***In Silico* Screening, Synthesis and *In Vitro* Evaluation of Some Quinazolinone and Pyridine Derivatives as Dihydrofolate Reductase Inhibitors for Anticancer Activity**

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Abstract: Dihydrofolate reductase (DHFR) is the important target for anticancer drugs belonging to the class of antimetabolites as the enzyme plays important role in the de novo purine synthesis. We here report the *in silico* screening to obtain best fit molecules as DHFR inhibitors, synthesis of some 'best fit' quinazolinone from 2-phenyl-3-(substituted-benzilidene-amino) quinazolinones (Quinazolinone Schiff's bases) **QSB₁₋₅** and pyridine-4-carbohydrazide Schiff's bases (**ISB₁₋₅**) derivatives and their *in vitro* anticancer assay. Synthesis of the molecules was performed using microwave assisted synthesis. The structures of these molecules were elucidated by IR and ¹H-NMR. These compounds were then subjected for *in vitro* anticancer evaluation against five human cancer cell-lines for anticancer cyto-toxicity assay. Methotrexate (MTX) was used as standard for this evaluation to give a comparable inhibition of the cell proliferation by DHFR inhibition. Paclitaxel, adriamycin and 5-fluoro-uracil were also used as standard to give a comparable activity of these compounds with other mechanism of anticancer activity. **ISB₃** (4-(N, N-dimethyl-amino)-phenyl) Schiff's base derivative of pyridine carbohydrazide showed equipotent activity with the standards used in *in vitro* anticancer assay as per the NCI (National Cancer Institute) guidelines.

Keywords: *In silico* screening, Docking and Pyridines, Quinazolinones, Schiff's bases, DHFR, Anticancer activity.

Introduction

In silico screening methods such as docking have a great advantage as compared to 2D similarity and 3D pharmacophore search methods as it utilizes the 3D receptor structure in a quantitative way¹. Docking calculations alone or combined with the virtual screening has been carried out to develop the DHFR and tyrosine kinase inhibitors *etc*²⁻⁸.

Dihydrofolate reductase (E.C.1.5.1.3) functions as the catalyst for the reduction of the dihydrofolate to tetrahydrofolate that generates reduced folate carriers of one carbon fragments and it is the important co-factor in the biosynthesis of nucleic acids and amino acids. The inhibition DHFR leads to the partial depletion of intracellular reduced folates with the subsequent limitation of cell growth⁹. MTX is a potent inhibitor of DHFR¹⁰. The quinazoline moiety has the potential for the DHFR inhibition and several 2, 4-diamino-quinazoline analogues. Isoniazid a pyridine Carbohydrazide derivative is hypothesized to act on *M. Tuberculi* DHFR¹¹. Thus Schiff's bases of quinazolinones and pyridine carbohydrazide were designed and *in silico* screened, amongst which the best fit molecules selected for synthesis and *in vitro* anticancer evaluation.

Experimental

Molecular docking

Molecular docking was performed for quinazolinones and pyridine analogues using the GLIDE[®] integrated Maestro[®] 7.5 interface on the Linux operation system. The high-resolution (2.10 Å X-ray structure of human DHFR complex with inhibitor (RCSB PDB id code 1BOZ) was imported into GLIDE module and the docking simulations were carried out without cofactor NADPH to explore the binding interactions of quinazolinone Schiff's bases (**QSB₁₋₅**) and pyridine-4-carbohydrazide-Schiff's bases (**ISB₁₋₅**) derivatives with amino acid residues of the enzyme. MTX was included in the docking run. The low energy conformations of these ligands were generated by LigPrep module with OPLS-2005 force field.

Scoring function

GLIDE module uses force field based MCSA minimization with free energy scoring. This produces G-Scores and ΔG Energy of binding scores. These scores are mentioned in Table 1.

Validation of the docking protocol

Validation of the docking protocol was carried out by correlation coefficient method^{12,13} and by pose regeneration of the same ligand structure as seen in the crystallized PDB.

Table 1. G-Scores and ΔG binding energy scores.

S. No.	Ar	G-Score	$\Delta G^*_{\text{Energy}}$	H-Bonds
QSB ₁	2,6-(Cl) ₂ -C ₆ H ₃	-11.0	-76.6	2
QSB ₂	4-Cl-C ₆ H ₄	-10.10	-78.6	2
QSB ₃	2-OH-4-OCH ₃ -Ph	-9.80	-48.3	2
QSB ₄	C ₆ H ₄	-9.70	-50.0	2
QSB ₅	3,4-(OCH ₃) ₂ -C ₆ H ₃	-9.50	-50.5	2
ISB ₁	Fuan-2-yl	-8.35	-104.0	3
ISB ₂	3- OH-C ₆ H ₄	-8.22	-102.6	3
ISB ₃	(CH ₃) ₂ -N-C ₆ H ₄	-8.18	-102.0	2
ISB ₄	3- NO ₂ -C ₆ H ₄	-8.12	-98.7	3
ISB ₅	4- Cl-C ₆ H ₄	-8.10	-100.0	3
MTX	-----	-10.10	-163.3	7

*Kcal/mole

Synthesis of the molecules (*QSB*₁₋₅ and *ISB*₁₋₅)

¹H-NMR spectra were recorded in CDCl₃ for quinazolinones and DMSO-d₆ for pyridines, on a Varian Mercury-300 MHz Tetramethylsilane (TMS) as internal standard (chemical shifts in δ ppm). The IR spectra were recorded on a Spectrum RX-1 Perkin Elmer FT-IR spectrometer in potassium bromide discs. MerckGF₂₅₄ pre-coated silica plates were used for TLC. The melting points are uncorrected and taken on digital melting point apparatus. The compounds were synthesized using the Scheme 1 and 2. Samsung domestic microwave oven M-183DN was used for microwave irradiated (MWI) synthesis.

Scheme 1

Scheme 2.

3-Amino-2-phenyl-quinazolin-4-(3H)-one (**1**)

Equimolar quantities of 2-phenyl-3,1-benzoxazine and hydrazine hydrate (99%) were taken in pyridine and irradiated at 200 watts for 1.5 minutes. Upon completion of the reaction, 100 mL of cold water was added and the precipitate obtained was recrystallized from 99% absolute ethanol. m.p: 196-198°C. ¹H-NMR: δ 7.92 (m, 9H, Ar-H quinazolinone-H), 5.67 (2H, -NH₂ of quinazoline), ¹HMR (D₂O): δ 7.92 (m, 9H Ar-H quinazolinone). IR (KBr cm⁻¹): 3300 (sharp, -NH₂), 3200 (NH-H), 2954 (CH, aromatic str), 1680 (C=O, characteristic quinazolinone carbonyl str), 1605 (C=N of the quinazolinone).

General procedure for synthesis of Schiff's bases of 3-amino-2-phenyl-quinazolin-4-(3H)-one (*QSB*₁₋₅)

Equimolar quantities of **2** and aldehyde were taken in 10 mL of ethanol and one drop of concentrated sulphuric acid was added to the mixture and irradiated at 300 watts for 1.5 minutes. The reaction was monitored by TLC, the solid obtained was washed with water, ethanol and columned over silica gel 120 column and purified (chloroform : ethanol (9:1) solvent system). Recrystallized from ethanol:water (95:5). The physical data of these compounds is mentioned in Table 2.

Spectral data of selected compounds

3-[(2,6-Dichloro-phenyl)methylene]amino-2-phenylquinazolin-4(3H)-one (*QSB*₁)

¹H-NMR: δ 7.43-8.28 (m, 12H, quinazolinone-H and Ar-H), 9.37 (s, 1H, N=CH). IR (KBr): 3055 (quinazolinone-H, Ar-H), 1680 (C=O), 1607, 1587 (C=N), 1566-1472 (C=C, benzene and quinazolinone rings), 770, 690 (1,2,3-trisubstituted benzene), 766, 690 (mono-substituted benzene).

3-[(4-Chloro-phenyl)methylene]amino-2-phenylquinazolin-4(3H)-one (*QSB*₂)

¹H-NMR: δ 7.0-8.40 (m, 13H, Ar-H and Quinazolinone-H), 9.40(s, 1H, N=CH).

Table 2. Physical constants of the compounds and yields.

Code name.	Ar	M.pt °C	%Yield	MWl* Time in min
QSB ₁	2,6-(Cl) ₂ -C ₆ H ₃	176-178	70	1.00
QSB ₂	4-Cl-C ₆ H ₄	196-198	89	1.00
QSB ₃	2-OH-4-OCH ₃ -Ph	132-134	70	1.00
QSB ₄	C ₆ H ₄	196-198	85	1.00
QSB ₅	3,4-(OCH ₃) ₂ -C ₆ H ₃	179-181	78	1.00
ISB ₁	Furan-2-yl	266-268	90	2.50
ISB ₂	3- OH-C ₆ H ₄	235-237	80	2.50
ISB ₃	(CH ₃) ₂ -N-C ₆ H ₄	223-225	85	2.50
ISB ₄	3- NO ₂ -C ₆ H ₄	229-231	80	2.50
ISB ₅	4- Cl-C ₆ H ₄	184-186	80	2.50

*Microwave irradiation

3-[[(4-Hydroxy-2-methoxy-phenyl)methylene]amino]-2-phenyl-quinazolin-4(3H)-one (QSB₃)

¹H-NMR: δ 3.81 (s, 3H, methoxy protons), 6.92 -8.3 (m, 12H, quinazolinone-H and Ar-H), 9.40 (s, 1H, -H-C=N-), 11.2 (-OH). IR (KBr): 3305.8, 3215.1, 1749.3, 1712.7, 1664.5, 1575.7, 1467.7, 1377.1.

3-[[(3,4-Dimethoxy-phenyl)methylene]amino]-2-phenyl-quinazolin-4(3H)-one (QSB₅)

¹H-NMR: 3.85 (2s, 6H 2 x OCH₃), 7.33-8.28 (m, 12H, quinazolinone-H and Ar-H), 9.40 (s, 1H, N=CH).

General procedure for synthesis of Schiff's bases of pyridine-4-carbohydrazide (ISB₁₋₅)

Equivalent quantities of isoniazid and aryl aldehydes were dissolved in ethanol and 3 drops of concentrated sulphuric acid or ZnCl₂ (250 mg /mol) or LaCl₃ (300 mg/mol) was added and mixture was irradiated in Domestic Microwave at 150 watts for 15-20 minutes. The reaction was monitored with TLC using chloroform and ethanol (9:1) as eluent. On completion of the reaction the solvent was removed under reduced pressure on rotary evaporator. The solid obtained washed with Pet. ether:ethyl acetate (1:5), ethanol or passed over silica gel column to obtain pure compounds. Recrystallize from 99% absolute ethanol. Physical data of these compounds is given in Table 2.

Spectral data of selected compounds

Isonicotinic acid (furan-2-yl-methylene) hydrazide (ISB₁)

¹H-NMR: δ 12.00 (-NH of Hydrazide), 8.96 (d, 2H, -CH of pyridine), 8.5 (-N=CH imine), 7.9 (d, 2H, pyridine), 6.3 (d, 1H, CH of Furan), 6.6 (d, 1H, CH of Furan) 7.4 (s, 1H, -CH of Furan). IR (KBr): 3300(-NH), 2900(-CH aromatic str.), 1710 (carbonyl of amide), 1605, 1545 (C=N pyridine and imine), 1445(aromatic CH str.).

Isonicotinic acid-(m-hydroxy-benzilidene) hydrazide (ISB₂)

¹H-NMR: δ 12.00 (-NH of Hydrazide), 9.3 (-OH), 8.80 (d, 2H, -CH of pyridine), 8.4 (-N=CH imine), 8.00(d, 2H, -CH of pyridine), 7.2 (d, 1H, Ar-H), 7.0 (d, 1H, Ar-H), 6.80 (d, 1H, Ar-H). IR (KBr): 3300, 2875, 1710, 1601, 1584, 1435.

Isonicotinic acid (p-(N, N-dimethyl-amino)-benzilidene) hydrazide (ISB₃)

¹H-NMR: δ 12.00 (-NH of Hydrazide), 8.80 (d, 2H, -CH of pyridine), 8.6 (-N=CH imine), 7.80 (d, 2H, -CH of pyridine), 6.4 (d, 2H, Ar-H), 7.4 ((d, 2H, Ar-H), 3.0(2 x CH₃).

Isonicotinic acid ((m-nitro-benzilidine) hydrazide (ISB₄))

¹H-NMR: δ 12.00 (-NH of Hydrazide) , 8.80 (d , 2H,-CH of Pyridine), 8.6 (-N=CH imine), 8.2 (d,2H,-CH of pyridine), 7.8-7.4(m, 4H,Ar-H).

Isonicotinic acid ((p-chloro-benzilidine) hydrazide (ISB₅))

¹H-NMR: δ 12.00 (-NH of Hydrazide) , 8.80 (d , 2H,-CH of Pyridine), 8.6 (-N=CH imine), 8.2 (d,2H,-CH of pyridine), 7.8-7.4 (4H,m,Ar-H).

In vitro anticancer evaluation

The study was performed at Indian Institute of Integrative Medicine, Jammu. The cancer cell-line cytotoxicity assay was performed on five human cancer cell-lines for the percent total growth inhibition (TGI₅₀) values. The standards such as MTX, 5-fluorouracil, paclitaxel and adriamycin were used in the assay to give a comparable value of the inhibition of DHFR, thymidylate synthase, DNA topoisomerase and microtubulin inhibition. The data of the compounds is given in Table 3.

Results and Discussion

Docking of the molecules and synthesis

MTX has seven inter hydrogen bondings with amino acid residues of DHFR and the ΔG energy of binding for MTX being -163.3 kcal/mol. The ligands interacting with at least one of these residues having comparable G-Scores, and Δ G energy of binding lower than MTX were selected for synthesis and *in vitro* human cancer cell-line cyto-toxicity assay. The molecules were synthesized by microwave irradiation procedure. The ¹H-NMR of the molecules showed the characteristic -N=CH proton shift at 9.40 ppm and for pyridine Shift's bases at 8.5 ppm. Other aromatic protons were present in 7.00-9.00 ppm region. IR spectrum showed the presence of the amide group of 1745cm⁻¹ for the pyridines and 1680 cm⁻¹ for the quinazolinones and peaks at 1601 cm⁻¹ and 1605 cm⁻¹ 1585 cm⁻¹ for C=N were observed for quinazolinones and pyridines.

Table 3. Cancer cell-line cyto-toxicity assay and G-scores.

Name	Conc, M	CoLo- 205	HCT-15	IMR-32	Hep-2	IGR- OV-1	G-Score
% Growth inhibition							
ISB ₁	1x10 ⁻⁵	11	0	38	15	0	-8.35
ISB ₂	1x10 ⁻⁵	11	0	30	0	5	-8.22
ISB ₃	1x10 ⁻⁵	65	50	75	33	52	-8.18
ISB ₄	1x10 ⁻⁵	10	0	37	10	5	-8.12
ISB ₅	1x10 ⁻⁵	11	0	30	0	5	-8.10
QSB ₁	1x10 ⁻⁵	16	5	32	15	13	-11.00
QSB ₂	1x10 ⁻⁵	16	6	25	6	22	-10.10
QSB ₃	1x10 ⁻⁵	5	1	20	0	10	-9.80
QSB ₄	1x10 ⁻⁵	12	3	7	0	0	-9.70
QSB ₅	1x10 ⁻⁵	15	3	9	0	0	-9.50
5-Fu [#]	2x10 ⁻⁵	52	49	-	-	-	-
Adriamyc	1x10 ⁻⁶ M	-	-	62	-	-	-
MTX*	1x10 ⁻⁵	-	-	-	56	-	-10.10
Paclitaxel	1x10 ⁻⁵	-	-	-	-	54	-

*Methotrexate , [#] 5-Fluorouracil.

In vitro anticancer evaluation

The quinazolinone Schiff's bases (**QSB₁₋₅**) did not show anticancer activity but showed comparable inhibition. Amongst pyridine carbohydrazide Schiff's bases **ISB₁₋₅**, one molecule was found to be equipotent with Methotrexate showing the possibility of inhibition of DHFR. The other results were comparable with paclitaxel and adriamycin as per the NCI guidelines for this molecule.

Conclusion

ISB₃, (CH₃)₂-N-C₆H₄ Schiff's base of pyridine-4-carbohydrazide with G-Score -8.10, (approximately similar for all the compounds from this series) was found to be active as per NCI guidelines. Thus molecule may not only act by DHFR inhibition but also by other mechanisms of anticancer activity.

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