compound **10** was conducted with LiOH to minimize possible epimerization of the *tert*-butyl chiral center. Thus, heating of **10** at 40 °C for four days resulted in 50% conversion to desired product **11**, which was separated from starting ester **10** by preparative reverse-phase chromatography in a 50% yield and a 1% overall yield from **1**.

The synthesis of our second probe, **16**, is shown in Scheme 2. Brequinar (**13**) was readily synthesized from ketone **12** under classic Pfitzinger conditions.[10,4a] The desired cationic fragment **15** was generated through nucleophilic displacement of bromide **14** with triphenylphosphine. Amide coupling of **13** with **15** required extensive investigation to find conditions that allowed for easy purification of probe **16**. While standard coupling conditions with HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate) and HOBt/EDC (EDC = *N*-Ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide) worked well, product purification was best facilitated by simple acid chloride generation of **13** followed by reaction with amine **15**. Probe **16** was generated in 26% overall yield from **12**.

Probe **11** containing the crucial carboxylic acid maintained excellent potency ($IC_{50} = 0.093 \pm 0.04$ μM) in the DHODH assay (Figure 2C). However, in cells, **11** did not inhibit cell growth and showed an $IC_{50} > 30$ μM in HCT-116, a colon cancer cell line that is sensitive to DHODH inhibition, and > 50 μM in Mia-Paca-2. Conversely, the methyl ester **10** was more potent in HCT-116 cells ($IC_{50} = 6.8 \pm 2.9$ μM in HCT-116), which may be a result of superior cellular permeability in comparison to **11**. Ester **10** may be hydrolyzed to the carboxylic acid inside the cell, which may explain its cellular toxicity. This pro-drug approach would significantly improve target exposure and may also explain the difference between **10** and **11**'s cellular activi-

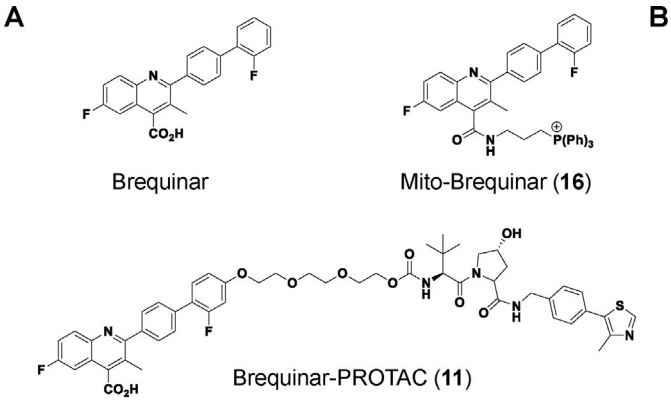


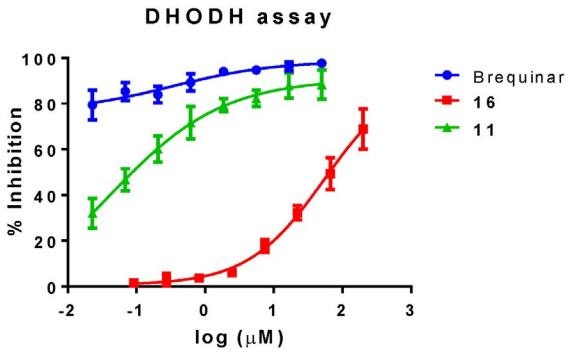

**Scheme 1.** Synthesis of brequinar-PROTAC probes **10** and **11**.




**Scheme 2.** Synthesis of mitochondria-directed brequinar **16.**

ty. Interestingly, HCT-116 dosed with **10** completely blocked colony formation at 2.5 μM and fewer colonies were formed than were observed at a four-fold higher dose of brequinar (Figure 2D). The results of **10** in the clonogenic assay differed from the MTT, which may have occurred for a few reasons. In the clonogenic assay, cells were treated with **10** for twice as long as they were in the MTT assay, which may be more favorable towards ester hydrolysis to yield **11**. However, **10** hindered new colony formation better than brequinar and has inspired us to pursue a more in-depth biological characterization of **10**. Unfortunately, protein degradation was not observed with either **10** or **11** using western blot (see the Supporting Information, Figure 1). There are several possible explanations for this lack of activity. First, there are currently no published PROTAC probes that induce protein degradation with mitochondrial targets. The protein ubiquitination system within the mitochondria may be significantly different than the cytosol or utilize a different E3 ligase. Interestingly, Azzu and Brand established that the inner mitochondrial protein UCP2 can be degraded using cytosolic proteasomal machinery, but it is unclear which E3 ligase performs ubiquitination.[12] It is possible that a ligand for an E3 ligase that targets mitochondrial proteins is necessary for degradation. Additionally, the linker between brequinar and the VHL ligand could require optimization. We incorporated an ethylene glycol linker to keep the ClogP low with **11** having a predicted ClogP of 8.04 (free acid). If replaced

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| | $IC_{50}$ (μM) | | |
|---|---|---|---|
| **Compound** | **DHODH** | **HCT116** | **MiaPaCa-2** |
| Brequinar | < 0.02 | 0.94 ± 0.3 | 30.0 ± 14 |
| **10** | > 200 | 6.8 ± 0.9 | > 50 |
| **11** | 0.093 ± 0.04 | > 30 | > 50 |
| **16** | 68 ± 19 | 5.2 ± 0.3 | 3.7 ± 0.7 |

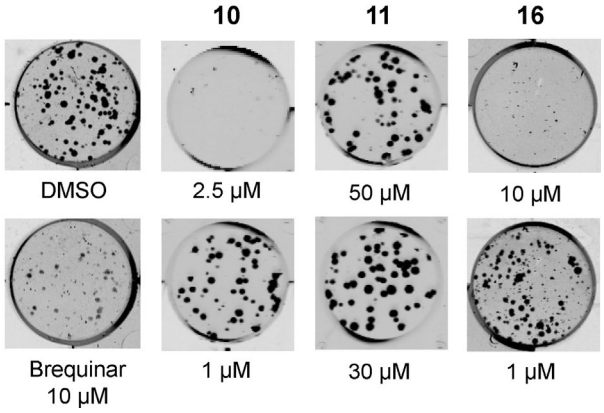


**Figure 2.** (A) Structures of brequinar and new probes; (B) dose–response curves from the DHODH assay; (C) $IC_{50}$ values from brequinar and new probes tested in DHODH and MTT assay; (D) HCT-116 colonies treated with varying doses of brequinar probes.

with an aliphatic chain of similar length, the ClogP would be 11.77 (free acid). Finally, other DHODH inhibitors may be better suited for PROTACs. An imatinib-based PROTAC probe did not induce degradation of BCR-ABL despite binding. However, dasatinib- and bosutinib-based PROTACs probes did induce BCR-ABL degradation.[13] This suggests that other structurally distinct DHODH inhibitors could induce degradation. Despite this, probe **10** did possess excellent cytotoxicity in HCT-116 that was superior to brequinar in the colony formation assay.

Probe **16** was not observed to inhibit DHODH at high concentrations ($IC_{50} > 50$ μM), which was expected, but it did display an $IC_{50} = 5.2 \pm 0.3$ μM in HCT-116 and was the most potent in MiaPaca-2 ($IC_{50} = 3.7 \pm 0.7$ μM). Significant cytotoxicity of **16** in MiaPaca-2 suggested that better mitochondrial accumulation may improve the therapeutic relevance of DHODH inhibition in pancreatic cancer. Probe **16** must be hydrolyzed within the mitochondria to induce cytotoxicity through DHODH inhibition. A $t_{1/2}$ of 277 min was observed for **16** in mouse-liver microsomes, which suggests that active metabolites may be generated during the dosing period. We found the TPP-amide linker **15** did not possess significant cytotoxicity ($IC_{50} > 100$ μM) and that **16** followed similar trends to brequinar (lower $IC_{50}$ value for continuous vs. 24 h treatment). These data support the pro-drug mechanism of action and suggest that further pro-drug analogues are warranted. We acknowledge that an ester attachment may increase the chances of hydrolysis; however, our initial efforts to generate an ester analogue of **16** were thwarted by poor compound stability. This proof of concept study has directed us to pursue SAR studies focusing on stable TPP pro-drugs that can be cleaved by mitochondrial esterases.

In summary, we present the synthesis of two new brequinar-based probes to evaluate mechanistic details of DHODH inhibition. We have developed novel synthetic routes to brequinar probes with two different attachment sites off the quinoline scaffold, and have validated that the incorporation of targeting headpieces maintains cytotoxicity in cell lines. Future studies will focus on ligand/linker optimization and further evaluation of these probes.

## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** brequinar · DHODH · PROTAC · triphenylphosphonium

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