_N_-Phenyl Ureidobenzenesulfonates, a Novel Class of Promising Human Dihydroorotate Dehydrogenase Inhibitors

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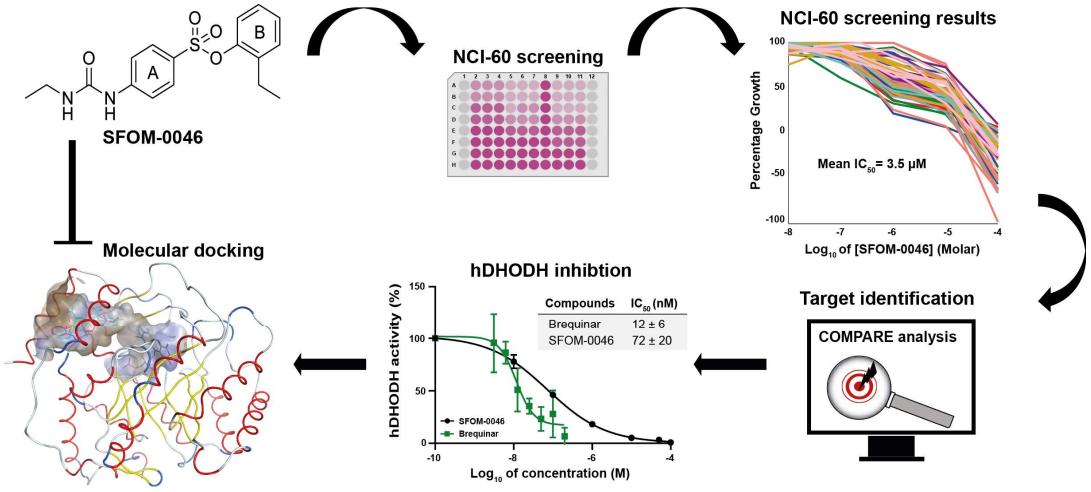
GRAPHICAL ABSTRACT



ABSTRACT

N-phenyl ureidobenzenesulfonates (PUB-SOs) is a new class of promising anticancer agents inducing replication stresses and cell cycle arrest in S-phase. However, the pharmacological target of PUB-SOs was still unidentified. Consequently, the objective of the present study was to identify and confirm the pharmacological target of the prototypical PUB-SO named 2-ethylphenyl 4-(3-ethylureido)benzenesulfonate (SFOM-0046) leading to the cell cycle arrest in S-Phase. The antiproliferative and the cytotoxic activities of SFOM-0046 were characterized using the NCI-60 screening program and its fingerprint was analyzed by COMPARE algorithm. Then, human dihydroorotate dehydrogenase (hDHODH) colorimetric assay, uridine rescuing cell proliferation and molecular docking in the brequinar-binding site were performed. As a result, SFOM-0046 exhibited a means antiproliferative activity of 3.5 μM in the NCI-60 screening program and evidenced that leukemia and colon cancer cell panels were more sensitive to SFOM-0046. COMPARE algorithm showed that the SFOM-0046 cytotoxic profile is equivalent to the ones of brequinar and dichloroallyl lawsone, two inhibitors of hDHODH. SFOM-0046 inhibited the hDHODH in the low nanomolar range (IC_{50} = 72 nM) and uridine rescued the cell proliferation of HT-29, HT-1080, M21 and MCF-7 cancer cell lines in the presence of SFOM-0046. Finally, molecular docking showed binding pose of SFOM-0046 interacting with Met43 and Phe62 present in the brequinar-binding site. In conclusion, PUB-SOs and notably SFOM-0046 are new small molecules hDHODH inhibitors triggering replication stresses and S-Phase arrest.

KEY WORDS anticancer agents; human dihydroorotate dehydrogenase inhibitors; *N*-phenylureidobenzenesulfonates; PUB-SOs; SFOM-0046

ABBREVIATIONS

DCIP, 2,6-dichlorophenolindophenol; DHO, dihydroorotate; DSBs, double-strand breaks; DTP, Developmental Therapeutics Program; DUQ, decylubiquinone; FMN, flavin mononucleotide; hDHODH, human dihydroorotate dehydrogenase; PUB-SOs, N-phenyl ureidobenzenesulfonates; SFOM-0046, 2-ethylphenyl 4-(3-ethylureido)benzenesulfonate; TCA, trichloroacetic acid.

1. Introduction

It is estimated that 18.1 million people would be affected by cancer in 2018 causing 9.6 million deaths worldwide.[1] In the United States, these alarming numbers of death ranks cancer as the second-leading cause of death after heart pathologies.[2] In the last decades, research in molecular oncology has led to the emergence of new targeted drugs against cancer.[3] Despite all the progress made so far, standard chemotherapy still has a very important role in the first-line treatments of cancers. However, standard chemotherapy is mostly aggressive approaches that bring deleterious effects and impede the quality of patients' lives.[4] For this reason, it is still crucial to develop new chemotherapeutic agents that are more selective and less toxic to improve cancer prognosis.

The human dihydroorotate dehydrogenase (hDHODH) is a key enzyme in the synthesis of pyrimidines and DNA. It is encoded by the DHODH gene on chromosome 16 and it catalyzes the fourth limiting steps in the *de novo* pyrimidine synthesis. hDHODH transforms the dihydroorotate to orotate with FMN and ubiquinone (CoQ) acting as cofactors. This reaction ultimately leads to uridine monophosphate (UMP) formation, DNA replication and protein synthesis.[5] For cells with increased proliferation rate, the role of hDHODH is fundamental to cell integrity and survival.[6] As expected, hDHODH inhibition leads to UMP deficit that activates p53 responsible for the arrest of the cell cycle progression in S-phase and replication stresses.[7] Double strand-breaks are also recurrent effects usually seen while using hDHODH inhibitors.[8] In summary, pharmacological inhibition of hDHODH starves cancer cells of essential nucleotides and impede their rapid proliferation blocking the cell cycle progression in the S-phase.[9]

hDHODH is a druggable enzyme and the development of new, more effective and less toxic hDHODH inhibitors as innovative alternatives in chemotherapy is in constant demand and is a

current issue.[10, 11] For example, several potent hDHODH inhibitors such as ASLAN003, BAY2402234 and brequinar sodium (DUP-785, NSC 368390) are currently studied in preclinical and clinical trials for various hematologic malignancies and inflammatory pathologies (Fig. 1a-c). Moreover, the importance of hDHODH inhibitors is confirmed by leflunomide and teriflunomide that are two hDHODH inhibitors FDA-approved for the treatment of rheumatoid arthritis or multiple sclerosis.[12]

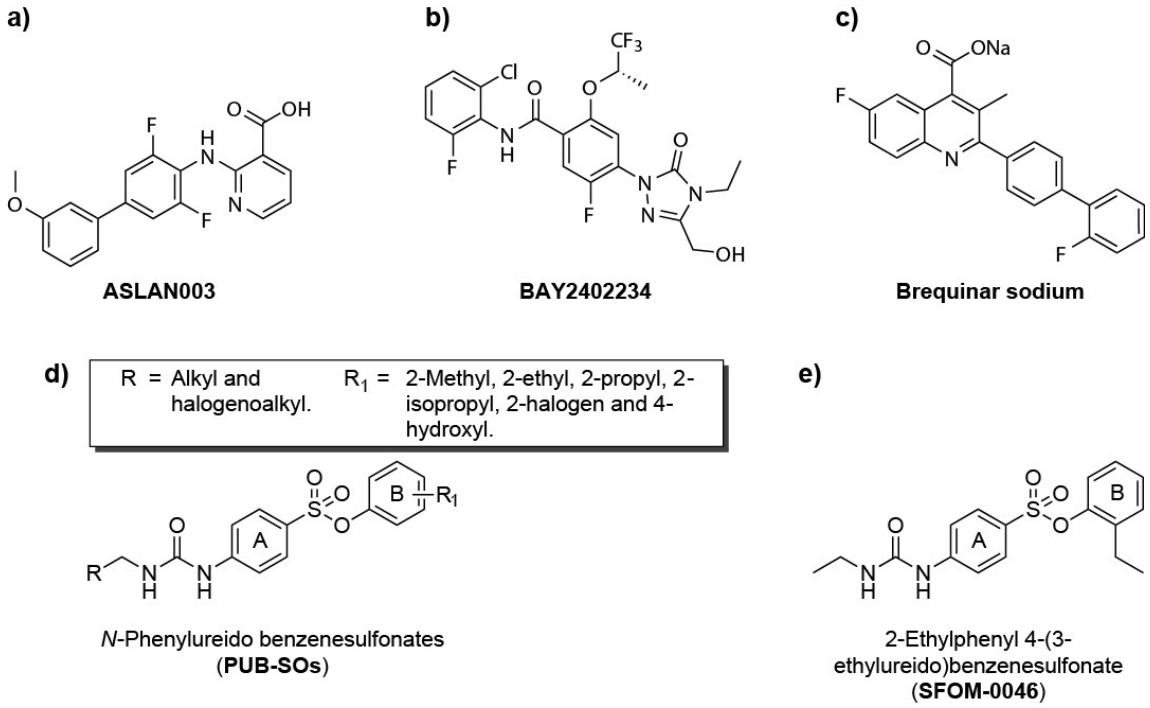


Fig. 1. Molecular structures of **a)** ASLAN003, **b)** BAY2402234, **c)** brequinar sodium, **d)** *N*-phenyl ureidobenzenesulfonates (PUB-SOs) and **e)** 2-ethylphenyl 4-(3-ethylureido)benzensulfonate (SFOM-0046).

It is in this context that we developed a new class of anticancer agents designed as *N*-phenyl ureidobenzenesulfonates (PUB-SOs, Fig. 1d).[13-16] PUB-SOs are small molecules composed of 2 aromatic rings (A and B) moieties bound through a sulfonate bridge. Our structure-activity relationship studies identified 2-ethylphenyl 4-(3-ethylureido)benzenesulfonate (SFOM-0046,

Fig. 1e) as one of the most promising hit compounds studied so far. SFOM-0046 has exhibited antiproliferative activity in the micromolar level on our panel of human cancer cell lines comprising fibrosarcoma HT-1080, skin melanoma M21, breast adenocarcinoma MCF-7 and colorectal adenocarcinoma HT-29. In addition, SFOM-0046 has exhibited a potent antitumoral activity similar to cisplatin on human HT-1080 fibrosarcoma tumors grafted onto the chorioallantoic membrane of chick embryos and low toxicity toward the embryos.[15] SFOM-0046 is characterized by induction of phosphorylation of histone 2AX (γ-H2AX) as well as phosphorylation of RAD51 and 53BP1, suggesting the involvement of replication stresses and DNA double-strand breaks (DSBs).[17, 18] SFOM-0046 was also found to activate ATM (*ataxia telangiectasia mutated*), ATR (*ataxia telangiectasia and Rad3-related protein*), checkpoint 1 (Chk1) and checkpoint 2 (Chk2) kinases.[19, 20] The activation of these signal pathways is known to lead to the arrest of cell division in S-phase.

Altogether, these results strongly suggest the involvement of replication stresses in the mechanism of action of SFOM-0046. However, the molecular target responsible for the replication stresses is still unidentified. The identification of such target is a prerequisite to the rational development of our new class of compounds. Consequently, the aim of the present study was to identify and validate the pharmacological target of PUB-SOs using SFOM-0046 as a prototypical compound. To that end, SFOM-0046 was first assessed using the National Cancer Institute's (NCI) Developmental Therapeutics Program (DTP) to screen its anticancer activity on their panels of 60 human tumor cell lines (NCI-60 screening program). Then, COMPARE algorithm from the DTP was used to identify potential pharmacological targets of SFOM-0046.[21] Thereafter, colorimetric enzymatic assay and antiproliferative rescuing experiments were performed to confirm its pharmacological target. Finally, molecular docking experiments were conducted to ascertain the binding mode of SFOM-0046 into the binding site of the pharmacological target found.

2. Materials and methods

2.1. Cells and materials

HT-29 human colon carcinoma, M21 human skin melanoma, HT-1080 human fibrosarcoma and MCF7 human breast carcinoma were purchased from the American Type Culture Collection (Manassa, VA, USA). SFOM-0046 was prepared according to the method described by Turcotte *et al.*[13] Brequinar used as hDHODH positive control was purchased from Tocris (Oakville, ON, Canada). Recombinant hDHODH was obtained from Abcam (Cambridge, MA, USA). Tris-HCl was supplied by Fisher Scientific (Edmonton, AB, Canada). All other chemicals were supplied by MilliporeSigma Canada Co. (Oakville, ON, Canada) or VWR International (Mont-Royal, QC, Canada) and used as received.

2.2. NCI-60 human tumor cell lines screen

SFOM-0046 was selected to integrate the NCI developmental therapeutic drug screening program to evaluate the cytotoxic activity of new compounds against 60 human cancer cell lines including leukemia, colon cancers, non-small cell lung cancers, central nervous system (CNS) cancers, melanoma, ovarian cancers, renal cancers, prostate cancers and breast cancers. The experiments were performed as described in the NIH/NCI-60 screening methodology.[22] Briefly, after cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, and 100% relative humidity for 24 h prior to the addition of experimental drugs. Following drug treatment, the plates were incubated for an additional 48 h at 37 °C, 5% CO_2. At the end of the incubation period, the cells were fixed *in situ* by the gentle addition of 50 µL of cold 50 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 min at 4 °C. The supernatant was discarded, and the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µL) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by

washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM Trizma® base, and the absorbance was read on an automated plate reader at a wavelength of 515 nm. For suspension cells, the same methodology was used but the cells were fixed with 50 µL of 80 % TCA (final concentration, 16% instead of 10% TCA). Using the absorbance measurements, concentration causing 50% of cell growth inhibition (IC_{50}), total growth inhibition (TGI) and lethal concentration causing 50% of the cell death (LC_{50}) were calculated.

2.3. COMPARE analysis for SFOM-0046 target prediction

The COMPARE analysis is based on the results provided by the NCI. The COMPARE algorithm can be found under the databases and tools section of the National Cancer Institute-DTP website (https://dtp.cancer.gov/databases_tools/compare.htm). Firstly, "Private COMPARE" was selected to find the NCI-60 screen related to SFOM-0046 in the NCI database. Then, the NSC number was entered and the DTP screening data GI_{50} endpoint were selected to run a standard COMPARE analysis. The standard and synthetic databases were selected to expand our research to a broad number of known chemicals. The IC_{50} parameters of SFOM-0046 with a minimum deviation of 0.2, standard deviation of 0.05 and minimum count of common cell lines of 55 were chosen.

2.4. hDHODH colorimetric assay

hDHODH colorimetric assay was used to assess the ability of SFOM-0046 to inhibit hDHODH enzyme and was based on the method described by Knecht *et al.* with slight modifications.[23] SFOM-0046 and brequinar used as positive control were freshly solubilized in DMSO. 99.5 µL of a buffer solution containing 50 mM Tris-HCl, 150 mM KCl, 0.1% Triton X-100 (pH 8), 0.1 mM decylubiquinone (DUQ), 1 mM DHO and 0.06 mM DCIP was added in a 96-well plate. Then, 0.25 µL of drug solution at the proper concentration followed by 0.25 µL (25 ng) of

hDHODH were added to the mixture to initiate the reaction. The absorbance was read every 10 min for 90 min with a SpectraMax® i3x (Molecular Devices, San Jose, CA, USA) at 610 nm. The initial rate of the reaction for SFOM-0046 at 100, 50, 1.0, 0.10 and 0.010 μM and brequinar at 200, 100, 50, 25, 12.5, 6.3 and 3.2 nM were calculated and the percentage of the activity was determined relatively to the enzyme activity with the vehicle (0.25% DMSO) which is considered as 100% activity. Finally, the IC_{50} was calculated using nonlinear regression by plotting the percentage of activity against log concentrations with GraphPad Prism 8.0.

2.5. Uridine rescue

HT-29, M21, HT-1080 and MCF7 cells were seeded at 3.5 x 10^5 cells per well in 96-well plates and incubated overnight at 37 °C, 5% CO_2, and 100% relative humidity. Uridine was freshly dissolved in water at a concentration of 40 mM and supplemented into the tissue culture media at 100 μM (final concentration).[24] Brequinar, SFOM-0046 and leflunomide in DMSO were diluted in supplemented DMEM and added to the cells with final concentrations ranged from 25 to 0.2 μM, 50 to 0.39 μM and 100 to 0.78 μM, respectively. DMSO concentration was kept constant at 0.5% (v/v) to avoid any related toxicity. SRB assay as described before was used to investigate the cell proliferation with and without addition of exogenous uridine. Concentration causing 50% of cell growth inhibition (IC_{50}) was calculated. The experiment was repeated at least twice in triplicate.

2.6. Molecular docking of SFOM-0046 in the brequinar-binding site

Docking experiments were performed using the Molecular Operating Environment (MOE) drug discovery software platform from Chemical Computing Group (version 2019.01).[25] Firstly, the X-ray crystallographic structure of the hDHODH complexed with 6-fluoro-2-[2-methyl-4-phenoxy-5-(propan-2-yl)phenyl]quinoline-4-carboxylic acid (C44), a brequinar analog (ID: 4IGH at 1.3 Å resolution) were obtained from the RCSB protein data bank and loaded to MOE

software. SFOM-0046 was drawn with ChemBioDraw 13.0 software and imported in MOE as SDF file for docking experiment. The protein was prepared using the QuickPrep tool with default settings to add hydrogens and partial charges, correct Asn/Gln/His orientations, optimize the H-bond network (protonate 3D), delete water molecules farther than 4.5 Å from the ligand and the receptor, and perform an energy minimization (RMS gradient of 0.1 kcal/mol/Å). The ligand was then isolated and the binding site was defined with amino acid near to 4.5 Å from brequinar and flavin mononucleotide (FMN). Then, energy minimization of brequinar-binding site with ligands was realized prior docking hDHODH inhibitors. The default settings apply to all atoms and include the absence of restriction, a force field Amber10: ETH; R-Field 1:80; Cutoff [8, 10], system appearing reasonable for the charges, the rigidity of the water molecules and a gradient of 0.1 RMS kcal/mol/Å^2. The surface hydrophobicity (brown) and hydrophilicity (blue) of the hDHODH binding site was mapped using the Surface and Map tool with default setting. Finally, SFOM-0046 was docked in the brequinar-binding site using the selected atoms from the receptor and the selected residues from the binding site. The different conformations, the interactions with the amino acids of the active site and the energies of the complexes were analyzed and recorded. Pictures of the most stable conformer in the brequinar-binding site was taken in the 2D and 3D models.

2.7. Data analysis

The human dihydroorotate dehydrogenase (hDHODH) enzymatic activity was realized with two independent experiments using at least 7 different points in the curve. The IC_{50} values represent the half-maximal inhibitory concentration. They were calculated using a nonlinear regression by plotting the percentage of activity against the log concentrations using GraphPad Prism 8.0. The standard deviation values were also calculated using GraphPad Prism 8.0.

3. Results and discussion

3.1. NCI-60 human tumor cell lines screen

The NCI-60 human tumor cell lines screen is an important service provided by the DTP at the cancer research community to assess the potential anticancer activity of synthetic, semi synthetic or purified natural products.[26] SFOM-0046 was sent to DTP and screened on the 60 cancer cell lines of NCI-60 screening program. The results are summarized in Fig. 2 and confirm the anticancer activity of SFOM-0046 on all cancer cell lines assessed with a mean antiproliferative activity of 3.5 μM. The complete NCI-60 results are shown in supplemental data (Fig. S1-S3). Additionally, the results show a higher activity of SFOM-0046 toward leukemia and colon cancer cells as compared to other cancer cell panels with an average IC_{50} of 0.81 and 1.5 μM, respectively. TGI and LC_{50} are mostly not reached at the higher concentration tested showing that SFOM-0046 is mainly a cytostatic rather than a cytotoxic agent. This difference in sensitivity between all cell lines tested in the NCI-60 drug screening program provides a unique anticancer activity profile of SFOM-0046 that can be used in the COMPARE analysis algorithm to identify its pharmacological target.

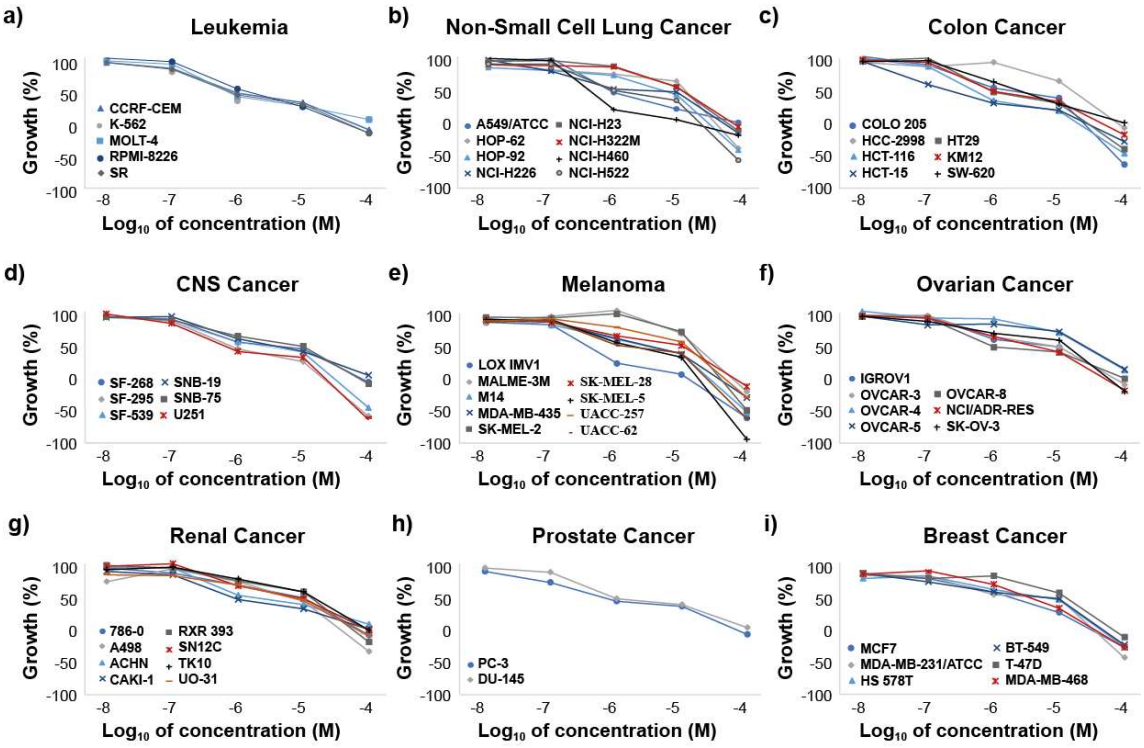


Fig. 2. Antiproliferative activity of SFOM-0046 against panels of cell lines in the NCI-60 screening assay[22]: **a)** Leukemia: CCRF-CEM, K-562, MOLT-4, RPMI-8226 and SR; **b)** Non-small cells lung cancers: A549/ATCC, HOP-62, HOP-92, NCI-H226, NCI-H23, NCI-H322M, NCI-H460 and NCI-H522; **c)** Colon cancers: COLO 205, HCC-2998, HCT-116, HCT-15, HT29, KM12 and SW-620; **d)** Central nervous system (CNS) cancers: SF-268, SF-295, SF-539, SNB-19, SNB-75 and U251; **e)** Melanoma: LOX IMV1, MALME-3M, M14, MDA-MB-435, SK-MEL-2, SK-MEL-28, SK-MEL-5, UACC-257 and UACC-62; **f)** Ovarian cancers: IGROV1, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, NCI/ADR-RES and SK-OV-3; **g)** Renal cancers: 786-0, A498, ACHN, CAKI-1, RXR 393, SN12C, TK10 and UO-31; **h)** Prostate cancers: PC-3 and DU-145 and **i)** Breast cancers: MCF7, MDA-MB-231/ATCC, HS 578T, BT-549, T-47D et MDA-MB-468.

3.2. COMPARE Analysis

The very distinct antiproliferative responses (IC_{50}, TGI and LC_{50}) toward either synthetic, natural or pharmaceutical products constituted a specific and unique pattern of drug activity named drug "fingerprint". Basically, the cellular resistance and sensitivity correlate with molecular target expression and provide the analytical basis for the COMPARE algorithm.[27] The COMPARE results from the analysis of SFOM-0046 is presented in Table 1. The COMPARE results from the standard database showed a strong similarity with two anticancer agents, namely brequinar and dichloroallyl lawsone (NSC 126771, Fig. 3a) exhibiting Pearson correlation coefficients of 0.815 and 0.794, respectively. Moreover, the COMPARE results from the synthetic database also showed high similarity with 2-(4-cyclohexylphenyl)-6-fluoro-3-methyl-4-quinolinecarboxylic acid (NSC 339768) a brequinar analog and the sodium salt of the dichloroallyl lawsone (NSC 136592, Fig. 3b) exhibiting Pearson correlation coefficients of 0.875 and 0.869, respectively. An example of the COMPARE analysis fingerprint is presented in the Fig. 4 showing the superposition of the antiproliferative profile of SFOM-0046 to that of brequinar. These results suggest that SFOM-0046 may act as brequinar and dichloroallyl lawsone, two hDHODH inhibitors.

Table 1

Highest Compounds Score and Pearson Correlation Coefficients Obtained From COMPARE Analysis of SFOM-0046 using Standard and Synthetic Databases.

Standard database		**Synthetic database**	
Brequinar	**Dichloroallyl lawsone**	**2-(4-Cyclohexyl phenyl)-6-fluoro-3-methylquinoline-4-carboxylic acid**	**Sodium dichloroallyl lawsone**
0.815	0.794	0.875	0.869

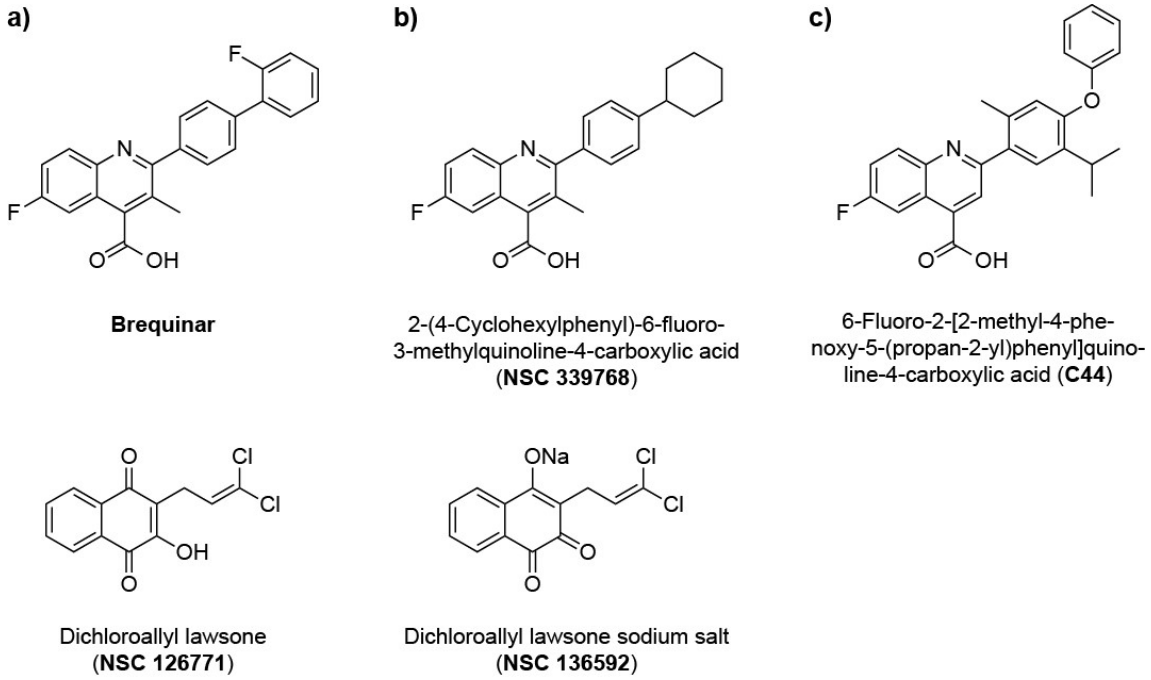


Fig. 3. Molecular structures of **a)** brequinar and dichloroallyl lawsone (highest COMPARE score of standard database), **b)** 2-(4-cyclohexylphenyl)-6-fluoro-3-methylquinoline-4-carboxylic acid and sodium dichloroallyl lawsone (highest COMPARE score of synthetic database) and **c)** 6-fluoro-2-[2-methyl-4-phenoxy-5-(propan-2-yl)phenyl]quinoline-4-carboxylic acid (C44) used in molecular modeling experiments.

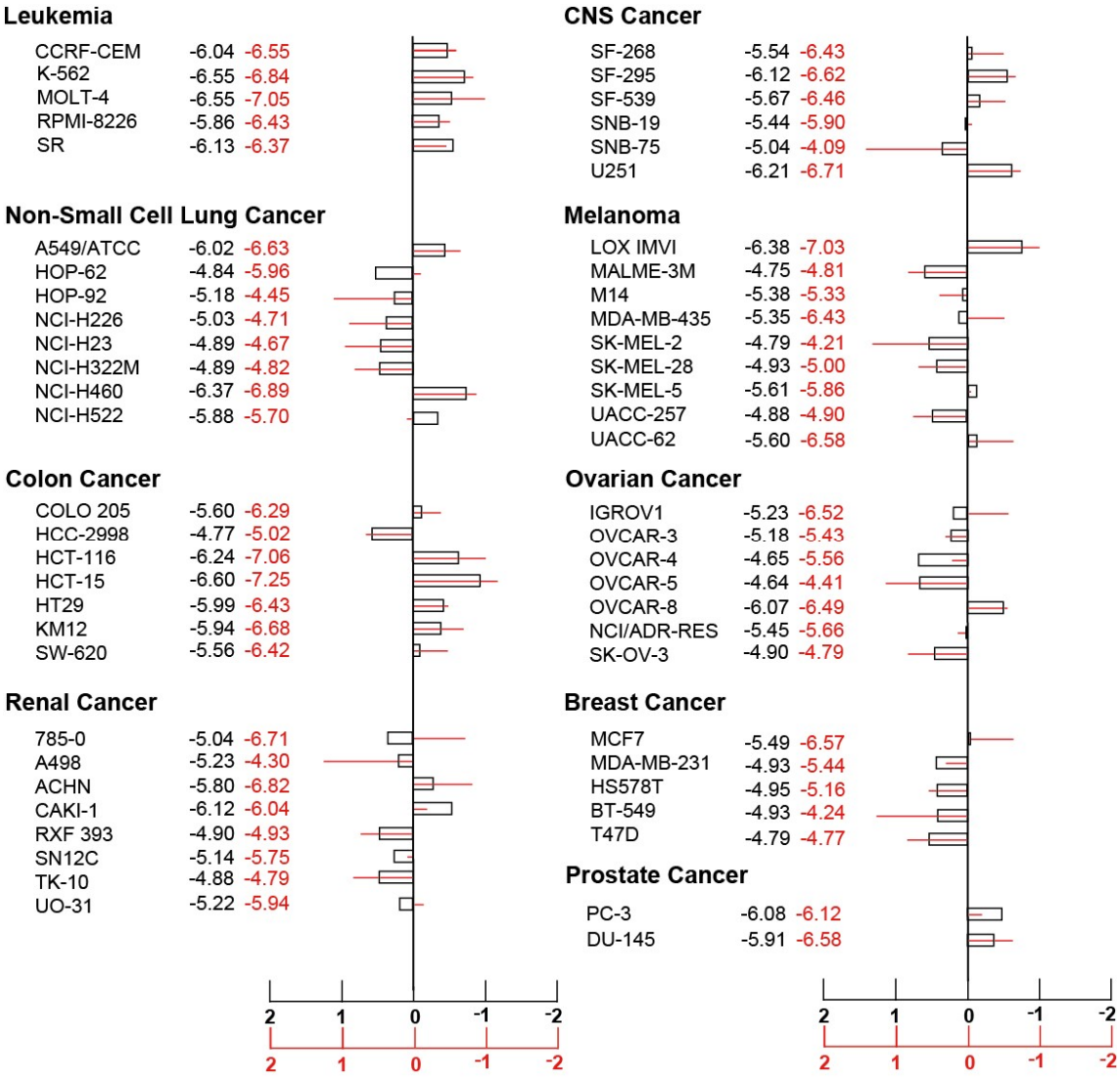


Fig. 4. Superposition of the antiproliferative profiles of SFOM-0046 (in black) and brequinar (in red) against the panels of 60 human tumor cell lines of the NCI-60 screening program.

3.3. SFOM-0046 inhibits the hDHODH

Our hypothesis was ascertained using hDHODH enzyme colorimetric inhibition assay. This inhibition assay is based on the conversion of DHO to orotate by hDHODH, reducing consecutively the chromogenic molecule DCIP to the colorless $DCIPH_2$. The reduction of DCIP into DCIPH2 is quantifiable by UV absorbance spectroscopy at 610 nm and correlates proportionally to the activity of hDHODH. First, the optimal concentration of DMSO showing

no interference with hDHODH activity was assessed and established at 0.25%. As shown in Fig. 5**,** the hDHODH catalytic activity is affected by the presence of both SFOM-0046 and brequinar. Both molecules are exhibiting IC_{50} of 72 nM and 12 nM, respectively. Moreover, our results show that both brequinar and SFOM-0046 affect the hDHODH activity in a concentration-dependent manner. Consequently, these results confirm that hDHODH is the pharmacological target of SFOM-0046.

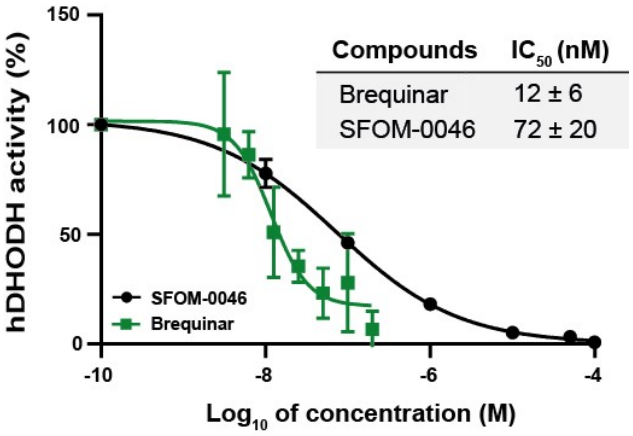


Fig. 5. Effect of SFOM-0046 and brequinar on hDHODH activity.

3.4. Exogenous uridine rescue cell proliferation

Nucleic acid biosynthesis machinery present an important cellular metabolism in cancer cells.[28] hDHODH being a major actor in the *de n*ovo pyrimidine synthesis and may constitute the Achilles heel for cell proliferation. In fact, its inhibition blocks the formation of orotate, a uridine precursor which ultimately leads to nucleotide starvation.[29] To further confirm SFOM-0046 as an hDHODH inhibitor, we used 100 µM of exogenous uridine to retrieve the pyrimidine biosynthesis in drug-treated cells. As shown in Table 2, the antiproliferative activity of SFOM-0046 as well as brequinar in HT-29, HT-1080, M21 and MCF-7 cancer cells is highly affected by the presence of the exogenous uridine. In fact, these molecules are exhibiting IC_{50} in the low micromolar range in absence of uridine. However, the IC_{50} values increase significantly in

presence of uridine. Leflunomide, known as a less potent hDHODH inhibitor and the prodrug of teriflunomide has no significant effect on cells after addition of uridine.[10] Moreover, our results show that exogenous uridine retrieve the proliferative activity in a cell type-independent manner after a 48 h incubation. Consequently, the *in vitro* rescuing experiment using exogenous uridine validates the hDHODH as the pharmacological target of SFOM-0046. hDHODH plays an important role in malignant neoplastic diseases and in several other diseases such as autoimmune diseases, immune and inflammatory diseases, destructive bone disorders, angiogenic-related disorders, viral diseases and infectious diseases. Therefore, hDHODH are currently being studied to treat these pathologies.[9, 11] In this context, the identification of hDHODH as pharmacological target of SFOM-0046 opens the door to new applications for this new class of small-molecule drugs.

Table 2

Antiproliferative Activity (IC_{50}) of Brequinar, SFOM-0046 and Leflunomide on HT-29, HT-1080, M21 and MCF7 Human Cancer Cell Lines in Presence and Absence of Exogenous Uridine.

		IC_{50} (µM)			
	Uridine (µM)	**HT-29**	**HT-1080**	**M21**	**MCF-7**
Brequinar	0	0.59	0.21	0.57	5.1
	100	24	17	25	25
SFOM-0046	0	0.99	0.45	3.0	5.9
	100	27	7.4	30	18
Leflunomide	0	75	30	83	55
	100	75	38	83	57

3.5. Molecular modeling of SFOM-0046 in the brequinar-binding site

The brequinar-binding site in our model is consisted of 39 amino acids distributed near to 4.5 Å of brequinar and FMN and include: Tyr38, Leu42, Leu46, Leu50, Pro52, Glu53, Ala55,

His56, Leu58, Thr63, Leu67, Leu68, Ala95, Ala,96, Phe98, Lys100, Met111, Gly119, Ser120, Val134, Arg136, Val143, Asn145, Tyr147, Phe149, Asn284, Leu309, Val333, Gly334, Gly335, Val336, Leu355, Tyr356, Thr357, Ala358, Leu359, Thr360, Gly363 and Pro364. The energy docking results of the 5 most favorable poses of SFOM-0046 in the brequinar-binding site are presented in Table 3. Of note, the brequinar-binding site is known to possess an intrinsic plasticity and can accommodate diverse molecular scaffolds.[30] The most favorable docking pose of SFOM-0046 showing the lowest S-score (-7.42 kcal/mol) is depicted in Fig. 6a. Moreover, the superposition of this SFOM-0046 pose and the brequinar pose is shown in Fig. 6b. First, our results show that the molecular scaffold of SFOM-0046 has the potential to dock into brequinar-binding site and to interact with its key amino acids within the brequinar-binding site. Second, the position of SFOM-0046 in the binding site comparatively to brequinar analog C44 (Fig. 3c) is as follows: 1) the 1-ethylphenylurea moiety (ring A) of SFOM-0046 is positioned close to the fluoroquinolinecarboxylic acid moiety of C44 near to the FMN cofactor and 2) the ethylphenyl moiety (ring B) of SFOM-0046 is positioned close to the phenoxy group of C44. Fig. 7a and 7b illustrate the 2D interaction diagrams of C44 and SFOM-0046, respectively. As depicted in Table 4, SFOM-0046 exhibits 2 interactions with the brequinar-binding site while C44 makes 10 main interactions with its binding site. The oxygen of the sulfonate group forms a hydrogen bond with the SH group of Met43 while the aromatic ring interacts with Phe62 making a pi-H interaction. For the C44, the carboxylic acid makes hydrogen bonds and ionic interactions with Gln47 and Arg136. The phenyl rings of quinoline and the phenoxy group make pi-H interaction with Thr360 and Phe62, respectively. As a conclusion, these molecular modeling experiments showed that SFOM-0046 has the molecular properties to interact efficiently with the brequinar-binding site. They also showed the potential active conformation of SFOM-0046 and its important interactions with key amino acids for its biological activity.

Table 3

Docking Energy Score of the 5 Most Favorable Poses of SFOM-0046 in the Brequinar-Binding Site.

Pose	**S** (kcal/mol)	**RMSD refine** (Å)
1	-7.42	1.13
2	-7.41	1.06
3	-7.34	1.60
4	-7.10	2.05
5	-6.85	2.11

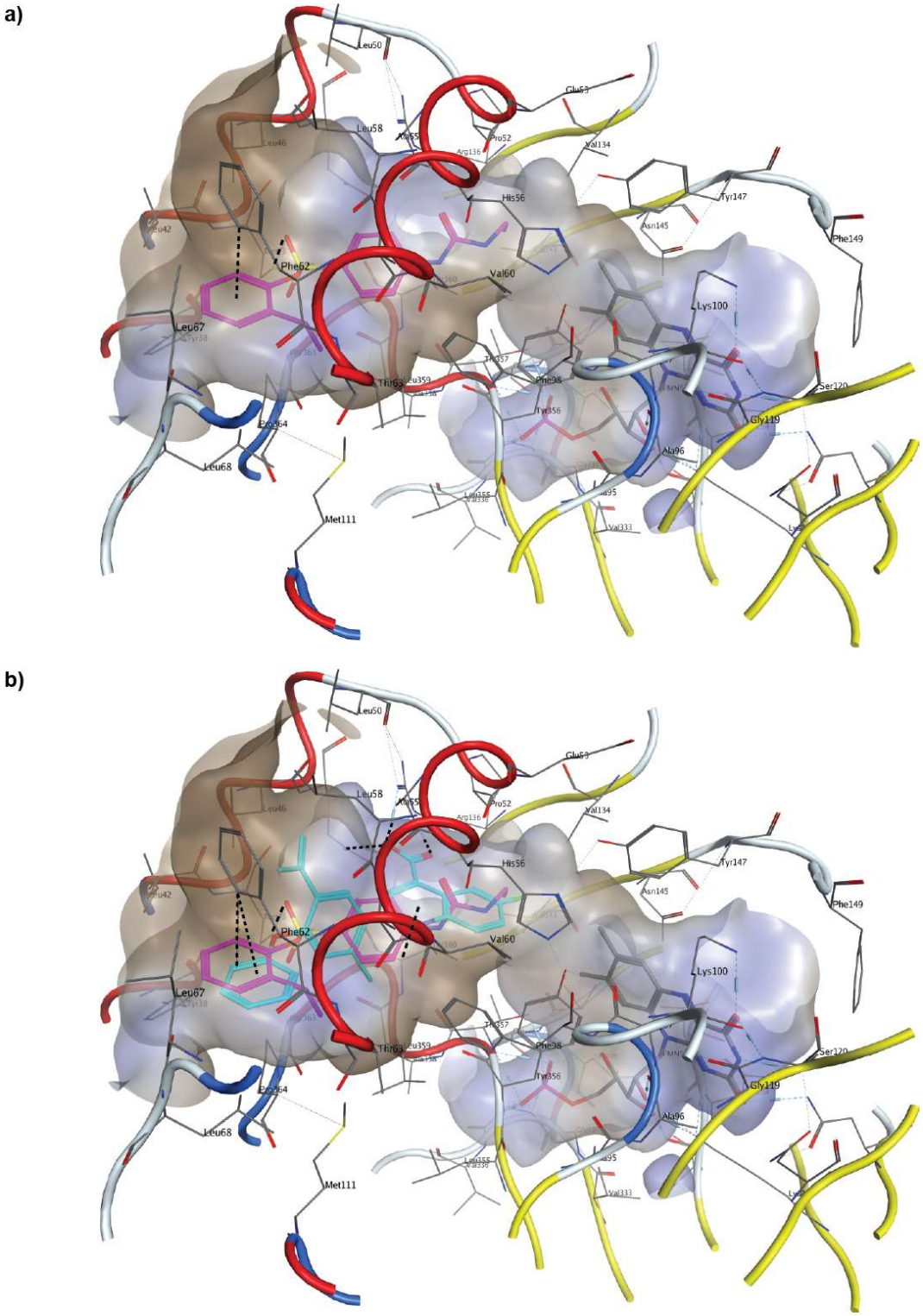


Fig. 6. Docking of the most stable conformation of **a)** SFOM-0046 (pink) and **b)** SFOM-0046 (pink) overlapped with CD44 (turquoise) in the brequinar-binding site. Main ligand interactions are in dashed lines.

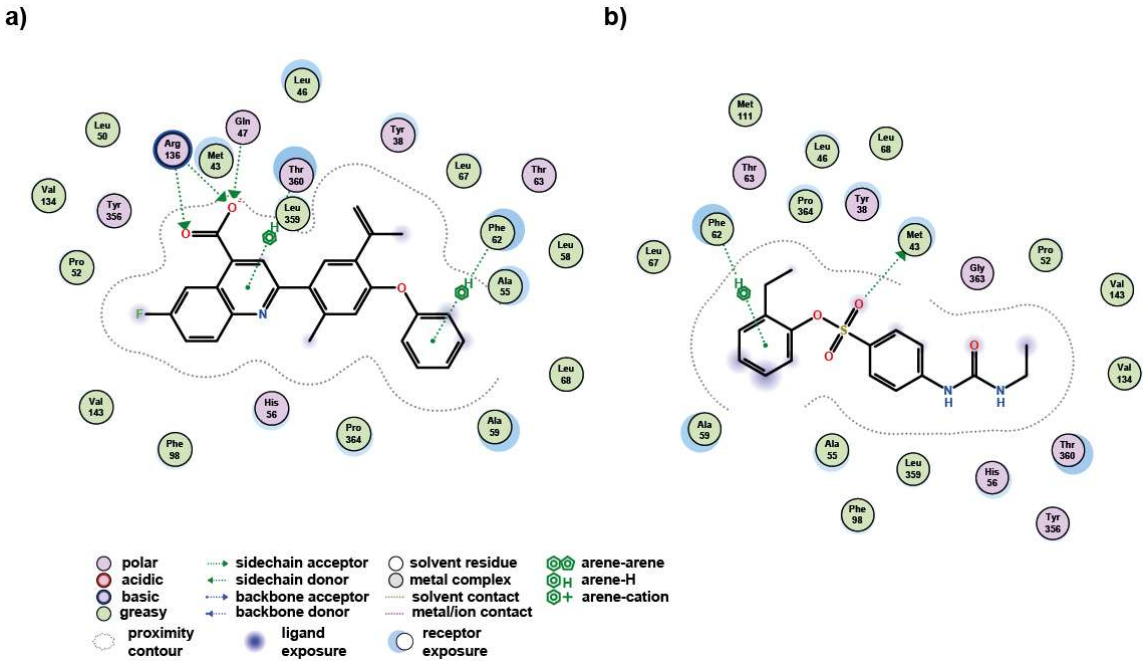


Fig. 7. 2D interaction diagrams of **a)** brequinar and **b)** SFOM-0046 within the brequinar-binding site.

Table 4

Interactions Summary of SFOM-0046 and 6-Fluoro-2-[2-methyl-4-phenoxy-5-(propan-2-yl)phenyl]quinoline-4-carboxylic Acid (C44) with the Amino Acids of the Brequinar-Binding Site.

	Ligand	**Receptor**	**Interaction**		**Distance**	**Energy**
	Atom	Atom	Amino Acid	Type	(Å)	(Kcal/mol)
SFOM-0046	O14	SD	Met43	H-donor	3.56	-1.2
	6-ring	CD2	Phe62	pi-H	3.72	-1.3
C44	O1	NE2	Gln47	H-acceptor	2.75	-2.3
	O1	NE	Arg136	H-acceptor	3.28	-0.5
	O1	NH2	Arg136	H-acceptor	2.69	-6.2
	O2	NE	Arg136	H-acceptor	2.74	-9.4
	O1	NE	Arg136	Ionic	3.28	-2.8
	O1	NH2	Arg136	Ionic	2.69	-6.9
	O2	NE	Arg136	Ionic	2.74	-6.4
	O2	NH2	Arg136	Ionic	3.32	-2.7
	6-ring	CD2	Phe62	pi-H	4.01	-1.0
	6-ring	CA	Thr360	pi-H	4.07	-0.6

4. Conclusion

The identification of the pharmacological target of a new class of molecules is necessary for rational development of a new drug and the process is not straightforward involving much effort to achieve that goal. We describe herein the approach that led to the identification of the pharmacological target of PUB-SOs as new hDHODH inhibitors. First, we used a prototype compound SFOM-0046 which is one of the most promising molecules regarding the antiproliferative activity, the cell cycle arrest in S-phase and the potency to generate replication stresses. Second, SFOM-0046 was integrated to the NCI-60 screening program and confirms an antiproliferative activity in the low micromolar range against the panels of 60 cancer cell lines. The cytotoxic fingerprint of SFOM-0046 generated by the NCI-60 screening program was used in the COMPARE algorithm and showed strong similarity to brequinar and dichloroallyl lawsone, two hDHODH inhibitors. The *in vitro* hDHODH colorimetric inhibition assay confirmed that SFOM-0046 is a hDHODH inhibitor with an IC_{50} of 72 nM. This result was further validated using the uridine rescue assay. The docking experiments also showed that SFOM-0046 possesses the molecular structure and the property to interact with the brequinar-binding site. Finally, PUB-SOs comprise several derivatives and analogs showing similar antiproliferative potential, cellular effects and replication stresses to SFOM-0046 prototype.[13-16] To that end, the molecular architecture of PUB-SOs is completely new and innovative as compared to current hDHODH inhibitors studied in clinics. In addition, the molecular structure of PUB-SOs is easily pharmacomodulable at low costs and could be valuable to circumvent eventual problems that might be encountered in clinic during their development. In this context, PUB-SOs are a novel class of very promising hDHODH inhibitors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online.

References

1. The Union for International Cancer Control's (UICC), New global cancer data GLOBOCAN 2018, https://www.uicc.org/new-global-cancer-data-globocan-2018. Accessed: November 28, 2019.
2. Heron M, Anderson RN. Changes in the leading cause of death: recent patterns in heart disease and cancer mortality. *NCHS Data Brief.* 2016;1-8.
3. Sawyers C. Targeted cancer therapy. *Nature.* 2004;432:294-297.
4. Gerhards NM, Rottenberg S. New tools for old drugs: functional genetic screens to optimize current chemotherapy. *Drug Resist Updat.* 2018;36:30-46.

5. Huang M, Graves LM. De novo synthesis of pyrimidine nucleotides; emerging interfaces with signal transduction pathways. *Cell Mol Life Sci.* 2003;60:321-336.

6. Reis RAG, Calil FA, Feliciano PR, Pinheiro MP, Nonato MC. The dihydroorotate dehydrogenases: past and present. *Arch Biochem Biophys.* 2017;632:175-191.

7. Mohamad Fairus AK, Choudhary B, Hosahalli S, Kavitha N, Shatrah O. Dihydroorotate dehydrogenase (DHODH) inhibitors affect ATP depletion, endogenous ROS and mediate S-phase arrest in breast cancer cells. *Biochimie.* 2017;135:154-163.

8. Liu L, Dong Z, Lei Q, Yang J *et al.* Inactivation/deficiency of DHODH induces cell cycle arrest and programed cell death in melanoma. *Oncotarget.* 2017;8:112354-112370.

9. Madak JT, Bankhead A, 3rd, Cuthbertson CR, Showalter HD, Neamati N. Revisiting the role of dihydroorotate dehydrogenase as a therapeutic target for cancer. *Pharmacol Ther.* 2019;195:111-131.

10. Munier-Lehmann H, Vidalain PO, Tangy F, Janin YL. On dihydroorotate dehydrogenases and their inhibitors and uses. *J Med Chem.* 2013;56:3148-3167.

11. Lolli ML, Sainas S, Pippione AC, Giorgis M *et al.* Use of human dihydroorotate dehydrogenase (hDHODH) inhibitors in autoimmune diseases and new perspectives in cancer therapy. *Recent Pat Anticancer Drug Discov.* 2018;13:86-105.

12. Sykes DB. The emergence of dihydroorotate dehydrogenase (DHODH) as a therapeutic target in acute myeloid leukemia. *Expert Opin Ther Tar.* 2018;22:893-898.

13. Turcotte V, Fortin S, Vevey F, Coulombe Y *et al.* Synthesis, biological evaluation, and structure-activity relationships of novel substituted *N*-phenyl ureidobenzenesulfonate derivatives blocking cell cycle progression in S-phase and inducing DNA double-strand breaks. *J Med Chem.* 2012;55:6194-6208.

14. Gagne-Boulet M, Moussa H, Lacroix J, Cote MF *et al.* Synthesis and biological evaluation of novel *N*-phenyl ureidobenzenesulfonate derivatives as potential anticancer agents. Part 2. Modulation of the ring B. *Eur J Med Chem.* 2015;103:563-573.

15. Pauty J, Cote MF, Rodrigue A, Velic D *et al.* Investigation of the DNA damage response to SFOM-0046, a new small-molecule drug inducing DNA double-strand breaks. *Sci Rep.* 2016;6:23302.

16. Gagné-Boulet M, Bouzriba C, Godard M, Fortin S. Preparation, characterization and biological evaluation of new *N*-phenyl amidobenzenesulfonates and *N*-phenyl ureidobenzenesulfonates inducing DNA double-strand breaks. Part 3. Modulation of ring A. *Eur J Med. Chem.* 2018;155:681-694.

17. Panier S, Boulton SJ. Double-strand break repair: 53BP1 comes into focus. *Nat Rev Mol Cell Biol.* 2014;15:7-18.

18. Chapman JR, Taylor MR, Boulton SJ. Playing the end game: DNA double-strand break repair pathway choice. *Mol Cell.* 2012;47:497-510.

19. Marechal A, Zou L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb Perspect Biol.* 2013;5:

20. Zeman MK, Cimprich KA. Causes and consequences of replication stress. *Nat Cell Biol.* 2014;16:2-9.

21. National Cancer Institute (NCI), Developmental Therapeutics Program (DTP), https://dtp.cancer.gov/. Accessed: November 28, 2019.

22. National Cancer Institute (NCI), Developmental Therapeutics Program (DTP), NCI-60 Human Tumor Cell Lines Screen, https://dtp.cancer.gov/discovery_development/nci-60/default.html. Accessed: November 28. 2019.

23. Knecht W, Bergjohann U, Gonski S, Kirschbaum B, Loffler M. Functional expression of a fragment of human dihydroorotate dehydrogenase by means of the baculovirus

expression vector system, and kinetic investigation of the purified recombinant enzyme. *Eur J Biochem.* 1996;240:292-301.

24. Koundinya M, Sudhalter J, Courjaud A, Lionne B *et al.* Dependence on the pyrimidine biosynthetic enzyme DHODH is a synthetic lethal vulnerability in mutant KRAS-driven cancers. *Cell Chem Biol.* 2018;25:705-717.e711.
25. Chemical Computing Group, Molecular Operating Environment (MOE), https://www.chemcomp.com/Products.htm. Accessed: November 28, 2019.
26. Yamori T. Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics. *Cancer Chemother Pharmacol.* 2003;52 Suppl 1:S74-79.
27. Holbeck SL, Collins JM, Doroshow JH. Analysis of Food and Drug Administration-approved anticancer agents in the NCI60 panel of human tumor cell lines. *Molecular cancer therapeutics.* 2010;9:1451-1460.
28. Roesch A. Tumor heterogeneity and plasticity as elusive drivers for resistance to MAPK pathway inhibition in melanoma. *Oncogene.* 2015;34:2951-2957.
29. Vyas VK, Ghate M. Recent developments in the medicinal chemistry and therapeutic potential of dihydroorotate dehydrogenase (DHODH) inhibitors. *Mini Rev Med Chem.* 2011;11:1039-1055.
30. Walse B, Dufe VT, Svensson B, Fritzson I *et al.* The structures of human dihydroorotate dehydrogenase with and without inhibitor reveal conformational flexibility in the inhibitor and substrate binding sites. *Biochemistry.* 2008;47:8929-8936.