Journal Pre-proofs


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PII: S0960-894X(20)30700-9
DOI: https://doi.org/10.1016/j.bmcl.2020.127589
Reference: BMCL 127589

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 5 June 2020
Revised Date: 22 September 2020
Accepted Date: 24 September 2020

Please cite this article as: DeRatt, L.G., Christine Pietsch, E., Tanner, A., Shaffer, P., Jacoby, E., Wang, W., Kazmi, F., Zhang, X., Attar, R.M., Edwards, J.P., Kuduk, S.D., A Carboxylic Acid Isostere Screen of the DHODH Inhibitor Brequinar, *Bioorganic & Medicinal Chemistry Letters* (2020), doi: https://doi.org/10.1016/j.bmcl.2020.127589

# A Carboxylic Acid Isostere Screen of the DHODH Inhibitor Brequinar

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Dihydroorotate dehydrogenase (DHODH) enzymatic activity impacts many aspects critical to cell proliferation and survival. Recently, DHODH has been identified as a target for acute myeloid differentiation therapy. In preclinical models of AML, the DHODH inhibitor Brequinar (BRQ) demonstrated potent anti-leukemic activity. Herein we describe a carboxylic acid isostere study of Brequinar which revealed a more potent non-carboxylic acid derivative with improved cellular potency and good pharmacokinetic properties.

Acute myeloid leukemia (AML) is a cancer of the blood and bone marrow resulting from mutations that occur in normal blood stem cells.[1] In AML, leukemic cells lose the ability to differentiate into adult white blood cells. This leads to accumulation of abnormal myeloid progenitor cells (myeloblasts) in the bone marrow, which are characterized by excessive proliferation and disrupt the production of normal blood cells.[2]

The chemotherapeutic standard of care for AML has undergone little change over the last four decades.[3] Although a rare disease, AML is the most common type of acute leukemia in adults with >20,000 new cases reported per year in the US.[4] The rapid progression of untreated AML results in mortality within weeks to months and, along with the poor survivorship of treated patients (5 year survival rate <30%), highlights an unmet medical need for improved therapeutics.

With the success of differentiation therapy in acute promyelocytic leukemia (APL), which represents a small subset (10-15%) of all AML patients, this approach represents a powerful treatment strategy for the remaining 90% of the patient population.[5] In 2016, reports by Sykes et. al.[6] demonstrated the central role that dihydroorotate dehydrogenase (DHODH) plays in AML by regulating myeloid differentiation in both *in vitro* and *in vivo* models across a range of AML subtypes independent of oncogenic mutations. DHODH are flavin mononucleotide (FMN)-binding flavoproteins that catalyze the conversion of L-

dihydroorotate (DHO) to orotate (ORO), which represents the rate limiting step in the *de novo* pyrimidine biosynthesis pathway.[7] As such, inhibition of DHODH in AML represents a metabolic vulnerability that leads to differentiation and/or apoptosis.[8]

With the discovery of the role of DHODH in AML, a renewed energy has been focused on identifying novel DHODH inhibitors as therapeutic agents for cancer treatment.[9] Brequinar (BRQ), a biphenyl quinoline carboxylic acid with sub-nanomolar hDHODH enzyme inhibition, was the first DHODH inhibitor to enter clinical trials for oncology indications, albeit for solid tumors and not heme malignancies.[10] The results of several Phase II studies revealed no objective responses in the majority of patients and the drug had a narrow therapeutic window halting further clinical development.

Previous SAR studies surrounding BRQ focused on modification of the bulky biaryl subunit and quinoline core that exemplified the importance of the carboxylic acid moiety at C4 (Figure 1).[11] This carboxylate forms a key salt bridge interaction with Arg 136 (*vide infra*) in the hDHODH protein leading to a high affinity of BRQ for hDHODH. However, an exhaustive isostere study focused on replacement of the carboxylic acid functionality has not been reported.[12] It is well known that the presence of a carboxylic acid in a drug or drug candidate can be responsible for limited permeability, metabolic stability, and toxicities.[13]

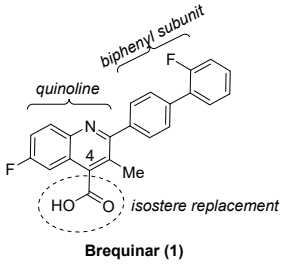



**Figure 1.** Brequinar (BRQ), a potent DHODH inhibitor

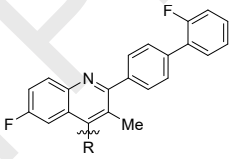
To date, carboxylic acid and salts thereof have been the only acceptable substituents at the C4 position of BRQ and its structural derivatives due to the aforementioned interactions with Arg136. Other functionalities, such as the ethyl ester or primary amide, drastically reduce or completely abolish the inhibitory activity.[14] Herein, structure activity relationship (SAR) studies on the replacement of the carboxylic acid with isosteres on the BRQ core structure are disclosed.

In our attempt to identify acceptable replacements for the carboxylic acid functionality, a number of novel and previously reported[14] DHODH inhibitors were prepared and evaluated in both an enzymatic and cellular assay. In our hDHODH enzymatic assay, BRQ **1** was confirmed to have exceptional inhibitor activity with an $IC_{50}$ = 0.48 nM (Table 1). However, in a cellular proliferation assay with MOLM-13 AML cells, a large 125-fold shift was observed, with a measured $IC_{50}$ of 60 nM. The large difference can likely be attributed to the low permeability due to the lipophilic carboxylic acid nature of BRQ. Consistent with findings in an earlier report,[14] the hydroxymethyl compound **2** led to a large (>300-fold) loss in biochemical activity, but only a 11-fold loss in cellular activity. Additionally, derivatives with fluorinated substituents at the C4 position (compounds **3**-**5**) were not well-tolerated and diminished activity was observed. A characteristic replacement of the carboxylic acid group in the form of a tetrazole (**6**) showed reduced enzymatic activity ($IC_{50}$ = 223 nM) and complete loss of activity in the cellular assay. Similarly, the oxadiazolone **7** was largely inactive in both assays. Interestingly, the incorporation of a

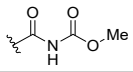
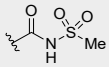
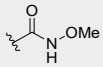
primary amide **8** provided a compound with an $IC_{50}$ of 47 nM in the enzymatic assay (100-fold loss vs BRQ), but only a 2-fold loss of activity was observed in the cellular assay ($IC_{50}$ = 128 nM) showing a substantial reduction in shift between the two assays.[14]

Efforts to modulate the pKa of the amide nitrogen to engage key hydrogen bonding interactions were then pursued. Both the cyanoacetamide **9** and the acetylcarbamate **10** did not provide an improvement in the inhibitory activity. The sulfonamide **11** was similarly potent in the biochemical assay, yet the cellular potency was reduced ~2-fold as compared to the primary amide **8**. Surprisingly, the *N*-hydroxyacetamide **12** demonstrated excellent potency with an $IC_{50}$ of 1.4 nM in the enzymatic assay, and only a 16-fold shift in the cellular potency ($IC_{50}$ = 23 nM). This derivative is more potent in MOLM-13 than BRQ and marks a substantial improvement in the shift between biochemical and cellular assays as well. Moreover, the *N*-methoxyacetamide **13** further improved the biochemical activity and cellular potency to $IC_{50}$ = 0.12 nM and $IC_{50}$ = 9.0 nM, respectively. This represents ~6-fold potency enhancement in the MOLM-13 cellular assay compared to BRQ. Further SAR showed that activity was reduced modestly when elongating the alkyl substituent on the hydroxyamide to *N*-ethoxyacetamide **14**, but still an improvement compared to BRQ. Lastly, the *N*-hydroxy-*N*-methylacetamide **15** was prepared to study the effects of the disruption of potential hydrogen bonding interactions. Interestingly, **15** maintained activity in the biochemical assay though this result may be attributed to the observed hydrolytic instability of **15.**[15] Additionally, BRQ and **13** were tested using a THP-1 cell line and shown to have a measured proliferation $IC_{50}$ = 300 nM and 50 nM, respectively. This result confirms the MOLM-13 observation and demonstrates that the trend in cellular activity is not restricted to one cell type.

**Table 1.** Effects of Carboxylic Acid Replacement on Enzymatic and Cellular Potency[a]



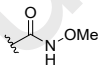

| Compound | R = | DHODH Enzymatic Assay $IC_{50}$ (nM) | MOLM-13 Cellular Assay $IC_{50}$ (nM) |
|---|---|---|---|
| **1 (BRQ)** |  | 0.48 | 60 |
| **2** |  | 166 | 700 |
| **3** |  | 160 | >3000 |
| **4** |  | 808 | 640 |
| **5** |  | 74 | >3000 |
| **6** |  | 223 | >3000 |
| **7** |  | 1560 | >3000 |
| **8** |  | 47 | 128 |
| **9** |  | 116 | >3000 |

| | | | |
|---|---|---|---|
| **10** |  | 1250 | 403 |
| **11** |  | 32 | 300 |
| **12** |  | 1.4 | 23 |
| **13** |  | **0.12** | **9** |
| **14** |  | 4.2 | 33 |
| **15** |  | 7.7 | 5100 |

[a]Values represent the average of n ≥ 2 experiments. Inter-assay variability <30%. DCIP absorbance assay has a LLOQ of 0.5 nM.

The smaller shift in biochemical versus cellular activity is worth noting for the amide class of compounds which can be attributed to the reduced lipophilicity leading to better permeability. For reference, the LogP of BRQ was calculated to be 6.39. The lower cLogP for amide **8** (5.36) results in a more permeable analog with a corresponding reduced shift between the biochemical and cellular assays. For compounds **12** and **13**, the LogP was calculated to be 5.08 and 5.43, respectively. As shown in Table 2, the permeability of **13** was greatly improved, resulting in less of a shift between assays. Additionally, good human microsomal metabolic stability was observed, although the mouse showed higher clearance for all carboxamides relative to BRQ.

**Table 2.** Physicochemical Properties of BRQ Derivatives.

| Compound | R = | MOLM-13 Cellular Assay $IC_{50}$ (nM) [a] | A→B (+Pgp inh.)[b] | cLogP | Human/Mouse LM $t_{1/2}$ (min) |
|---|---|---|---|---|---|
| **1 (BRQ)** |  | 60 | 8.99 | 6.39 | >180/>180 |
| **8** |  | 128 | 19.7 | 5.36 | 135/65 |
| **12** |  | 23 | 8.43 | 5.08 | >180/36 |
| **13** |  | 9 | 22.8 | 5.43 | >180/57 |
| **14** |  | 33 | 11.1 | 5.96 | >180/18 |

[a]Values represent the average of n ≥ 2 experiments. Inter-assay variability <30%. [b]MDCK cells.

With the improved cellular activity observed for the *N*-methoxyamide **13**, the pharmacokinetic profile was obtained in mouse for comparison with BRQ. Compound **13** demonstrated high oral bioavailability, a larger Vdss compared to BRQ, and a $t_{1/2}$ of 2.35 h. The clearance was still low, which was notable considering the stability in mouse liver microsomes was considerably lower ($t_{1/2}$ = 57 min) compared to BRQ ($t_{1/2}$ > 180 min). Additionally, the plasma protein binding (PPB) was high for both compounds.

Overall, this shows that good pharmacokinetics and improved potency can be achieved with derivatives that lack the carboxylic acid functionality found in BRQ.

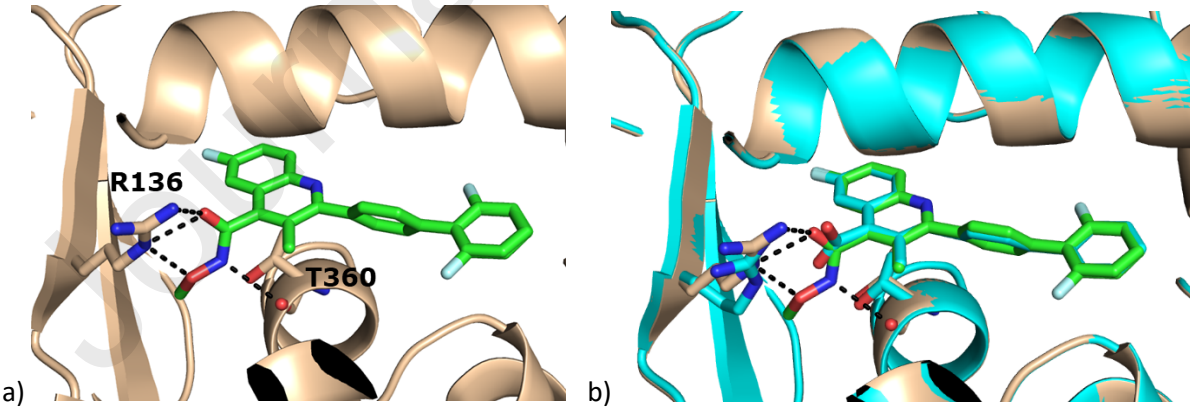
**Table 3.** Mouse pharmacokinetics of BRQ and **13**.[a]

| Compound | F (%) | $t_{1/2}$ (h) | Cl (ml/min/kg) | Vdss (L/kg) | PPB h/m (% free) |
|---|---|---|---|---|---|
| **1 (BRQ)** | 72 | -* | 0.2 | 0.3 | 0.39/0.73 |
| **13** | 108 | 2.35 | 5.09 | 1.15 | 0.55/0.77 |

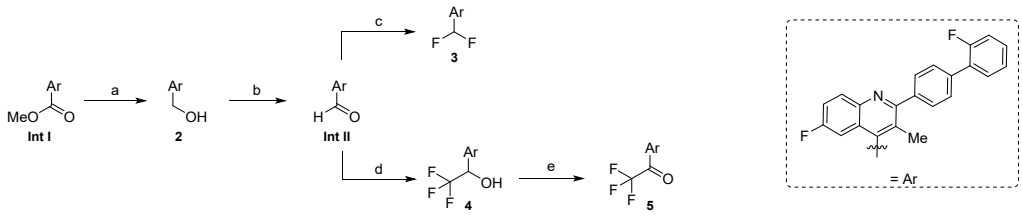
[a] C57 Mice (n = 6). Oral dose 10 mg/kg, PEG400/water (70:30) vehicle; IV dose = 2 mg/kg, PEG400/water (70:30) vehicle. *not determined due to high extrapolated AUC.

To further understand the improvement in activity of **13**, a crystal structure with the DHODH protein was obtained through a co-crystallization experiment (pdb 7K2U) (Figure 2a). The electron density for most of the inhibitor was excellent except for the methoxy group which was much weaker, perhaps indicating some mobility of this group in the binding site. The fluorophenyl is modeled in two conformations that were supported by the electron density. Comparing the structure of DHODH bound to **13** with the published structure of hDHODH bound to des-fluoro-BRQ analog (pdb 1D3G) shows **13** adopts a very similar binding mode with only slight rearrangement of the sidechain of Arg136 in the binding site (Figure 2b). The carbonyl of the amide of **13** made two hydrogen bonds with the sidechain of Arg136, similar to what is observed with BRQ. The nitrogen of the amide donated a hydrogen bond to the backbone carbonyl of Thr360, an interaction not observed with BRQ. Additionally, the oxygen of the methoxy made a hydrogen bond with the sidechain of Arg136. This slight change in binding pattern can be rationalized in part by the difference in pKa of the *N*-methoxyamide (measured: 7.31 ± 0.01; calculated: 8.6) compared to the carboxylic acid (measured: 4.15 ± 0.05; calculated: 2.4).[16] Analogous to BRQ, numerous hydrophobic interactions with many regions of the protein were also observed.

**Figure 2.** a) Crystal structure of **13** (7K2U) b) Overlay of **13** (7K2U) and des-fluoro-BRQ analog (1D3G). DHODH/compound **13** are shown in wheat/green, respectively. 1D3G overlay shown in cyan. Dashed lines represent hydrogen bonds between **13** and DHODH.

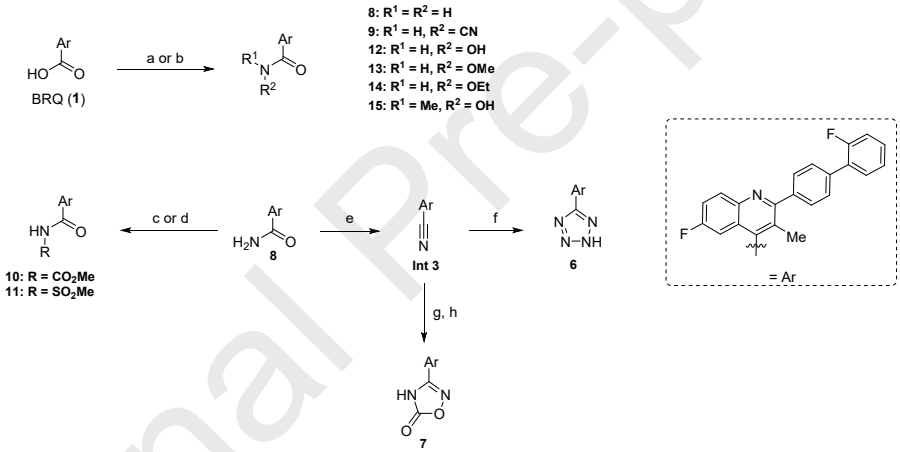



The synthesis of the target compounds **2-5** is described in Scheme 1. Compound **2** was prepared from the requisite BRQ methyl ester via reduction with DIBAL. Oxidation using DMP afforded the aldehyde intermediate, which was treated with DAST and $TMSCF_3$ to provide **3** and **4**, respectively. Further oxidation of **4** with DMP afforded ketone **5**.



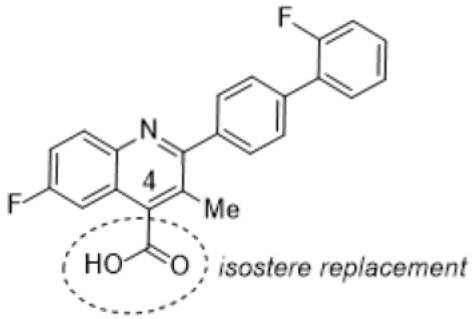

**Scheme 1.** Synthesis of compounds **2-5**. Reagents and conditions: a) DIBAL, toluene, DCM, -78 °C to r.t., 99%. b) DMP, DCM, r.t., 87%. c) DAST, DCM, -78 °C to r.t., 80%. d) $TMSCF_3$, $K_2CO_3$, DMF, r.t. then 1N HCl, 89%. e) DMP, DCM, r.t., 76%.

Amides **8**, **9**, **12**, and **13** were synthesized using PyBOP as a coupling agent with the appropriate amine (Scheme 2). Compounds **14** and **15** were prepared through conversion of BRQ to the acid chloride then treatment with the corresponding amine. The primary amide **8** was then treated with NaH and MsCl to generate compound **10**. Compound **11** was formed in an analogous fashion using NaH, LiOtBu and $CO(OMe)_2$. Additionally, compound **8** was treated with TFA in pyridine to form the nitrile intermediate which was further converted to tetrazole **6**. Lastly, oxadiazolone **7** was obtained through a two-step process from the nitrile intermediate.




**Scheme 2.** Synthesis of compounds **6-15**. Reagents and conditions: a) $(COCl)_2$, DMF, DCM then either $NH_2OEt$ or NHMeOH, 73% and 28% over two steps, respectively. b) PyBOP, $Et_3N$, DMF and either $NH_4Cl$, $NH_2CN$, $NH_2OH$•HCl, or $NH_2OMe$, 18%, 34%, 55%, and 38%, respectively. c) NaH, MsCl, THF 0 °C to r.t., 44%. d) NaH, Li*O*tBu, $CO(OMe_2)$, THF, 0 °C to r.t. ,37%. e) TFA, pyridine, 1,4-dioxane, 0 °C to r.t., 73%. f) $NaN_3$, $NH_4Cl$, DMF, 130 °C, 21%. g) $NH_2OH$ (50% aqueous), EtOH, r.t. to 80 °C. h) CDI, DBU in 1,4-dioxane, 100 °C, 27% over two steps.

In summary, an SAR study on the carboxylic acid moiety of Brequinar revealed a subset of isosteres that led to improved activity in both biochemical and cellular assays. Additionally, the enhanced permeability of these compounds corresponded to a lower shift between the two assays. In particular, *N*-methoxyamide **13** exhibited good pharmacokinetic properties in mouse and represents a compelling non-carboxylic acid starting point for further modifications of the aromatic regions of the compound to further reduce lipophilicity.




**DHODH inhibitor**
**Brequinar**

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