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Synthesis of 2-,4,-6-, and/or 7-substituted quinoline derivatives as human dihydroorotate dehydrogenase (hDHODH) inhibitors and anticancer agents: 3D QSAR-assisted design

Vivek K. Vyas[a,*], Gulamnizami Qureshi[a], Drashti Oza[a], Hardik Patel[b], Krupali Parmar[b], Palak Patel[b], Manjunath D. Ghate[a]

[a] Department of Pharmaceutical Chemistry, Institute of Pharmacy, Nirma University, Ahmedabad 382481, Gujarat, India
[b] Institute of Science, Nirma University, Ahmedabad 382481, Gujarat, India

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ABSTRACT

Following our research for human dihydroorotate dehydrogenase (hDHODH) inhibitors as anticancer agents, herein we describe 3D QSAR-based design, synthesis and *in vitro* screening of 2-,4,-6-, and/or 7-substituted quinoline derivatives as hDHODH inhibitors and anticancer agents. We have designed 2-,4,-6-, and/or 7-substituted quinoline derivatives and predicted their hDHODH inhibitory activity based on 3D QSAR study on 45 substituted quinoline derivatives as hDHODH inhibitors, and also predicted toxicity. Designed compounds were docked into the binding site of hDHODH. Designed compounds which showed good predictive activity, no toxicity, and good docking score were selected for the synthesis, and *in vitro* screening as hDHODH inhibitors in an enzyme inhibition assay, and anticancer agents in MTT assay against cancer cell lines (HT-29 and MDA-MB-231). Synthesized compounds **7** and **14** demonstrated IC_{50} value of 1.56 μM and 1.22 μM, against hDHODH, respectively, and these are our lead compounds for the development of new hDHODH inhibitors and anticancer agents.

Flavoenzyme dihydroorotate dehydrogenase (DHODH) [EC 1.3.99.11] catalyzes oxidation of dihydroorotate (DHO) to orotate (ORO), and reduction of flavin mononucleotide (FMN) to dihydroflavin mononucleotide ($FMNH_2$).[1] This is a fourth step in *de novo* pyrimidine biosynthesis. Pyrimidine bases are important precursors for the biosynthesis of DNA, RNA, glycoproteins and phospholipids and required for the cellular metabolism and cell growth.[2] For DNA and RNA biosynthesis in cells, salvage pathway or *de novo* synthesis provides nucleosides. Salvage pathway does not produce enough nucleosides for survival of rapidly proliferating cells such as cancer cells and *T*-lymphocytes, thus progression of tumor, proliferation and replication of DNA are sensitive to *de novo* pyrimidine biosynthesis inhibition, and makes them ideal targets for the development of new drug candidates against cancer, rheumatoid arthritis and multiple sclerosis.[3,4] Brequinar[5] and leflunomide (Arava ®)[6] are two examples of such compounds (Fig. 1). Brequinar (6-fluoro-2-(2′-fluoro-[1,1′-biphenyl]-4-yl)-3-methylquinoline-4-carboxylic acid) is an antitumor agent and leflunomide (5- methyl-N-[4-(trifluoromethyl)phenyl]-1,2-oxazole-4-carboxamide) is a prodrug, which converts into active its metabolite teriflunomide (Aubagio ®/A77 1726), and used as immunosuppressive drug. Brequinar is a quinoline carboxylic acid derivative and a potent inhibitor of hDHODH. Brequinar was developed as a potential anticancer agent in clinical trials, however it was not approved as anticancer drug. Leflunomide is an isoxazole-based drug, and it was reported on melanoma cells proliferation.[6] Recently many small molecules as inhibitors of DHODH are designed, synthesized and evaluated as anticancer agents,[7] antimalarial agents, antiviral agents, antibacterials, and for the treatment of immunological disorders. Combined *in silico* approaches were used for the identification of these lead compounds as hDHODH inhibitors.[8–11] Following our research for the identification of hDHODH inhibitors, in the year 2014,[12] we designed and synthesized quinoline-2-carboxamide derivatives as hDHODH inhibitors and anticancer agents. Comprehensive *in silico* methods both structure- and ligand-based were used for the design of quinoline-2-carboxamide derivatives. Very recently, in the year 2017,[13] we have designed and synthesized 1,2,5-trisubstituted benzimidazole derivatives using liquid phase combinatorial synthesis method as hDHODH inhibitors. In continuation of work for the generation of new lead compounds as hDHODH inhibitors and anticancer agents, in this work, we performed 3D QSAR study (CoMFA and CoMSIA models) on the substituted

* Corresponding author.
E-mail address: vivekvyas@nirmauni.ac.in (V.K. Vyas).

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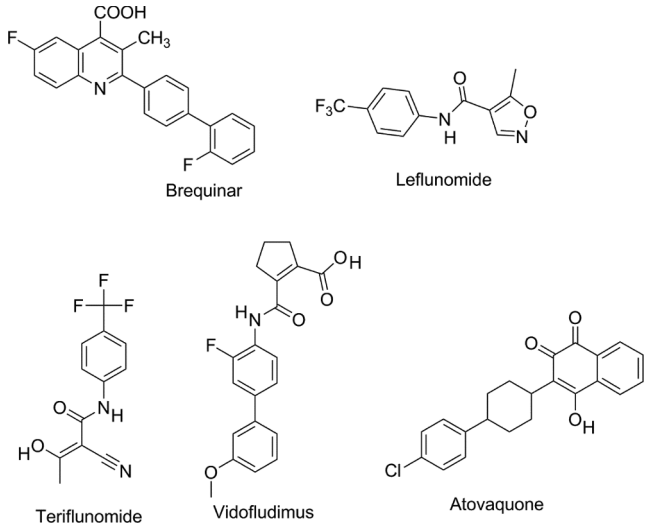


Fig. 1. Human dihydroorotate dehydrogenase (hDHODH) inhibitors.

quinoline derivatives as a computational design strategy for the design of hDHODH inhibitors. Contour maps generated through CoMFA and CoMSIA analysis were used for the selection of substituents on quinoline ring. In the designed compounds, quinoline scaffold was maintained as a core structure and substituents which were found significant with the contour maps analysis were selected. Furthermore, 3D QSAR models helped in the prediction of the activity of designed compounds. *In silico* toxicities were also predicted for designed compounds. Prior to their selection for the synthesis and activity, designed compounds were docked in the hDHODH active site. Selected designed compounds (good predicted activity and docking score) were synthesized as 2,4,-6-,and/or 7-substituted quinoline derivatives. Synthesized compounds were screened for hDHODH inhibition activity in a colorimetric assay, and as antiproliferative agents in MTT assay. Overall, a combined strategy was employed in this work, which resulted in lead compounds, which were targeted against hDHODH enzyme as anticancer agents.

3D QSAR was performed on reported hDHODH inhibitors[12,14] (Table S1 under supplementary data). 3D QSAR models were validated on statistical parameters calculated using PLS analysis implemented in sybyl × software (Fig. S1 and Table S2 under supplementary data). Align 3 (Distill) was selected as a best method of alignment based on highest value of q^2, r^2cv, r^2_{ncv}, r^2_{pred}, SEE and F value in CoMFA and CoMSIA models (Table S2 under supplementary data). An external test set of 9 compounds was used for the external validation of models in which CoMFA and CoMSIA model showed r^2_{pred} value of 0.823 and 0.782, respectively.

CoMFA and CoMSIA contour maps are demonstrated using template compound **Tr7** inside the electrostatic, steric, hydrophobic, hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA) fields. CoMFA stearic map (Fig. S2A under supplementary data) is shown in green coloured (contributed 80%) sterically favoured regions, and yellow coloured (contributed 20%) sterically disfavoured areas. In steric contour map, two small green coloured contour were present; one was above the −C4 carboxylate position of quinoline ring, and second was present near the −C6 position of quinoline ring. Both the contour suggested presence of bulky group at these positions (expansion of functional group) in order to design hDHODH inhibitors. Sterically disfavoured yellow coloured contour was present on terminal phenyl ring of biphenyl ring system at −C2 position of quinoline ring. This suggested that in the design compounds further extension of single phenyl ring at −C2 position of quinoline ring might leads to decrease in the activity. CoMFA electrostatic map (Fig. S2B under supplementary data) is shown in blue coloured (contributed 80%) electrostatic favoured regions, and red coloured (contributed 20%) electrostatic disfavoured areas. A large blue coloured contour covered −C8 position of quinoline ring, which suggested that the presence of electropositive groups at these positions would increase the inhibitory activity of designed compounds. A large red coloured contoured cover the terminal phenyl ring at −C2 position of quinoline ring, which revealed that the presence of electronegative groups on terminal phenyl ring of biphenyl ring system or extension of first phenyl ring with the electronegative groups at 4th position would favour the inhibitory activity of the compounds. CoMSIA model calculated additionally hydrophobic, HBD and HBA fields. Hydrophobic contour map of CoMSIA analysis is shown in Fig. S3A under supplementary data, represented by yellow (contributed 80%, hydrophobic favoured), and grey (contributed 20%, hydrophobic disfavoured) coloured contours. Yellow coloured contours positioned same as green coloured contour in CoMFA steric map, and suggested the extension of substitution at this position (−C4 carboxylate) by hydrophobic groups for the design of inhibitors. Grey coloured contour covered the −N1 of quinoline ring, which represented those regions of the molecules where hydrophobic groups resulted in decreased activity. CoMSIA hydrogen bond acceptor (HBA) contour map (Fig. S3B under supplementary data) is shown in magenta (contributed 80% HBA favoured), and red (contributed 20% HBA disfavoured) coloured contours. Red coloured contour covered the −N1 position of quinoline ring, and magenta coloured contour covered the −C4 carboxylic acid functional group, which suggested the presence of HBA group at this position. CoMSIA hydrogen bond donor (HBD) contour map (Fig. S3C under supplementary data) is shown in cyan (contributed 80%, HBD favoured), and purple (contributed 20%, HBD disfavoured) coloured contours. HBD disfavoured purple coloured contour, and HBA magenta coloured contour are placed at same position (−C4 carboxylic acid) which suggested the need of HBA group at this position in the design of new compounds. HBD favoured cyan contour covered the terminal phenyl ring of biphenyl ring system, which suggested the presence of HBD group on terminal phenyl ring or at the −C4 position of first phenyl ring system for the design of compounds.

Contour maps representation obtained by the 3D QSAR study was taken as the design rational, and 30 substituted quinoline derivatives (**1d–30d**) were designed (Table S3 under supplementary data) as hDHODH inhibitors and anticancer agents. Quinoline ring was maintained as a main scaffold in the designed compounds. CoMFA steric counter map and CoMSIA hydrophobic map suggested the extension of functional group (bulky and hydrophobic) at −C4 position of quinoline ring, thus phenyl, benzyl and biphenyl ring were selected, and ester and ether spacers were introduced between quinoline and phenyl, benzyl and biphenyl rings at −C4 position of quinoline ring system as per CoMSIA HBA and HBD contour maps for H-bonding with the active site of hDHODH. CoMFA electrostatic counter map suggested the presence of electropositive groups at −C8 position (substituted) of quinoline ring. Electron withdrawing groups $-NO_2$, −Cl, −F, −Br and methoxy were selected at −C'4 position of first phenyl ring, which was substituted at −C2 position of quinoline ring as per CoMFA electrostatic counter. As per the CoMFA steric counter map $-CH_3$, $-C_2H_5$, $-CF_3$, $-OCF_3$, $-C_3H_7$, −Cl, and −diCl groups were selected at −C6 and −C7 positions of quinoline ring. Overall design rational of the molecules on the basis of 3D QSAR contour maps is shown in Fig. 2.

3D QSAR models (CoMFA and CoMSIA) were used for the prediction of hDHODH inhibition activity of designed compounds. Predicted activity (pIC_{50}) of designed compound is shown in Table S3 under supplementary data. Compound **28d** (synthesized compound number **14**) was predicted with highest activity (pIC_{50}) of 8.056 and 8.166 using CoMFA and CoMSA models, respectively, and compounds **1d, 2d, 4d, 17d,** and **19d** also showed good predicted activity (pIC_{50}). Substitution of electron withdrawing groups specially −Cl at R^1-position of phenyl ring, along with the presence of small alkyl group $-6CH_3$, and −6,7-diCl at R-position of quinoline ring were found important groups in the designed compounds with good predicted activity. Prediction of toxicity risk (*in silico*) was carried out for the toxicity factors like

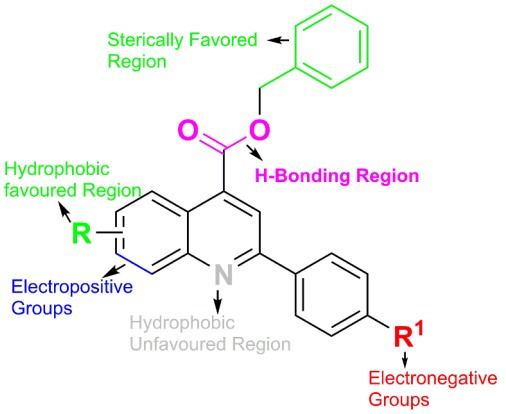


Fig. 2. Design strategy for the design of 2-,4,-6-,and/or 7-substituted quinoline derivatives using 3D QSAR generated CoMFA and CoMSIA contour maps.

mutagenicity, tumorigenicity, irritant and reproductive effects. Designed compounds were predicted with no toxicity for all these toxicity risk factors.

Human DHODH is a flavoenzymes which catalyze stereoselective oxidation of L-dihydroorotate (DHO) to orotate (ORO). In this electron transfer reaction, flavin mononucleotide (FMN) serve as an intermediate. Human DHODH exhibits a two-site ping-pong mechanism with FMN. Human DHODH belongs to family-2 enzymes, which transfer electrons to ubiquinone (CoQ) for reoxidizing FMN. Crystal structures of hDHODH in complexes with inhibitors helped in the structure-based design of specific inhibitors. Docking study with designed compounds was performed to further scrutinise the designed compounds for their selection as candidates for the synthesis and hDHODH inhibition activity. Brequinar was also docked as standard reference compound, and binding interactions of designed compounds were compared with brequinar at the binding site of hDHODH. In hDHODH structure a tunnel is formed from two α-helices, which represent the active site of hDHODH, and described as the target site of hDHODH inhibitors. Brequinar showed highest docking score of 58.92, and formed interactions with Leu42, Pro52, Arg136, Tyr365 and Leu68 (Fig. S4 under supplementary data) at the active site of hDHODH. Carboxyl group of brequinar formed H-bond interaction with the side chain of Arg136. Designed compounds showed interaction with Met43, Leu46, Gln47, Pro52, Glu53, Ala55, Thr63, Leu67, Arg136, Tyr356, Leu359, and Pro364 residues. The designed compounds also showed a water (HOH444) mediated interaction with Thr360. Due to absence of carboxyl group in the designed compounds, they did not showed brequinar-like binding mode. Docking results of the designed compounds are shown in Table S3 under supplementary data. Most of the compounds showed good docking score, compounds **4d (8)** and **15d (10)** showed highest docking score of 56.76 and 55.75, respectively.

Binding modes of compounds **4d (8)** and **15d (10)** are shown in Fig. 3. Compound **4d (8)** showed binding interactions at the active site with Leu42, Met43, Gln47 and Leu67 as shown in Fig. 3(A), and compound **15d (10)** showed binding interactions with Met43, Leu46, Ala59, Gln47, Leu67, Thr356 and Pro364 as shown in Fig. 3(B). Docking revealed a clear preference of electron withdrawing −Cl and -F groups in the designed compounds.

Quinoline ring was synthetically derivatized at −C2, −C4 and −C6-, and/or −C7 positions to obtain a series of 2,4,6-,and/or 7 substituted quinoline derivatives, and characterized as target compounds. In the first step of synthesis, 2-(4-subsitutedphenyl)-6,7-substituted-quinoline-4-Carboxylic acid (**4a–e**) were synthesized based on Doebner reaction using substituted anilines (**3**) with substituted benzyldehydes (**1**) and pyruvic acid (**2**) (Scheme 1). In the second step of reaction, substituted-quinolin-4-ylmethanol derivatives (**5a–e**) were synthesized. Substituted quinoline-4-carboxylic acid derivatives (**4a–e**) were reacted with lithium aluminum hydride ($LiAlH_4$) in the presence of tetrahydrofuran (THF). Target compounds **(6–10)** (ester derivatives) were synthesized *via* the reaction of **4a–e** with benzylbromide in the presence of sodium hydride (NaH) and dimethylformamide (DMF). Other target compounds (ether derivatives) (**11–15**) were synthesized using Williamson ether synthesis *via* reaction of substituted-quinolin-4-ylmethanol (**5a–e**) derivatives in DMF with benzyl bromide.

Chemical structures of all the compounds were confirmed using FTIR, ^{1}H NMR, ^{13}C NMR, mass spectra and elemental analysis data (Supplementary data). Ester and ether derivatives were prepared, and observed with significant features in FTIR spectra. Compounds demonstrated characteristic O−H (−COOH) stretching peaks near broad rang 3500–3205 cm^{-1}, −C═O stretching peaks (−COOH) near 1720–1780 cm^{-1} whereas compounds showed O−H stretching peaks ($-CH_2OH$) at 3500–3200 cm^{-1}, aliphatic C−H stretching peaks near 2950–2870 cm^{-1}, and −C═O stretching peaks were absent. Mass analysis of synthesized compounds correlated with M^{+1} peaks as stable base peaks (Supplementary data). The significant features of ^{1}H NMR spectra of the synthesized compounds were observed as the aromatic region showed sets of aromatic protons in the range of δ 6.8–8.4 ppm and correlated with C atoms (δ 120–138 ppm) in ^{13}C NMR spectrum, C-6 methyl group in the quinoline ring showed a resonance peak in the range δ 2.4–2.5 ppm in ^{1}H NMR spectra and correlated with C atoms (δ 21 ppm) in ^{13}C NMR spectra (Supplementary data), signal of aliphatic ester as a linker at −C4 position of quinoline ring observed in the region δ 5.4–5.5 ppm in ^{1}H NMR spectra, and correlated with C atoms (δ 67 ppm) in ^{13}C NMR spectra.

Synthesized compounds were screened for hDHODH colorimetric enzyme inhibition assay with recombinant hDHODH enzyme. All the synthesized compounds were also screened for antiproliferative activity against two different human cancer cell lines, colorectal adenocarcinoma cell line (HT-29) and breast cancer cell line (MDA-MB-231) to further obtain biologically relevant information as anticancer agents.

Chromogen reduction method with 2,6-dichlorophenolindophenol (DCIP) dye was used to perform hDHODH enzyme inhibition assay. Chromogen reduction was coupled with the oxidation of L-dihydroorotate (L-DHO) as well as reduction of ubiqinone (CoQ). Enzyme inhibition was resulted in loss of chromogen absorbance at 600 nm. Absorbance of each well was measured at 600 nm and quantified in triplicate using a 96 microplate reader (Bio-Rad-680 instrument) and the data were exported to an Excel (Microsoft) spreadsheet for analysis. The percentage enzyme inhibition was measured from 0.01 μM to 30 μM concentration of synthesized compounds, along with the standard brequinar sodium. The IC_{50} value (Table 1) as a practical readout of the enzyme inhibition of synthesized compounds under comparable conditions was obtained from dose-response plot (Fig. 4) using GraphPad. Most of the synthesized compounds showed comparative activity, and the best compounds of ester series were **7** and **8,** which showed IC_{50} value of 1.56 μM and 1.96 μM, respectively against hDHODH enzyme, and in ether series compound **14** showed highest hDHODH inhibition activity with an IC_{50} value of 1.22 μM. Presence of −Cl group at R^1 position of phenyl ring, which is substituted at −C2 position of quinoline ring along with 6,7-diCl substituents in quinoline ring were found best substitutions in the ether series (**14**). In the ester series of compounds presence of $-CH_3$ at −R position (−C6) (**7, 8**) along with of –F group at R^1 position in **7**, and $-OCH_3$ in **8** contributed to the potency of synthesized compounds against hDHODH enzyme. The activity data revealed a clear preference of electron withdrawing −Cl and −F groups at phenyl ring substituted at −C2 position of quinoline ring for the potency against hDHODH enzyme. Compounds (**8, 10**) with $-OCH_3$ group at R^1 positions also showed better activity. Furthermore the presence of −6,7diCl in compounds **10** and **14** have a marked impact on activity against hDHODH enzyme. The results of hDHODH enzyme inhibition activity are in agreement with the *in silico* results (predicted activity and docking score).

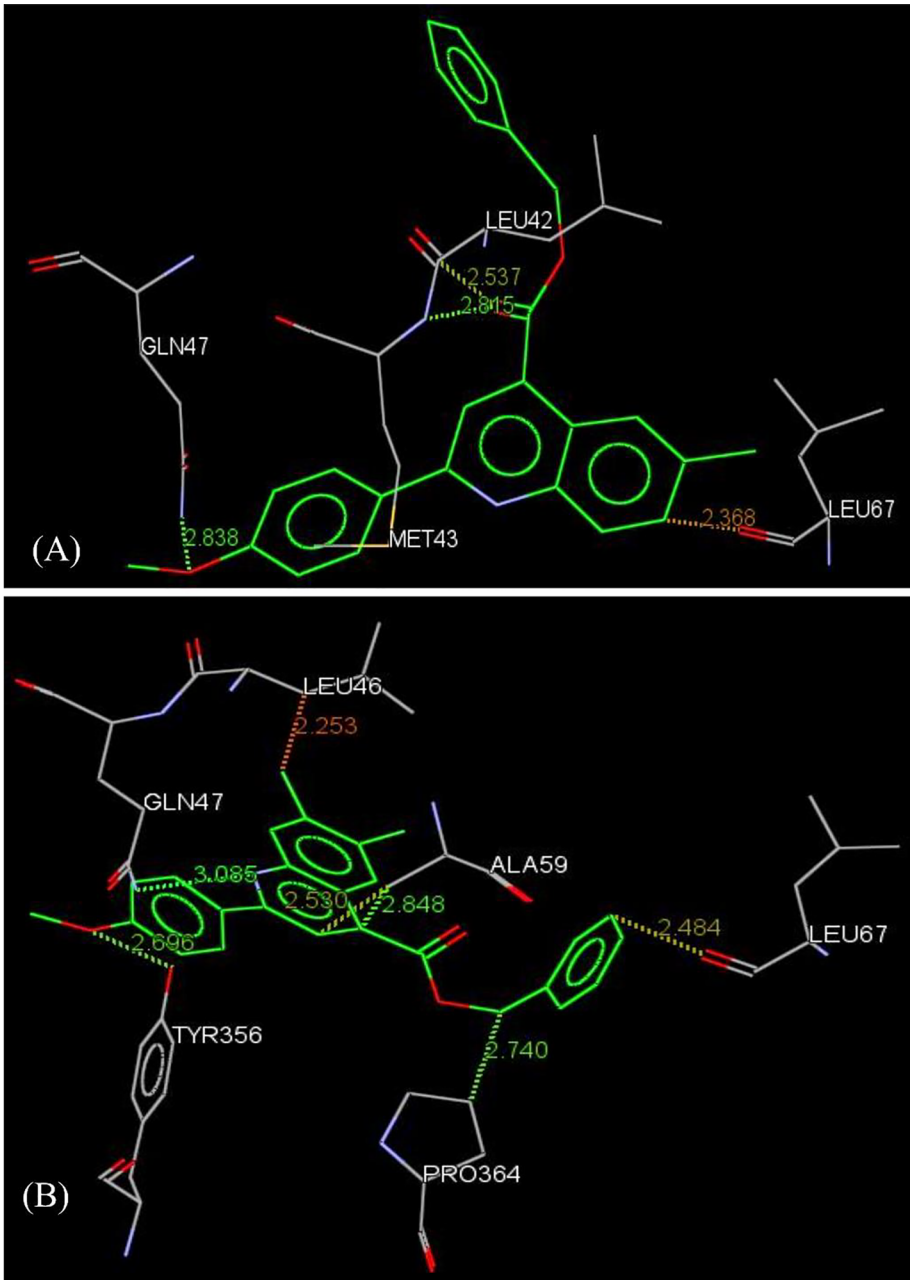
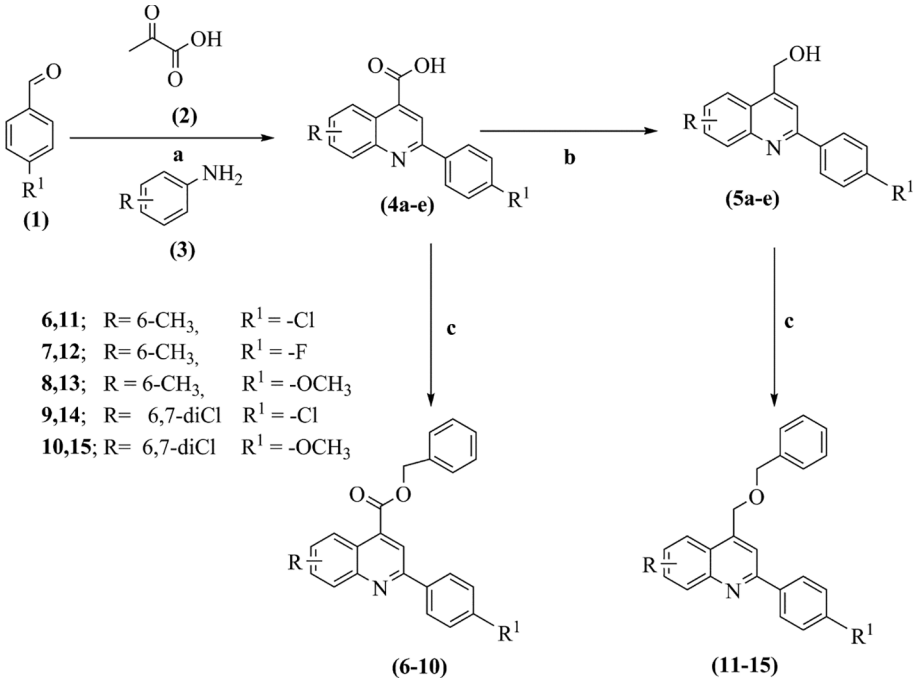


Fig. 3. Docking pose of compound **8** (A), **10** (B), in human DHODH enzyme. Compounds **8**, and **10** are in wire frame with C-atom in light green colour and other atoms by their colour, H-bond interactions were highlighted using light green dotted lines, and distance of H-bonding between the atoms of ligand and residues of protein in Å. The labelled protein residues are capped in stick model with colour by atom.

All the synthesized compounds (**6–15**) along with two standards brequinar sodium and paclitexal were evaluated for antiproliferative activity against two different cancer cell lines, named breast cancer (MDA-MB-231) and colorectal adenocarcinoma (HT-29). The IC_{50} value (cell growth inhibition) of synthesized compounds were determined from dose-response curve (Table 2). Compounds **7** (IC_{50} = 15.06 μM against HT-29, and IC_{50} = 13.16 μM against MDA-MB-231), and **8** (IC_{50} = 17.29 μM against HT-29, and IC_{50} = 20.52 μM against MDA-MB-231) with $-CH_3$ group at –R position (−C6) (**7, 8**) along with of –F group at R^1 position in **7**, and $-OCH_3$ in **8** were found the most potent compounds in MTT assay. Compound **14** showed IC_{50} value of 16.78 μM against HT-29. Compound **7** and **8** exhibited good antiproliferative activity against both the cancer cell lines, which were almost equally potent as brequinar sodium and paclitaxel.

In the present study, we described 3D QSAR-based design, synthesis and *in vitro* hDHODH inhibition and antiproliferative activity, of 2-,4,-6-,and/or 7-substituted quinoline derivatives. We designed 30 substituted quinoline derivatives using the information obtained from the generated CoMFA and CoMSIA contour maps. The designed compounds showed good predicted activity using ligand-based 3D QSAR models, and no *"in silico"* toxicity. In addition to ligand-based QSAR method a structure-based method (molecular docking) was used to select designed compounds as candidates for the synthesis and activity, based on the docking results and predicted activity. Compounds 4d (**8**), and 15d (**10**) (ester-linker), and 27d (**15**) and 28d (**14**) (ether-linker) showed very good results in "*in silico*" studies. Synthesized compounds were evaluated in an *in vitro* hDHODH inhibition assay, and also screened for antiproliferative activity using MTT assay on cancer cell lines.



Scheme 1. Synthetic strategy for the synthesis of substituted quinoline derivatives via Doebner reaction, $LiAlH_4$ catalyzed reduction and Williamson ether synthesis reactions. Reagents and conditions (a) ethanol, reflux 3–4 h (b) $LiAlH_4$, THF, 0°C (c) benzyl bromide, DMF, NaH, 0 °C.

Table 1
Human DHODH enzyme inhibitory activity of 2-,4,-6-,and/or 7-substituted quinoline derivatives.

Compound	IC_{50} (μM)	Compound	IC_{50} **(μM)**
6	7.37	**13**	nd
7	1.56	**12**	13.44
8	1.96	**14**	1.22
9	7.48	**15**	15.48
10	3.34	**Brequinar sodium**	0.016
11	nd		

IC_{50} values are determined as the mean ± SD of three independent experiments performed.
nd = not determined.

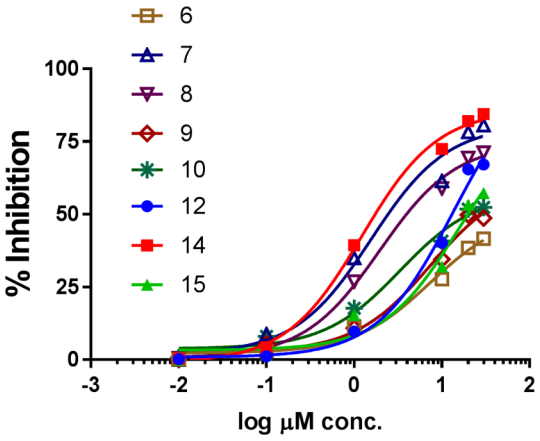


Fig. 4. Human DHODH inhibition dose–response curve of synthesized compounds. Six different concentrations 0.01 μM, 0.1 μM, 1 μM, 10 μM, 20 μM, 30 μM of the compounds were tested and IC_{50} values were determined.

Compounds **7**, **8** and **14** exhibited good hDHODH inhibition activity, and *in vitro* anticancer activity against HT-29 and MDA-MB-231 cell lines.

However, in comparison with standard brequinar, the synthesized compounds showed weak hDHODH inhibition. Even the most potent compound **14** showed IC_{50} value of 1.22 μM, whereas the IC_{50} value of brequinar was 0.016 μM (Table 1). We investigated the reason of potential differences in the hDHODH inhibitory activity and found that all the compounds in the training set (**Tr1–Tr37**) (Table S1 under supplementary data), where brequinar belongs, are characterized by the presence of a carboxylic group, which is mainly involved in a key polar interaction with *h*DHODH active site (Arg 136 and Gln47). Other known more potent hDHODH inhibitors are also involved in such kind of interaction with the active site of hDHODH, as the well structure activity relationship studies of known hDHODH suggested the presence of an acidic moiety in the compound to retain the activity. 3D QSAR model was not able to predict in the carboxyl area (−C4 position of quinoline ring) any other influence different from the presence of a carboxyl group type of interaction (HBD and HBA). This is because all the training set compounds were lacking chemiodiversity in that specific region, so the synthesized compounds did not adopted a brequinar-like binding mode. This might be the reason of weak hDHODH inhibitory activity of the synthesized compounds. On the other hand, the inhibitory activities of synthesized compounds toward the proliferation of two cancer cells were comparable to those of brequinar. Recently, Wong et al.[15] showed that DHODH inhibitors induce cell cycle arrest in cancer cells via additional DHODH-independent pathway. Their study showed that DHODH inhibitors does not affect the inherent abundance or the expression of DHODH in the cancer cells although they observed significant decreased in enzymatic activity in all cell lines. So the anticancer activity of synthesized compounds might be due cell cycle arrest in cancer cell lines via additional DHODH-independent pathway. Comparison of *in silico* computational study and *in vitro* study revealed that the compounds which showed better results (predicted activity and docking score) were found as the potent compounds in both the screening methods. Overall, predicted activity (pIC_{50}), docking study, and *in vitro* activity data revealed a clear preference of electron withdrawing −Cl and −F groups. Results of both the screening assay validated the computational design of substituted quinoline derivatives. Finally, it is concluded that synthesized compounds **7** and **14** are lead compounds as hDHODH inhibitors for the development of new anticancer agents.

Table 2
Antiproliferative activity of substituted quinoline derivatives against HT-29 and MDA-MB-231 cancer cell lines with standards brequinar sodium and Paclitaxel.

Colorectal adenocarcinoma cell lines (HT-29)		Breast cancer cell lines (MDA-MB-231)	
Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
6	> 100	**6**	> 100
7	15.06	**7**	13.16
8	17.29	**8**	20.53
9	48.71	**9**	> 100
10	42.18	**10**	39.16
11	> 100	**11**	> 100
12	> 100	**12**	> 100
13	> 100	**13**	> 100
14	16.78	**14**	> 100
15	> 100	**15**	> 100
Paclitaxel	14.56	**Paclitaxel**	13.23
Brequinar Sodium	13.98	**Brequinar Sodium**	12.45

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2019.01.038.

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