Synthesis and Evaluation of Some New Compounds of 2-Methylthio-6-amino-4-(3H)-quinazolines as Dihydrofolate Reductase Inhibitors

Submitted by

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1426 H
2005 G
To Yara and Badr,  
and my beloved family
CONTENTS

List of Abbreviations ..................................................................................................... iii
List of Tables .................................................................................................................. iv
List of Figures.................................................................................................................. v
List of Schemes............................................................................................................... vi
Acknowledgments ......................................................................................................... vii
Abstract........................................................................................................................... ix

1. Introduction ............................................................................................................... 1
  1.1 Dihydrofolate Reductase (DHFR) .................................................................... 1
  1.2 The Folate Pathway .......................................................................................... 3
  1.3 Inhibitors of Dihydrofolate Reductase.............................................................. 8
    1.3.1 Classical DHFR Inhibitors .................................................................. 8
    1.3.2 Non-classical DHFR Inhibitors ......................................................... 10

2. Literature Background ........................................................................................... 11

3. Research Objectives and Rational Design ............................................................ 15

4. Results and Discussion ............................................................................................ 18
  4.1 Molecular Dynamic Studies............................................................................ 18
  4.2 Chemistry........................................................................................................ 33
  4.3 Biological Investigations ................................................................................ 38
    4.3.1 Dihydrofolate Reductase (DHFR) Inhibition Assay ......................... 38
    4.3.2 Antitumor Testing ............................................................................. 41

5. Conclusion ............................................................................................................... 45

6. Experimental ........................................................................................................... 47
  6.1 Molecular Dynamic Calculations ..................................................................... 48
    6.1.1 Molecular Structure of the Synthesized Quinazolines ...................... 48
    6.1.2 Docking and Molecular Dynamic Simulations .................................. 48
  6.2 Synthesis ......................................................................................................... 49
    6.2.1 2-Thioxo-3-substituted-6 or 7-nitro-3H-quinazolin-4-ones 
          (17,18,20). ......................................................................................... 49
    6.2.2 2-Methylthio-3-substituted-6 or 7-nitro-3H-quinazolin-4-ones (21, 
          22, 24) ................................................................................................. 49
    6.2.3 2-Methylthio-3-(phenyl or benzyl)-6 or 7-amino-3H-quinazolin-4- 
          ones (25–27) ......................................................................................... 50
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2.4</td>
<td>2-Methylthio-3-(phenyl or benzyl)-6-[(substituted)benzylidinamino]-3H-quinazolin-4-ones (28–33).</td>
<td>50</td>
</tr>
<tr>
<td>6.2.5</td>
<td>2-Methylthio-3-(phenyl or benzyl)-6-[N-(substituted benzyl)amino]-3H-quinazolin-4-ones (34–39).</td>
<td>51</td>
</tr>
<tr>
<td>6.2.6</td>
<td>2-Methylthio-3-(phenyl or benzyl)-6-[N-(substituted benzyl)-N-methylamino]-3H-quinazolin-4-ones (40–45).</td>
<td>52</td>
</tr>
<tr>
<td>6.2.7</td>
<td>2-Methylthio-3-(phenyl or benzyl)-6-[(substituted phenyl)sulphonylamino]-3H-quinazolin-4-ones (46–51).</td>
<td>53</td>
</tr>
<tr>
<td>6.2.8</td>
<td>N-Substituted-N'-[2-methylthio-3-(phenyl or benzyl)-3H-quinazolin-4-one-6-yl]thioureas (52–57).</td>
<td>54</td>
</tr>
<tr>
<td>6.2.9</td>
<td>2-Methylthio-3-(phenyl or benzyl)-6-[(substituted phenyl)carboxynamino]-3H-quinazolin-4-ones (58–69).</td>
<td>55</td>
</tr>
<tr>
<td>6.3</td>
<td>Dihydrofolate Reductase (DHFR) Inhibition Assay</td>
<td>56</td>
</tr>
<tr>
<td>6.4</td>
<td>Antitumor Screening</td>
<td>57</td>
</tr>
</tbody>
</table>

7. References.................................................................................................................. 58
List of Abbreviations

AICAR  aminoimidazole carboxamide ribonucleotide
AIDS  acquired immunodeficiency syndrome
DHFR  dihydrofolate reductase
DMF  dimethylformamide
DMSO  dimethyl sulfoxide
dTMP  deoxythymidine monophosphate
dUMP  deoxyuridine monophosphate
TEA  triethylamine
FA  folic acid
Fe  iron powder
FH₂  dihydrofolic acid
FH₄  tetrahydrofolic acid
FPGS  folypolyglutamate synthetase
GAR  glycinamide ribonucleotide
GI₅₀  median growth inhibition
HCl  hydrochloric acid
hDHFR  human dihydrofolate reductase
LC₅₀  median lethal concentration
MD  molecular dynamics
MS  mass spectrometry
MTX  methotrexate
MTXGₙ  MTX-polyglutamate
NaBH₄  sodium borohydride
NaCNBH₃  sodium cyanoborohydride
NADPH  nicotinamide adenine dinucleotide phosphate (reduced)
NCI  National Cancer Institute
NMR  nuclear magnetic resonance
PCP  Pneumocystis carinii pneumonia
PS  pico-second
PTM  pyrimethamine
PTX  piritrexim
RFC  reduced folate carrier
RMS  root mean square
TGI  total growth inhibition
TMP  trimethoprim
TMQ  trimetrexate
TMS  tetramethylsilane
TS  thymidylate synthetase
List of Tables

Table 1: Binding data for the quinazoline analogues 17-27 docked at hDHFR active site and their H-bond interaction with amino acid residues. .............. 23

Table 2: Binding data for the quinazoline analogues 28-33 docked at hDHFR active site and their H-bond interaction with amino acid residues. ............. 25

Table 3: Binding data for the quinazoline analogues 34-45 docked at hDHFR active site and their H-bond interaction with amino acid residues. ............. 27

Table 4: Binding data for the quinazoline analogues 46-51 docked at hDHFR active site and their H-bond interaction with amino acid residues. ............. 29

Table 5: Binding data for the quinazoline analogues 52-57 docked at hDHFR active site and their H-bond interaction with amino acid residues. ............. 31

Table 6: Binding data for the quinazoline analogues 58-69 docked at hDHFR active site and their H-bond interaction with amino acid residues. ............. 31

Table 7: Physicochemical properties and DHFR inhibition (IC_{50}, \mu M) of the newly synthesized compounds 17–27. ........................................................ 34

Table 8: Physicochemical properties and DHFR inhibition (IC_{50}, \mu M) of the newly synthesized compounds 28–33. ........................................................ 35

Table 9: Physicochemical properties and DHFR inhibition (IC_{50}, \mu M) of the newly synthesized compounds 34–45. .......................................................... 36

Table 10: Physicochemical properties and DHFR inhibition (IC_{50}, \mu M) of the newly synthesized compounds 46–69. .......................................................... 37

Table 11: In vitro three cell lines primary antitumor assay a result of the synthesized compounds. ......................................................................................... 41

Table 12: Growth inhibitory and lethal concentrations (GI_{50}, TGI_{50}, LC_{50}) of some selected in vitro cell lines (\mu M). a ................................................................. 43

Table 13: Median growth inhibitory concentration (GI_{50}, \mu M) of in vitro subpanel tumor cell lines................................................................. 44

Table 14: Total growth inhibitory concentration (TGI, \mu M) of in vitro subpanel tumor cell lines................................................................. 44
List of Figures

Figure 1: The folic acid pathway .......................................................................................... 4

Figure 2: Sites of action of antifolates ........................................................................... 5

Figure 3: Structures of the proposed target compounds ............................................. 16

Figure 4: A- Crystallographic structure of “MTX, 3” (stick model). (C, gray; N, blue; O, red, P, yellow-green). B- Energy minimized structure of compound 17 (stick model ). (C, gray; N, blue; O, red); C- Superposition of the X-ray structure of MTX, 3 (blue) and the energy minimized 17 (yellow). ............................................................................. 19

Figure 5: A-Flat-ribbon presentation of the crystallographic structure of ternary complex of MTX-NADPH-hDHFR showing the stick models of MTX, 3 and the cofactor NADPH (C, gray; N, blue; O, red). B- Crystal structure of MTX, 3 at the hDHFR binding pocket showing H-bonding interactions with the key residues. (Colored-stick, C, gray; N, blue; O, red). ................. 20

Figure 6: A-Flat-ribbon presentation of the lowest energy conformer of compound 17 docked in the hDHFR binding domain and the cofactor NADPH (C, gray; N, blue; O, red). B-Binding mode for compound 17 docked and minimized in the hDHFR binding pocket, showing residues involved in its recognition. Solid lines indicate the H-bond formation. Dashed lines indicate unstable H-bond. ............................................................................. 21

Figure 7: A- Flat-ribbon presentation of the lowest energy conformer of compound 26 docked in the hDHFR binding domain and the cofactor NADPH (C, gray; N, blue; O, red); B- Binding mode for compound 26 docked and minimized in the hDHFR binding pocket, showing residues involved in its recognition. Solid lines indicate the H-bond formation. Dashed lines indicate unstable H-bond. ............................................................................. 22

Figure 8: A- Flat-ribbon presentation of the lowest energy conformer of compound 43 docked in the hDHFR binding domain and the cofactor NADPH (C, gray; N, blue; O, red). B- Binding mode for compound 43 docked and minimized in the hDHFR binding pocket, showing residues involved in its recognition. Solid lines indicate the H-bond formation. Dashed lines indicate unstable H-bond. ............................................................................. 28

Figure 9: A-Flat-ribbon presentation of the lowest energy conformer of compound 66 docked in the hDHFR binding domain and the cofactor NADPH (C, gray; N, blue; O, red). B- Binding mode for compound 66 docked and minimized in the hDHFR binding pocket, showing residues involved in its recognition. Solid lines indicate the H-bond formation. Dashed lines indicate unstable H-bond. ............................................................................. 30
Figure 10: Inhibition (IC$_{50}$) of DHFR activity by methotrexate (MTX, 3). Each point represents a mean of 3 experiments. ............................................................. 39

Figure 11: Structures of the active DHFR inhibitors.............................................................. 45

Figure 12: Structures of the active antitumor agents.............................................................. 46

List of Schemes

Scheme 1: Synthesis of 2-Thioxo-3-substituted-6 or 7-nitro-3$H$-quinazolin-4-ones (17–20), 2-Methylthio-3-substituted-6 or 7-nitro-3$H$-quinazolin-4-ones (21–24), and 2-Methylthio-3-(phenyl or benzyl)-6 or 7-amino-3$H$-quinazolin-4-ones (25–27)... ....................................................................... 33

Scheme 2: Synthesis of 2-Methylthio-3-(phenyl or benzyl)-6-[(substituted)-benzylidinamino]-3$H$-quinazolin-4-ones (28–33), 2-Methylthio-3-(phenyl or benzyl)-6-[N-(substituted benzyl)amino]-3$H$-quinazolin-4-ones (34–39), and 2-Methylthio-3-(phenyl or benzyl)-6-[N-(substituted benzyl)-N-methylamino]-3$H$-quinazolin-4-ones (40–45)... ........................ 34

Scheme 3: Synthesis of 2-Methylthio-3-(phenyl or benzyl)-6-[(substituted phenyl)sulphonyl]amino-3$H$-quinazolin-4-ones (46–51), N-Substituted-N'-[2-methylthio-3-(phenyl or benzyl)-3$H$-quinazolin-4-one-6-yl]thioureas (52–57), and 2-Methylthio-3-(phenyl or benzyl)-6-[(substituted phenyl)carbonylamino]-3$H$-quinazolin-4-ones (58–69). ...... 36
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Abstract

Dihydrofolate reductase (DHFR) catalyzes the reduction of folate or dihydrofolate into tetrahydrofolate, which then converted into $N^5, N^{10}$-methylene tetrahydrofolate. This latter compound functions as the source of the methyl group to convert dUMP into dTMP. Inhibition of DHFR has long been an attractive goal for the development of chemotherapeutic agents against bacterial and parasitic infections as well as cancer. The aim of this research study is to locate a novel synthetic lead compound(s) for future development as DHFR inhibitors. A new series of quinazoline analogues is designed guided by molecular dynamic (MD) studies, in such a manner to imitate methotrexate (MTX,3), the classical inhibitor of DHFR, and fitted with functional groups believed to enhance the inhibition of the enzyme activity. Forty eight new compounds along with two reported derivatives belong to the aforementioned nucleus, guided with molecular modeling prediction, have been synthesized. Structure elucidation of the new compounds was fulfilled based on elementary analyses (C, H, N), $^1$H-NMR and Mass spectrometry. The synthesized compounds were evaluated for their in vitro inhibition of DHFR activity at the Department of Pharmacology, College of Pharmacy, King Saud University and for their in vitro antitumor activity at the National Cancer Institute (NCI), Bethesda, Maryland, USA.

As can be concluded from the obtained results, compounds 2-methylthio-3-benzyl-6-[N-(4-methoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (41), 2-methylthio-3-phenyl-6-[N-(3, 4-dimethoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (42), and 2-methylthio-3-benzyl-6-[N-(3, 4-dimethoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (43) are the most active members of this study as DHFR inhibitors with IC$_{50}$ values of 0.5, 0.4, and 0.4 $\mu$M, respectively.
Compounds 2-methylthio-3-benzyl-6-(3, 4-dimethoxy-benzylideneamino)-3H-quinazolin-4-one (31), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 20.1, 68.0, 97.8 \(\mu\text{M}\), respectively; 2-methylthio-3-benzyl-6-[N-(3, 4-dimethoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (43), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 23.5, 83.1, > 100.0 \(\mu\text{M}\), respectively; N-ethyl-N’-(2-methylthio-3-benzyl-3H-quinazolin-4-one-6-yl)thiourea (53), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 26.7, 74.7, 97.8 \(\mu\text{M}\), respectively; and 2-methylthio-3-benzyl-6-(phenylcarbonylamino)-3H-quinazolin-4-one (59), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 9.1, 39.5, 78.1 \(\mu\text{M}\), respectively; are the most active antitumor agents in this study, as compared with the known antitumor drug ‘Melphalan’ (GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 27.1, 35.3, 65.5 \(\mu\text{M}\), respectively). It seemed that compound 43 exerts its antitumor potency through DHFR inhibition mode of action, while the other active compounds, namely 31, 53, and 59, might exert their antitumor potency through DHFR inhibition and/or some other mechanism of action.

These studied quinazoline analogues could be considered as useful templates for future development and further derivatization or modification to obtain more potent DHFR inhibitors.
1. Introduction

The ultimate goal of modern drug discovery is to identify a therapeutic agent that is effective against a disease. Although the process of drug discovery is a complex issue, it may be divided into three main steps: i) development of relevant biological system for testing of the compounds \textit{in vitro} and \textit{in vivo}; ii) identification of lead compounds for concept test in the biological assays; iii) optimization of the lead structure to enhance the selectivity ratio, toxicity profile or pharmacokinetics, and ultimately furnish a candidate drug suitable for appropriate \textit{in vivo} studies and further clinical evaluation. The research study described herein mainly reports the design and synthesis of new ligands for the enzyme dihydrofolate reductase (DHFR).

1.1 Dihydrofolate Reductase (DHFR)

Dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate-NADP$^+$ oxidoreductase; EC 1.5.1.3) is an enzyme of pivotal importance in biochemistry and medicinal chemistry. DHFR functions as a catalyst for the reduction of dihydrofolate to tetrahydrofolate. Reduced folates are carriers of one-carbon fragments; hence they are important cofactors in the biosynthesis of nucleic acids and amino acids. The inhibition of DHFR leads to partial depletion of intracellular reduced folates with subsequent limitation of cell growth.\textsuperscript{1} DHFRs isolated from different species are relatively small proteins (18–25 KDa), which are easily available. DHFR has attracted attention of protein chemists as a model for the study of enzyme structure/functional relationships. The species-differences among the DHFRs,\textsuperscript{2} have been used to discover compounds with particular selectivity, e.g., that are lethal to bacteria but relatively harmless to mammals.\textsuperscript{3} Such selective inhibitors are trimethoprim (TMP, 1)
and pyrimethamine (PTM, 2), which are used in therapy for their antibacterial and antiprotozoal properties, respectively.

Compounds that inhibit DHFR exhibit an important role in clinical medicine as exemplified by the use of methotrexate (MTX, 3) in neoplastic diseases,\textsuperscript{4} inflammatory bowel diseases\textsuperscript{5} and rheumatoid arthritis,\textsuperscript{6} as well as in psoriasis\textsuperscript{7,8} and in asthma,\textsuperscript{9} TMP (1) in bacterial diseases;\textsuperscript{10} and PTM (2) in protozoal diseases.\textsuperscript{11,12} Lately, a new generation of potent lipophilic DHFR inhibitors such as trimetrexate (TMQ, 4) and piritrexim (PTX, 5) have shown antineoplastic\textsuperscript{13} and most importantly, antiprotozoal\textsuperscript{14} activities.
The enzymatic reduction involved in the inhibition of DHFR is a random process in which either the substrate (dihydrofolate) or the cofactor NADPH, forms a binary complex with the enzyme, with subsequent rapid binding of the inhibitor, to form ternary complex. A tremendous amount of work has been done regarding inhibitors of DHFR.\textsuperscript{15} The essence of the work is that all steric, electronic, and hydrophobic parameters have been effective in the inhibition of DHFR, depending upon the source of the enzyme and type of inhibitors.

1.2 The Folate Pathway

Folate coenzyme are involved with about 20 enzymatic reactions in mammalian metabolism.\textsuperscript{16} The reactions of folate metabolism are interconnected, and the inhibitors discussed within this thesis may also disturb metabolism at sites other than those designated. An intact enzyme pathway is necessary to maintain \textit{de novo} synthesis of the essential building blocks involved in DNA synthesis as well as of important amino acids.

Folic acid (FA, 6) is a water soluble B-vitamin that plays a crucial role in the biosynthesis of DNA. The vitamin consists of 2-amino-4-oxo-pteridine with a side-chain incorporating both \textit{p}-aminobenzoic acid and glutamic acid. Humans can not synthesize folic acid, and thus depend on a variety of dietary sources for the vitamin.
Food folates exist mainly as 5-methyl-tetrahydrofolate (N⁵-methyl-FH₃) and 5-formyl-tetrahydrofolate (N⁵-formyl-FH₄), Figure 1. The active form of FA is its reduced tetrahydro-form (FH₄), which is formed after reduction of dihydrofolic acid by DHFR. FH₄ functions as the ‘acceptor’ of single-carbon units and the tetrahydrofolate is subsequently transformed enzymatically from the appropriate cofactor to precursor molecules that lead to the synthesis of purine and pyrimidine (e.g., thymine) nucleotides necessary for DNA, and also to the synthesis of important amino acids, e.g. methionine (Figure 1).

**Figure 1:** The folic acid pathway.
An overview relating to the biochemistry of the folates will be described. Once FA (6) has been reduced to FH₄ (Figure 1 and 2), a myriad of enzymes are involved in the subsequent reactions, using different derivatives of FH₄ in one-carbon transfer reactions. N⁵-Formyl-FH₄ (folinic acid, leucovorin) is taken up actively into the cell by a reduced folate carrier (RFC) system where it is converted to N¹⁰-formyl-FH₄. In another enzymatic pathway, N⁵-formyl-FH₄ can, after transport into the cell, be converted to N⁵-methyl-FH₄. N⁵-Formyl-FH₄ may also be taken up actively and form fresh supplies of FH₄. The same entrance system is used by MTX (3), as these reduced tetrahydrofolates, although the latter seem to have a relatively low affinity for RFC in comparison to MTX (3).¹⁸

N¹⁰-Formyl-FH₄ is responsible for the donation of single carbon groups in the de novo biosynthesis of purine nucleotides in the reactions catalyzed by the enzymes glycaminamide ribonucleotide (GAR) and aminoimidazole carboxamide ribonucleotide.

**Figure 2:** Sites of action of antifolates.
(AICAR) transformylases. As well as being used directly in purine synthesis, \( N^{10} \)-formyl-FH\(_4\) is also converted, via \( N^5,N^{10} \)-methenyl-FH\(_4\), to \( N^5,N^{10} \)-methylene-FH\(_4\). The latter is converted, first to dihydrofolate (FH\(_2\)) by the action of thymidylate synthetase (TS) and then to tetrahydrofolate (FH\(_4\)) by DHFR. \( N^5,N^{10} \)-Methylene-FH\(_4\) donates a methyl group to the 5-position of the pyrimidine ring of deoxyuridylate (dUMP) in a reaction catalyzed by TS forming deoxythymidylate, which is used for the DNA synthesis. Thus, \( N^5,N^{10} \)-methylene-FH\(_4\) is the only \textit{de novo} source of cellular thymidylate. Inhibition of TS results in so called thymine-less death, which leads to disruption of the synthesis of dividing cells. During this process \( N^5,N^{10} \)-methylene-FH\(_4\) is oxidized to dihydrofolate and must be converted back to its tetrahydro form by DHFR in order to maintain the reduced folate pool.

Intracellularly, folic acid and derived coenzymes also have the ability to be converted to polyglutamated derivatives with additional glutamic acid residues linked by amide bonds by the enzyme folylpolyglutamate synthetase (FPGS). This is an important mechanism for trapping classical folates and antifolates within the cell, thus maintaining high intracellular concentration.\(^{19}\) The attachments are performed on the \( \gamma \)-carboxyl group of the glutamate residue. FPGS is able to attach up to six glutamate molecules to the pteridine ring of MTX (3).\(^{20,21}\) This polyglutamation reaction has several biologically important consequences. By polyglutamation the hydrophilicity of the molecule is enhanced (due to a high increase in negative carboxylate groups) and together with the increased size of the molecule a greater intercellular retention is enabled with decreased cellular efflux and trapping of the drug within the cell, with prolonged drug action as a consequence.\(^{22}\) The polyglutamates appear to be somewhat more efficient substrates for DHFR than the monoglutamated counterparts.\(^{23,24}\) MTX-Polyglutamates (MTXG\(_N\)) are direct inhibitors of DHFR and are thus less
reversible inhibitors than MTX (3) itself.\textsuperscript{21} Tissues with high FPGS activity, such as liver, accumulate and retain the polyglutamated MTX (3) for prolonged periods of time. Hence, this increased concentration of polyglutamated MTXG\textsubscript{N} is responsible for the hepatotoxicity observed after chronic administration of the drug. Compared to monoglutamated drugs, the polyglutamates also have increased affinity to other folate-dependent enzymes such as TS,\textsuperscript{25,26} and the transformylases of glycaminamide ribonucleotide (GAR), and 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)\textsuperscript{27,28}, Figure 2. AICAR is directly involved in de novo purine synthesis and its inhibition results in inhibition of such synthesis. As a consequence, the polyglutamation of MTX (3) plays an essential role in the development of side effects. Another hypothesis about the mechanism of action of MTX (3) suggests that by inhibiting AICAR transformylase, MTX (3) causes the accumulation of AICAR. This inhibition might result in the subsequent increase of the production of the anti-inflammatory autocoid adenosine,\textsuperscript{29} an endogenous anti-inflammatory cytokine. This hypothesis could be one of the explanations for the anti-inflammatory properties that are seen in therapy with MTX (3) in autoimmune disorders where much lower concentrations are needed for the effect compared to doses that generate effects through direct inhibition of DHFR. Nevertheless, certain publication reports that treatment with MTX (3) in inflammatory bowel diseases does not cause elevated concentrations of adenosine, neither in plasma nor at the site of the disease.\textsuperscript{30} However, several attempts with non-polyglutamated analogues have indicated a release of adenosine, suggesting that polyglutamation of the antifolate is not required for these functions.\textsuperscript{31}

The administration of exogenous reduced folates, such as leucovorin, effectively prevents MTX (3) cytotoxicity in mammalian cells (Figure 1). The amount of leucovorin required to prevent severe clinical toxicity in high-dose MTX (3)
chemotherapy regimens is directly proportional to the amount of MTX (3) circulating in plasma. Leucovorin does not compete with MTX (3) for binding to DHFR but directly overcomes MTX (3) blockade of the enzymes by increasing the intracellular pools of FH₄ and thereby rescues cells from death. Although effective, this regimen offers a very expensive therapy.

1.3 Inhibitors of Dihydrofolate Reductase

Inhibitors of DHFR are classified as either classical or non-classical antifolates. The classical antifolates are characterized by a p-aminobenzoylglutamic acid side-chain in the molecule and thus closely resemble folic acid itself. MTX (3) is the most well known drug among the classical antifolates. Compounds classified as non-classical inhibitors of DHFR do not possess the p-aminobenzoylglutamic acid side-chain but rather have a lipophilic side-chain.

1.3.1 Classical DHFR Inhibitors

MTX is N-{4-[[2,4-diamino-6-pteridyl)methyl]-N¹⁰-methyl-amino}benzoyl-glutamic acid (3). It is the most often used DHFR inhibitor in clinic today, together with the antibacterial drug TMP (1). The most common use of MTX (3) is as an anticancer drug, but lately the drug also is considered to have anti-inflammatory and immunosuppressive properties with accompanied activity against autoimmune disorders. MTX (3) serves as an antimetabolite, which means that it has a similar structure to that of a cell metabolite, resulting in a compound with a biological activity that is antagonistic to the metabolite, which in this case is folic acid.
MTX (3) is a competitive and reversible inhibitor of DHFR that binds tightly to the hydrophobic folate-binding pocket of the enzyme. The affinity of MTX (3) for DHFR increases considerably in the presence of the cofactor NADPH.

MTX (3) inhibits the synthesis of metabolites involved in one-carbon unit transfer reactions such as the biosynthesis of the important nucleotides (see Section 1.2). As a result of this process, the synthesis of DNA is disrupted. The polyglutamated derivatives of 3 have been postulated to be the probable cause of the anti-inflammatory properties associated with 3 due to its inhibition of AICAR transformylase. The immunosuppressive activity seen in MTX (3) treatment may be due to the induction of apoptosis (i.e., programmed cell death) of activated lymphocytes, e.g., the T-cells.32,33

Intrinsic and acquired resistance to MTX (3) and other antifolate analogues limit their clinical efficacy. Resistance has been attributed to several different mechanisms including a reduced level of cellular uptake of the drug,4 and to an increase in enzyme levels involved in the folic acid biochemistry. MTX (3) is transported into cells by the energy-dependent RFC carrier transport system (see Section 1.2) used for the active transport of reduced folates. Consequently, a poor ability to transport the drug into the cell can be one source of natural resistance associated with the drug.34,35 Major limitations, besides the development of resistance with MTX (3) treatment, are bone marrow toxicity, gastrointestinal ulceration, and kidney and liver damage. In high doses given intermittently, the adverse effect on the bone marrow is relieved by the periodic administration of leucovorin (see Section 1.2), enabling the blockade of tetrahydrofolic acid production to be by-passed (leucovorin rescue procedure).
1.3.2 Non-classical DHFR Inhibitors

The clinically useful properties of MTX (3) have stimulated the quest for a variety of new analogues with modifications within the molecule, without interfering too much with the pharmacophore of the original drug. Thus, research is enduring for a more selective drug, most importantly, without the severe side-effects often associated with MTX (3).

New more lipophilic antifolates have been developed in an attempt to circumvent the mechanisms of resistance, such as decreased active transport, decreased polyglutamation, DHFR mutations etc. These modified antifolates differ from the traditional classical analogues by increased potency, greater lipid solubility, or improved cellular uptake (see Section 2.0).
2. Literature Background

A variety of heterocycles are useful chemotherapeutic agents with known activity against bacterial and parasitic infections as well as cancer. Many of these compounds are inhibitors of the folic acid pathway of the invading organisms with the enzyme dihydrofolate reductase (DHFR) being a particular target. A fair amount of analogue work has attended this area with the aim of producing more effective chemotherapeutic agents.

Quinazolines have been reported to be biologically versatile compounds possessing variety of activity including anticancer activity. An extensive interest in quinazolines has been increased since the discovery of relitrexed (7) and thymitaq (8) and their activity as thymidylate synthase inhibitors. Literature citation showed that a variety of analogues of 2,4-diamino-6-[(aryl)thio]quinazolines (9) with known antimalarial properties, via the inhibition of DHFR enzyme, were prepared wherein the 4-amino group was replaced by hydrazino and hydroxylamino moieties. Such changes were found to markedly reduce the antimalarial and antitumor properties of this series.

Opportunistic infections with *Pneumocystis carinii* and *Toxoplasma gondii* are a major cause of morbidity and mortality in patients with the acquired immunodeficiency syndrome (AIDS). The current therapeutic regimens involve combinations of DHFR inhibitors, such as TMP (1) or PTM (2) with dihydropterate synthase inhibitors such as sulfonamides. These standard therapies have significant frequency and severity of toxicity. Some of the adverse reactions associated with combined therapy may be antibody-mediated, because the addition of leucovorin (see Section 1.2) does not prevent side effects such as neutropenia, others might be systemic adverse effects...
which in some cases may lead to permanent diabetes.\textsuperscript{42,43} Trimetrexate\textsuperscript{41} (TMQ, 4), an antineoplastic agent, is a second line drug for \textit{P. carinii} pneumonia (PCP) and could be a more effective option for initial as well as salvage therapy. It is a potent dihydrofolate reductase inhibitor with IC\textsubscript{50} 42 nM against \textit{P. carinii} dihydrofolate reductase. Piritrexim (PTX, 5), an antineoplastic drug, also inhibit \textit{P. carinii} and \textit{T. gondii} dihydrofolate reductase at IC\textsubscript{50} 19.3 and 17.0 nM, respectively.\textsuperscript{44} Tricyclic pyrimido[4,5-\textit{c}][2,7]naphthyridones (10) were synthesized as conformationally restricted inhibitors of DHFR and as antitumor and/or antiinfectious agents. These analogues proved to be more inhibitory against rat liver than both \textit{P. carinii} or \textit{T. gondii} dihydrofolate reductase, and thus lacked selectivity. In addition, they were less potent than the bicyclic compounds trimetrexate (4) and piritrexim (5).\textsuperscript{45}

The treatment of \textit{P. carinii} and \textit{T. gondii} infections with nonclassical antifolates such as compounds 1, 4 and 5 take advantage of the fact that these organisms are permeable to lipophilic, non-classical antifolate and, unlike mammalian cells, lack the carrier mediated active transport system (see Section 1.2), required for the uptake of
classical antifolates with polar glutamate side chains. In addition, host tissues can selectively be protected by the coadministration of leucovorin, which is taken up by mammalian cells and reverses toxicity associated with DHFR inhibitors. Further, these lipophilic agents can penetrate into the central nervous system (CNS) where T. gondii infections usually occur. As an attempt to improve the cell penetration of the highly selective pyrido[2,3-\(d\)]pyrimidines while maintaining their potency and selectivity as DHFR inhibitors, some quinazoline analogues of (11) were synthesized in which the N\(^8\)-nitrogen of the pyridopyrimidine ring was replaced with a carbon. This alteration resulted in an unanticipated loss of the selectivity, alternatively, those compounds showed remarkable antitumor activity.

Another series of tetrahydroquinazoline analogues of piritrexim (5) were prepared. The members of this series are characterized by six membered carbocyclic \(\beta\)-ring and one carbon bridge between the phenyl ring and the heterocyclic moiety (12). They showed DHFR inhibition activity at IC\(_{50}\) concentration range of 0.057–0.10 \(\mu\)M, and proved active against 13 different tumor cell lines at concentration range of 0.1–1.0 \(\mu\)M. As an attempt for the identification of more selective and potent DHFR inhibitors with the overall goal to improve therapy and to minimize the adverse effects, the soft drug concept was used for the design of new agents for inhalation therapy and could make this route available to safer DHFR inhibitors. These drugs are intended to undergo a fast metabolism, which minimizes toxicity to the host, for the treatment of P. carinii pneumonia (PCP). Compounds 13 and 14 proved to be potent DHFR inhibitor soft drugs.
PT523 or N$^\alpha$-(4-amino-4-deoxypteroyl)-N$^5$-hemophthaloyl-L-ornithine (15) is an unusually potent antifolate which exerts its potency through a tight binding to DHFR and efficient utilization of the reduced folate carrier (RFC), the membrane transport protein responsible for cellular uptake of both folates and classical antifolates.$^{53-56}$ Compound 15 could overcome a clinically relevant 10 to 30−fold level of resistance to the classical antifolate methotrexate (MTX, 3). Replacement of N$^5$, N$^8$ and N$^{10}$ of 15 by carbon atom produced a more potent antitumor analogue,$^57$ through DHFR inhibition.

Efforts still continue for the discovery of non-classical antifolates, with high selectivity toward the parasitic or the bacterial or even the tumor cell DHFR.$^{58-65}$
3. Research Objectives and Rational Design

Dihydrofolate reductase (DHFR) catalyzes the reduction of folate or dihydrofolate to tetrahydrofolate and intimately couples with thymidylate synthase (TS). Thymidylate synthase (TS) is a crucial enzyme that catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) utilizing $N^5,N^{10}$-methylene-tetrahydrofolate as a cofactor which functions as the source of the methyl group as well as the reductant. This is the exclusive de novo sources of dTMP, hence inhibition of DHFR or TS activity in the absence of salvage, leads to “thymine-less death.” Thus DHFR inhibition has long been an attractive goal for the development of chemotherapeutic agents against bacterial and parasitic infections as well as cancer (see Section 1.0).

The AIDS epidemic, cancer chemotherapy, and organ transplantation have significantly increased the number of patients with impaired immune systems who are suffering from severe opportunistic infections including pneumonia caused by the fungus *Pneumocystis carinii*. $P. carinii$ pneumonia (PCP) is a serious disease with high prevalence and constitutes the major cause of death in AIDS patients. However, with the widespread use of highly active antiretroviral therapy and prophylaxis in patients known to be at risk for developing PCP, the incidence of PCP in patients with AIDS has declined dramatically. Current treatment with trimethoprim (TMP, 1) a nonclassical inhibitor of DHFR in combination with sulfonamide is still the standard therapy for PCP. The severe side effects associated with sulfia drugs often lead to discontinuation of therapy. Trimetrexate (TMQ, 4) and piritrexim (PTX, 5), two lipophilic agents originally developed as anticancers, are now used in clinic as a second-line therapy in moderate to severe PCP. Although
compounds 4 and 5 are both potent inhibitors of DHFR from *P. carinii*, they are not selective and inhibit the mammalian enzyme even more efficiently.\textsuperscript{46,93} The clinical use of 4 and 5 is therefore limited because of their systemic host toxicity and requirement of expensive cotherapy with the rescue agent leucovorin,\textsuperscript{47,93–96} a classical folate cofactor for one-carbon metabolism, which could be taken up via active transport only by mammalian cells and reverse toxicity associated with the lipophilic DHFR inhibitors.\textsuperscript{46,47,51,52,97}

On the basis of these considerations, and in continuation to the previous efforts of our group in the area of antitumor agents,\textsuperscript{98–109} the aim of this research study is to locate a novel synthetic lead compound(s) and its *in vitro* testing for future development as competitive DHFR inhibitor(s). Compounds possessing such activity will be candidates for treating cancers, bacterial and parasitic infections. A new series of quinazoline analogues is designed to possess a sulfhydryl or thioether functions at

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Structures of the proposed target compounds.}
\end{figure}
position 2-, an alkyl, aryl or arylalkyl groups at position 3-, a nitro or amino functional groups at position 6-, (A). The 6- amino function will be used to introduce Schiff’s base (B), sulphonamide, amide or thioureido (D) functions. The Schiff’s base B will be utilized to produce a methotrexate (MTX, 3), like analogue (C). Thioether, sulphonamide, Schiff’s base, amide and thioureido functional groups are known to contribute to the enhancement of the antitumor activity. Combining the inherited DHFR inhibition activity of the quinazolines and the antitumor activity enhancer functional groups in one structure anticipated to produce a more active compounds. Most of the functional group designed to be accommodated on the quinazoline ring such as thioether, alkyl, aryl, arylalkyl, nitro, Schiff’s base, amide and thioureido are known to increase lipid solubility, a character very much needed in the non-classical dihydrofolate reductase inhibitors to treat pneumocystis carinii pneumonia (PCP).

Prediction of the affinity of a drug for a specific enzyme or receptor can be of a great value in the drug discovery process. Computational methods can be employed to investigate this affinity issue. Advances in molecular biology and improved methods for the crystallization of proteins provided knowledge about the three dimensional structure of specific enzymes and proteins. DHFR is an enzyme, which has been subjected to extensive characterization because of its relatively small size and ease of co-crystallization with new ligands. In the present study, molecular dynamics (MD) binding affinity predictions based on existing crystal structures of DHFR in complex with the new synthesized compounds as potential inhibitors were performed.
4. Results and Discussion

4.1 Molecular Dynamic Studies

The proposed target compounds A-D (Figure 3) have been comparatively evaluated in terms of their mode of binding to human dihydrofolate reductase (hDHFR) pocket. Molecular docking and molecular dynamic (MD) simulations have been performed for the proposed compounds (17-69), to evaluate their recognition profiles at the hDHFR binding-pocket. The idea behind this concept is based on the finding that synthetic hDHFR inhibitors fit at the enzyme pocket in a highly comparable manner to the dihydrofolate inhibitor MTX (3), Figure 4. It was found that hDHFR inhibitors display differences in their hydrogen bonding properties and their electrostatic interaction with the surrounding amino acid residues at the enzyme pocket. Furthermore, the hDHFR inhibition potency would be reflected by the magnitude of energy changes upon binding with the enzyme in their ternary complex (enzyme, NADPH, and inhibitor), Figure 5. Hydrogen bonds with a bond length up to 3 Å were considered. Once a reasonable complex was formed, the energy of interaction of each ligand with the enzyme was calculated. The extent of complementarities (binding) of analogues with the enzyme pocket was predicted by energy calculations. The greater the negative energy change induced by docking of analogue; the more favorable the interaction and the more stable the complex.

As a reference to our modeling and docking study, the ternary complex of hDHFR enzyme with NADPH (cofactor) and MTX, 3 (inhibitor) was used. Studying the pteridine ring H-bonding interaction of 3 with the hDHFR active site revealed that the nitrogen atoms at N1, N8 and the nitrogen atom of the 2-amino group contributed three
Figure 4: A- Crystallographic structure of “MTX, 3” (stick model). (C, gray; N, blue; O, red, P, yellow-green). B- Energy minimized structure of compound 17 (stick model ). (C, gray; N, blue; O, red); C- Superposition of the X-ray structure of MTX, 3 (blue) and the energy minimized 17 (yellow).

stable H-bonds with the key pocket residue Glu30. Besides, the nitrogen atom of the 4-amino group of the pteridine ring conferred trifurcated H-bonds with the ‘catalytic triad’ residues of hDHFR pocket Ile7, Tyr121 and Val115. Furthermore, the nitrogen and oxygen of the glutamine amide function performed two H-bonds with Asn64 (Figure 5). Molecular mechanic studies indicated that H-bonding interactions and conformational changes of the amino acid residues of the active site can be considered the main recognition elements for hDHFR-ligand systems.

For compounds 17-24, MD studies indicated that the quinazoline nitrogen atom at position 1- and the 2-sulfhydryl group were able to form bifurcated H-bonds with Glu30 in a manner which resembles that of MTX (3) interaction (Table 1). Besides, the 4-carbonyl oxygen of the quinazoline ring initially conferred trifurcated H-bonds with
The existence of the 3-benzyl function of the quinazoline derivatives 19 and 23 provides flexibility for rotation as compared with their 3-phenyl counterpart 18 and 22. The benzyl configuration resulted in improving the quality of binding and enhancing the
Figure 6: A-Flat-ribbon presentation of the lowest energy conformer of compound 17 docked in the hDHFR binding domain and the cofactor NADPH (C, gray; N, blue; O, red). B-Binding mode for compound 17 docked and minimized in the hDHFR binding pocket, showing residues involved in its recognition. Solid lines indicate the H-bond formation. Dashed lines indicate unstable H-bond.

The magnitude of binding-energy (Table 1). The moving of the 6-nitro functional group of compounds 19 and 23 to position 7- produced compounds 20 and 24, which were not able to perform H-bonding with Ser59. However, the quinazoline ring in 20 and 24 remained geometrically oriented to form the same H-bonds obtained with compounds 17-23. Methylation of the 2-sulfhydryl group of 17-19 produced compounds 21-23 with improved accommodation (electrostatic interaction) with the hydrophobic pocket, as evident by the magnitude of the binding energy achieved.
The conformational changes, due to the reduction of 6-nitro group of 22 and 23 into the 6-amino compounds 25 and 26 were studied. During the simulations for 25, the 6-amino group was finally oriented in the pocket cavity in such a way to form an H-bond with Asp21. Interestingly, compound 26 provided a new hydrogen bond with NADPH which was not stable by the end of simulation. These conformational changes allowed the 6-amino group to form H-bonding with Ser59 that was stable throughout the simulations. On the other hand, the 3-benzyl side chain performed rotation around -
Table 1: Binding data for the quinazoline analogues 17-27 docked at hDHFR active site and their H-bond interaction with amino acid residues.

![Quinazoline structure](image)

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^aE_{binding} = E_{complex} - (E_{ligand} + E_{enzyme}). ^b Glu30 performed two H-bonds with sulfur atom at position 2- and the 4-carbonyl oxygen. ^c Ile7 performed two H-bonds with the 4-carbonyl oxygen and nitrogen at position 3-. ^d Performed unstable H-bond with compounds 17-19 and 21-23 during simulations.
the CH₂- bond in a manner that permitted the nitrogen atom at position 3- to achieve an additional H-bond with Ile7. Collectively, these binding profiles for 26 led to an enhanced binding energy (Figure 7). Moving the amino function to position 7- produced compound 27 which missed the chance for recognition with Ser59, producing a relatively inferior binding energy (Table 1).

The presence of the 6-(4-methoxy-benzylidine)-amino moiety as in compounds 28 and 29, allowed its phenyl ring to rotate around the torsion angle. This privilege eventually enabled the 4-methoxy group to bind with Lys55 and Trp57 through a bifurcated H-bonding. Moreover, the other 5 H-bonds characteristic for the quinazoline ring were evidenced and remained stable throughout the simulations. The introduction of the 3-methoxy group, as in 30-33, provided two more additional H-bonds with the amino acid residues Thr56 and Asn64. However, the latter H-bond vanished by the end of the simulations. Compounds 32 and 33, which have a third methoxy group at position 5-, performed no additional hydrogen bonding (Table 2).

In another series of analogues, as exemplified by compounds 34 and 35, reduction of the azomethine function appears to have augmented the binding with the enzyme pocket. Thus, oxygen atom of the 4-methoxy group contributed a hydrogen bond with Lys55. The other 3-methoxy (36 and 37) and 5-methoxy groups (38 and 39) were, however, oriented too far from the amino acid residues to achieve H-bonding. In case of compounds 36 and 38, the presence of the 3-phenyl group provides a limited accommodation of these analogues (Table 3). Presence of 3-benzyl moiety, facilitate the conformational rotation to allow compounds 35, 37 and 39 to be accommodated within the enzyme cavity. Also, the 3-benzyl moiety provides lipophilic interaction with the surrounding amino acid residues giving enhanced energy level relative to the
Table 2: Binding data for the quinazoline analogues 28-33 docked at hDHFR active site and their H-bond interaction with amino acid residues.

![Chemical structure](image)

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$a E_{\text{binding}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{enzyme}})$.

$^b$ unstable H-bond during simulations.
corresponding 3-phenyl analogues 34, 36 and 38. In case of the 4-methoxy derivatives 34 and 35, the 4-methoxy function was recognized with Lys55 forming a H-bonding, but in case of the 3,4-dimethoxy analogues 36 and 37, or the 3,4,5-trimethoxy derivatives 38 and 39, the 3-methoxy function perform the H-bonding instead. It was noticed that the 3,4-dimethoxy analogues 36 and 37 always display more favorable binding energy rather than the corresponding 3,4,5-trimethoxy counterpart 38 and 39, (Table 3).

Comparing the performance of the 6-[N-(substituted benzyl)-amino]- (34-39) and the 6-[N-(substituted benzyl)-N-methyl-amino]-quinazoline analogues (40-45) revealed a favorable binding for the N-methylated derivatives due to the lipophilic interaction of this methyl group in the hydrophobic binding pocket of the enzyme. The quinazoline ring of those compounds behaved similarly to their counterparts in the previously discussed compounds. Compounds 40 and 41, owing to their 4-methoxy groups, performed a bifurcated H-bond with Asp21 and Lys55 amino acids. Compounds 42 and 43 have combined two favorable structural features, namely a 6-(N-methyl) - group and a 3, 4-dimethoxy group. Accordingly, the 3-methoxy oxygen bound with Thr56 and Trp57 via two stable H-bonds, whereas the 4-methoxy oxygen atom was hooked to phe58. Altogether, the 3, 4-dimethoxy substitutions contributed 3 H-bonds that also substantiated the interaction of these analogues with the active site of the enzyme as demonstrated by the binding energy (Table 3). In compounds 44 and 45, the binding profiles indicated that the 5-methoxy group had no chance to perform any hydrogen bonds with any of the surrounding residues (Figure 8).

Concerning compounds 46-51, the presence of 6-sulphonamido- group permitted bifurcated H-bonds with ser59, through their two oxygen atoms (Table 4). In addition,
Table 3: Binding data for the quinazoline analogues 34-45 docked at hDHFR active site and their H-bond interaction with amino acid residues.

![Chemical Structure](image-url)

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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>37</td>
<td>Bn</td>
<td>H</td>
<td>3,4-(OCH₃)₂</td>
<td>-45.0</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>38</td>
<td>Ph</td>
<td>H</td>
<td>3,4,5-(OCH₃)₃</td>
<td>-34.2</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>39</td>
<td>Bn</td>
<td>H</td>
<td>3,4,5-(OCH₃)₃</td>
<td>-39.9</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>Ph</td>
<td>CH₃</td>
<td>4-OCH₃</td>
<td>-42.0</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>41</td>
<td>Bn</td>
<td>CH₃</td>
<td>4-OCH₃</td>
<td>-54.9</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>42</td>
<td>Ph</td>
<td>CH₃</td>
<td>3,4-(OCH₃)₂</td>
<td>-59.3</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>43</td>
<td>Bn</td>
<td>CH₃</td>
<td>3,4-(OCH₃)₂</td>
<td>-64.5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>44</td>
<td>Ph</td>
<td>CH₃</td>
<td>3,4,5-(OCH₃)₃</td>
<td>-50.9</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>45</td>
<td>Bn</td>
<td>CH₃</td>
<td>3,4,5-(OCH₃)₃</td>
<td>-39.0</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

$E_{\text{binding}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{enzyme}})$.  
$^b$H-bond with the 4-methoxy oxygen in compounds 34, 36 and 38.  
$^c$H-bond with 3-methoxy oxygen in compounds 35, 37 and 39.  
$^d$Unstable H-bond during simulations.
all the triad residues (Ile7, Tyr121 & Val115) and Glu30 remained in a good H-bonding interaction with the quinazoline moiety. Further, the aryl and 4-substituted aryl group attached to the sulphonyl group served to enhance the aromatic/aromatic interaction with the hydrophobic enzyme pocket (Table 4).

Compounds 52 and 53 substituted with 6-ethyl-thioureido- groups were able to interact through nitrogen atom to afford a stable H-bond with Thr56 (Table 5). Analysis of the simulation data of compounds 56 and 57 indicated that the presence of the
6-benzyl-thioureido- group improved the accommodation of these ligands within the enzyme cavity and augmented their binding energy relative to the corresponding analogues that carry 6-phenyl-thioureido- substitution (54 and 55) or 6-ethyl-thioureido-substitution (52 and 53), (Table 5). All the ligands in this group were able to keep good recognition with Tyr121 and Val115 during simulations. However, the side chain of Ile7 amino acid was drifted away during simulations and lost its H-bond with the 4-carbonyl oxygen of the quinazoline ring.

Studying the docking and dynamics of compounds 58-63 indicated that the quinazoline ring was recognized by the key amino acid residues Glu30, Tyr121 and Val115, with absence of recognition with amino acid Ile7, (Table 6). The 6-benzamido-groups performed an additional H-bond with ser59. Introducing a 4-methoxy-benzamido- group (64 and 65), altered the rotation of the 3-benzyl moiety sitting, in

Table 4: Binding data for the quinazoline analogues 46-51 docked at hDHFR active site and their H-bond interaction with amino acid residues.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R₁</th>
<th>R₂</th>
<th>E_bind</th>
<th>H-bonds</th>
<th>Glu30</th>
<th>Ile7</th>
<th>Tyr121</th>
<th>Val115</th>
<th>Ser59</th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>Ph</td>
<td>H</td>
<td>-41.8</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>47</td>
<td>Bn</td>
<td>H</td>
<td>-51.8</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>48</td>
<td>Ph</td>
<td>Br</td>
<td>-56.5</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>49</td>
<td>Bn</td>
<td>Br</td>
<td>-48.6</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>50</td>
<td>Ph</td>
<td>CH₃</td>
<td>-49.6</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>51</td>
<td>Bn</td>
<td>CH₃</td>
<td>-46.9</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

\[ E_{binding} = E_{complex} - (E_{ligand} + E_{enzyme}). \]
such a way that enabled the oxygen of the 4-methoxy group to form an H-bond with Asp21. Additional 3-methoxy group (66 and 67) triggered another hydrogen bond with Lys55 (Figure 9). In compounds 68 and 69, the oxygen atoms of both 4- and 5-methoxy groups performed bifurcated hydrogen bonds with Asp21 (Table 6).

The overall outcome of this MD study revealed that: (i) quinazoline ring is an essential backbone for hDHFR inhibition as it carries the recognition feature with the key amino acid residues in the enzyme pocket. (ii) The presence of 2-sulphydryl or
Table 5: Binding data for the quinazoline analogues 52-57 docked at hDHFR active site and their H-bond interaction with amino acid residues.

![Quinazoline analogues structure diagram]

<table>
<thead>
<tr>
<th>Compd</th>
<th>R₁</th>
<th>R₂</th>
<th>( ^{a} E_{\text{bind}} )</th>
<th>H-bonds</th>
<th>Glu30</th>
<th>Ile⁷ᵇ</th>
<th>Tyr121</th>
<th>Val115</th>
<th>Thr56</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>Ph</td>
<td>Et</td>
<td>-38.4</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>53</td>
<td>Bn</td>
<td>Et</td>
<td>-42.0</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>54</td>
<td>Ph</td>
<td>Ph</td>
<td>-35.6</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>Bn</td>
<td>Ph</td>
<td>-37.8</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>56</td>
<td>Ph</td>
<td>Bn</td>
<td>-47.8</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>57</td>
<td>Bn</td>
<td>Bn</td>
<td>-50.8</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{a} E_{\text{binding}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{enzyme}})\). \(^{b}\) Unstable H-bond during simulations.

Table 6: Binding data for the quinazoline analogues 58-69 docked at hDHFR active site and their H-bond interaction with amino acid residues.

![Quinazoline analogues structure diagram]

<table>
<thead>
<tr>
<th>Compd</th>
<th>R₁</th>
<th>R₂</th>
<th>( ^{a} E_{\text{bind}} )</th>
<th>H-bonds</th>
<th>Glu30</th>
<th>Tyr121</th>
<th>Val115</th>
<th>Ser59</th>
<th>Asp21</th>
<th>Lys55</th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>Ph</td>
<td>H</td>
<td>-47.1</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>59</td>
<td>Bn</td>
<td>H</td>
<td>-39.6</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>60</td>
<td>Ph</td>
<td>4-Br</td>
<td>-69.9</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>61</td>
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</tr>
<tr>
<td>62</td>
<td>Ph</td>
<td>4-CH₃</td>
<td>-50.6</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>63</td>
<td>Bn</td>
<td>4-CH₃</td>
<td>-42.1</td>
<td>5</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>Ph</td>
<td>4-OCH₃</td>
<td>-58.9</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>65</td>
<td>Bn</td>
<td>4-OCH₃</td>
<td>-54.2</td>
<td>6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>66</td>
<td>Ph</td>
<td>3,4-(OCH₃)₂</td>
<td>-56.6</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>67</td>
<td>Bn</td>
<td>3,4-(OCH₃)₂</td>
<td>-54.5</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>68</td>
<td>Ph</td>
<td>3,4,5-(OCH₃)₃</td>
<td>-50.5</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>69</td>
<td>Bn</td>
<td>3,4,5-(OCH₃)₃</td>
<td>-53.7</td>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{a} E_{\text{binding}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{enzyme}})\).
2-methylthio functional group is important to mimic the 2-amino group of MTX (3). These groups interact with one of the key residue in the enzyme pocket (Glu30) to improve recognition and binding energy. (iii) The 3-benzyl group containing structures favor the binding and got the opportunity to be accommodated within the enzyme binding pocket without steric hindrance rather than the 3-phenyl counterparts. (iv) The 6-amino group containing compounds is more efficient in recognition with the surrounding amino acid rather than the 7-amino group counterparts, which loses the recognition with the active site residues. (v) The 3,4-dimethoxy function improves the binding performance with the active site residues, while the introduction of the third methoxy moiety at position 5- blunts the interaction with the enzyme pocket residues. (vi) The presence of 6-[N-(substituted benzyl)-amino]- augments the binding interaction within the enzyme pocket. Meanwhile, the presence of 6-[N-(substituted benzyl)-N-methyl-amino]- enhances the lipophilic interaction with the hydrophobic pocket.

In order to pursue these molecular modeling findings, it was decided to synthesize and test compounds 17-69. Details are in sections 4.2 and 4.3.
4.2 Chemistry

The synthetic strategy to synthesize the targets A–D (Figure 3) is depicted in schemes 1–3. Nitroanthranilic acids (16a,b) were allowed to react with ethyl, phenyl, and benzyl isothiocyanates to produce 2-thioxo-3-substituted-6 or 7-nitro-3H-quinazolin-4-ones (17–20) adopting reported procedure. The 2-thioxo function of 17–20 was then methylated using methyl iodide to give the S-methyl thioether derivatives 21–24. The 6- or 7-nitro function of 21–24 was subjected to metal reduction using Fe/HCl to afford the 6- or 7-amino compounds 25–27 (Scheme 1, Table 7).

The amino function of 25 and 26 was then reacted with 4-methoxy-, 3,4-dimethoxy-, and 3,4,5-trimethoxybenzaldehyde in boiling DMF to afford the benzylideneamino analogues 28–33 (Scheme 2, Table 8). The azomethine function in 28–33 was then subjected to NaBH₄ reduction to produce the secondary amines 34–39.

Scheme 1:
Table 7: Physicochemical properties and DHFR inhibition (IC$_{50}$, μM) of the newly synthesized compounds 17–27.

![Chemical structure image]

<table>
<thead>
<tr>
<th>Compd</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>Yield %</th>
<th>MP °C</th>
<th>Solvent</th>
<th>Molecular formula</th>
<th>m/z (%)</th>
<th>DHFR inhibition (IC$_{50}$, μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>Et</td>
<td>H</td>
<td>6-NO$_2$</td>
<td>69</td>
<td>269–71</td>
<td>EtOH/H$_2$O</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$O$_3$S</td>
<td>251 (100)</td>
<td>20.0</td>
</tr>
<tr>
<td>18</td>
<td>Ph</td>
<td>H</td>
<td>6-NO$_2$</td>
<td>78</td>
<td>&gt;300</td>
<td>EtOH</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$O$_3$S</td>
<td>299 (84)</td>
<td>30.0</td>
</tr>
<tr>
<td>19</td>
<td>Bn</td>
<td>H</td>
<td>6-NO$_2$</td>
<td>–</td>
<td>–</td>
<td>ref. No. 114</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Bn</td>
<td>H</td>
<td>7-NO$_2$</td>
<td>32</td>
<td>220–2</td>
<td>EtOH/H$_2$O</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$O$_3$S</td>
<td>313 (48)</td>
<td>30.0</td>
</tr>
<tr>
<td>21</td>
<td>Et</td>
<td>CH$_3$</td>
<td>6-NO$_2$</td>
<td>82</td>
<td>130–2</td>
<td>EtOH</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$O$_3$S</td>
<td>265 (30)</td>
<td>25.0</td>
</tr>
<tr>
<td>22</td>
<td>Ph</td>
<td>CH$_3$</td>
<td>6-NO$_2$</td>
<td>89</td>
<td>200–2</td>
<td>MeOH</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$O$_3$S</td>
<td>313 (100)</td>
<td>25.0</td>
</tr>
<tr>
<td>23</td>
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</tr>
<tr>
<td>24</td>
<td>Bn</td>
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<td>7-NO$_2$</td>
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<td>139–41</td>
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<td>327 (62)</td>
<td>30.0</td>
</tr>
<tr>
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<td>Ph</td>
<td>CH$_3$</td>
<td>6-NH$_2$</td>
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<td>175–6</td>
<td>EtOH/CHCl$_3$</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$OS</td>
<td>283 (100)</td>
<td>50.0</td>
</tr>
<tr>
<td>26</td>
<td>Bn</td>
<td>CH$_3$</td>
<td>6-NH$_2$</td>
<td>32</td>
<td>210–2</td>
<td>EtOH/CHCl$_3$</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$OS</td>
<td>297 (73)</td>
<td>10.0</td>
</tr>
<tr>
<td>27</td>
<td>Bn</td>
<td>CH$_3$</td>
<td>7-NH$_2$</td>
<td>36</td>
<td>160–2</td>
<td>EtOH/CHCl$_3$</td>
<td>C$<em>{16}$H$</em>{15}$N$_3$OS</td>
<td>297 (60)</td>
<td>50.0</td>
</tr>
</tbody>
</table>

*Analyzed for C, H, N; results are within ± 0.4% of the theoretical values for the formulae given.

Scheme 2:

![Scheme image]
**Table 8:** Physicochemical properties and DHFR inhibition (IC\(_{50}\), \(\mu\)M) of the newly synthesized compounds 28–33.

\[
\begin{array}{ccccccccc}
\text{Compd} & R_1 & R_2 & \text{Yield } % & \text{MP } ^\circ\text{C} & \text{Solvent} & \text{Molecular }^a \text{ formula} & \text{m/z } (\%) & \text{DHFR inhibition } (\text{IC}_{50}, \text{\(\mu\)M}) \\
28 & \text{Ph} & 4-\text{OCH} _3 & 75 & 220–2 & \text{DMF} & C_{25}H_{19}N_3O_2S & 401 (3) & 20.0 \\
29 & \text{Bn} & 4-\text{OCH} _3 & 62 & 109–10 & \text{DMF} & C_{25}H_{19}N_3O_2S & 415 (10) & 10.0 \\
30 & \text{Ph} & 3,4-(\text{OCH} _3) _2 & 59 & 118–20 & \text{DMF/H}_2\text{O} & C_{25}H_{21}N_3O_3S & 431 (6) & 50.0 \\
31 & \text{Bn} & 3,4-(\text{OCH} _3) _2 & 45 & 133–5 & \text{DMF/H}_2\text{O} & C_{25}H_{21}N_3O_3S & 445 (1) & 40.0 \\
32 & \text{Ph} & 3,4,5-(\text{OCH} _3) _3 & 67 & 113–5 & \text{DMF} & C_{25}H_{23}N_3O_3S & 461 (1) & 13.0 \\
33 & \text{Bn} & 3,4,5-(\text{OCH} _3) _3 & 73 & 188–90 & \text{DMF} & C_{26}H_{25}N_3O_3S & 475 (4) & 20.0 \\
\end{array}
\]

^a Analyzed for C, H, N; results are within ± 0.4% of the theoretical values for the formulae given.

The amino function of those secondary amines was then methylated using formaldehyde and NaCNBH\(_3\) to produce the N-methylated tertiary amines, 6-[N-(substituted benzyl)-N-methyl-amino]-3\(H\)-quinazolin-4-ones (40–45), (Scheme 2, Table 9).

The 2-methylthio-3-(phenyl or benzyl)-6-amino-3\(H\)-quinazolin-4-ones (25, 26) were reacted with a variety of substituted benzenesulphonyl chloride producing the 6-sulphonamido analogues 46–51 (Scheme 3, Table 10). The benzene sulphonamide moiety is carrying either 4-CH\(_3\) or 4-Br functions that represent electron donating and electron withdrawing groups, respectively; in order to evaluate their electronic effects on biological activity. The amino function of 25, 26 was also reacted with ethyl, phenyl or benzyl isothiocyanates to yield the 6-thioureido analogues 52–57 (Scheme 3, Table 10). Compounds 25, 26 were reacted with a variety of substituted benzoyl chlorides to produce the 6-acylamino-derivatives 58–69, adopting the same reaction conditions used to prepare the 6-sulphonamido analogues 46–51 (Scheme 3, Table 10). Structure elucidation of the synthesized intermediates and final products was attained by the aid of elementary analyses (C, H, N), NMR, and mass spectrometry.
Table 9: Physicochemical properties and DHFR inhibition (IC_{50}, μM) of the newly synthesized compounds 34–45.

<table>
<thead>
<tr>
<th>Compd</th>
<th>R_1</th>
<th>R_2</th>
<th>R_3</th>
<th>Yield %</th>
<th>MP °C</th>
<th>Solvent</th>
<th>Molecular (^a ) formula</th>
<th>m/z (%)</th>
<th>DHFR inhibition (IC_{50}, μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>Ph</td>
<td>H</td>
<td>4-OCH(_3)</td>
<td>48</td>
<td>120–2</td>
<td>EtOH/Hex</td>
<td>C_{23}H_{21}N_{3}O_{2}S</td>
<td>403 (9)</td>
<td>25.0</td>
</tr>
<tr>
<td>35</td>
<td>Bn</td>
<td>H</td>
<td>4-OCH(_3)</td>
<td>72</td>
<td>131–3</td>
<td>EtOH/Hex</td>
<td>C_{24}H_{23}N_{3}O_{2}S</td>
<td>417 (7)</td>
<td>10.0</td>
</tr>
<tr>
<td>36</td>
<td>Ph</td>
<td>H</td>
<td>3,4-(OCH(_3))(_2)</td>
<td>65</td>
<td>188–90</td>
<td>EtOH/Hex</td>
<td>C_{24}H_{23}N_{3}O_{2}S</td>
<td>433 (11)</td>
<td>20.0</td>
</tr>
<tr>
<td>37</td>
<td>Bn</td>
<td>H</td>
<td>3,4-(OCH(_3))(_2)</td>
<td>53</td>
<td>174–5</td>
<td>EtOH/Hex</td>
<td>C_{24}H_{23}N_{3}O_{2}S</td>
<td>447 (14)</td>
<td>15.0</td>
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<tr>
<td>38</td>
<td>Ph</td>
<td>H</td>
<td>3,4,5-(OCH(_3))(_2)</td>
<td>81</td>
<td>138–40</td>
<td>EtOH/Hex</td>
<td>C_{24}H_{23}N_{3}O_{2}S</td>
<td>463 (10)</td>
<td>30.0</td>
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<tr>
<td>39</td>
<td>Bn</td>
<td>H</td>
<td>3,4,5-(OCH(_3))(_2)</td>
<td>85</td>
<td>152–4</td>
<td>EtOH/Hex</td>
<td>C_{24}H_{23}N_{3}O_{2}S</td>
<td>477 (3)</td>
<td>30.0</td>
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<tr>
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<td>CH(_3)</td>
<td>4-OCH(_3)</td>
<td>64</td>
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<td>CHCl(_3)/Hex</td>
<td>C_{25}H_{25}N_{3}O_{2}S</td>
<td>417 (6)</td>
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<td>CHCl(_3)/Hex</td>
<td>C_{25}H_{25}N_{3}O_{2}S</td>
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<td>CH(_3)</td>
<td>3,4-(OCH(_3))(_2)</td>
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<td>CHCl(_3)/Hex</td>
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</tr>
<tr>
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<td>72–5</td>
<td>CHCl(_3)/Hex</td>
<td>C_{25}H_{25}N_{3}O_{2}S</td>
<td>491 (4)</td>
<td>10.0</td>
</tr>
</tbody>
</table>

\(^a\) Analyzed for C, H, N; results are within ± 0.4% of the theoretical values for the formulae given.

Scheme 3:
Table 10: Physicochemical properties and DHFR inhibition (IC$_{50}$, μM) of the newly synthesized compounds 46–69.

![Chemical structures](image)

<table>
<thead>
<tr>
<th>Compd</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>Yield %</th>
<th>MP °C</th>
<th>Solvent</th>
<th>Molecular a formula</th>
<th>m/z (%)</th>
<th>DHFR inhibition (IC$_{50}$, μM)</th>
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<tr>
<td>46</td>
<td>Ph</td>
<td>H</td>
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<td>112–4</td>
<td>MeOH</td>
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<td>423 (2)</td>
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<tr>
<td>47</td>
<td>Bn</td>
<td>H</td>
<td>86</td>
<td>188–90</td>
<td>EtOH</td>
<td>C$<em>{22}$H$</em>{19}$N$_5$O$_3$S$_2$</td>
<td>437 (4)</td>
<td>10.0</td>
</tr>
<tr>
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<td>Ph</td>
<td>Br</td>
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<td>240–2</td>
<td>EtOH/H$_2$O</td>
<td>C$<em>{21}$H$</em>{16}$BrN$_5$O$_3$S$_2$</td>
<td>502 (1)</td>
<td>2.0</td>
</tr>
<tr>
<td>49</td>
<td>Bn</td>
<td>Br</td>
<td>79</td>
<td>258–60</td>
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<td>88</td>
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<tr>
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<td>Bn</td>
<td>Et</td>
<td>89</td>
<td>169–70</td>
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<td>Ph</td>
<td>93</td>
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<td>CHCl$_3$/EtOH</td>
<td>C$<em>{22}$H$</em>{19}$N$_5$O$_3$S$_2$</td>
<td>418 (4)</td>
<td>5.0</td>
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<tr>
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<td>195–7</td>
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<tr>
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<td>Bn</td>
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<td>CHCl$_3$/EtOH</td>
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<td>432 (1)</td>
<td>10.0</td>
</tr>
<tr>
<td>57</td>
<td>Bn</td>
<td>Bn</td>
<td>81</td>
<td>203–5</td>
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<td>C$<em>{24}$H$</em>{21}$N$_5$O$_3$S$_2$</td>
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<td>8.0</td>
</tr>
<tr>
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<td>Ph</td>
<td>H</td>
<td>65</td>
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<td>EtOAc</td>
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<td>387 (6)</td>
<td>20.0</td>
</tr>
<tr>
<td>59</td>
<td>Bn</td>
<td>H</td>
<td>58</td>
<td>192–5</td>
<td>EtOAc</td>
<td>C$<em>{23}$H$</em>{19}$N$_2$OS$_2$</td>
<td>401 (8)</td>
<td>15.0</td>
</tr>
<tr>
<td>60</td>
<td>Ph</td>
<td>4-Br</td>
<td>73</td>
<td>&gt; 300</td>
<td>CHCl$_3$/Hex</td>
<td>C$<em>{22}$H$</em>{18}$BrN$_2$OS$_2$</td>
<td>466 (2)</td>
<td>50.0</td>
</tr>
<tr>
<td>61</td>
<td>Bn</td>
<td>4-Br</td>
<td>86</td>
<td>&gt; 300</td>
<td>CHCl$_3$/Hex</td>
<td>C$<em>{23}$H$</em>{19}$BrN$_2$OS$_2$</td>
<td>480 (1)</td>
<td>8.0</td>
</tr>
<tr>
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<td>Ph</td>
<td>4-CH$_3$</td>
<td>78</td>
<td>125–7</td>
<td>DMF</td>
<td>C$<em>{23}$H$</em>{20}$N$_2$OS$_2$</td>
<td>401 (4)</td>
<td>10.0</td>
</tr>
<tr>
<td>63</td>
<td>Bn</td>
<td>4-CH$_3$</td>
<td>82</td>
<td>238–40</td>
<td>DMF</td>
<td>C$<em>{24}$H$</em>{21}$N$_2$OS$_2$</td>
<td>415 (1)</td>
<td>7.0</td>
</tr>
<tr>
<td>64</td>
<td>Ph</td>
<td>4-OCH$_3$</td>
<td>90</td>
<td>214–6</td>
<td>DMF</td>
<td>C$<em>{23}$H$</em>{18}$N$_2$OS$_2$</td>
<td>417 (1)</td>
<td>70.0</td>
</tr>
<tr>
<td>65</td>
<td>Bn</td>
<td>4-OCH$_3$</td>
<td>64</td>
<td>105–7</td>
<td>DMF</td>
<td>C$<em>{24}$H$</em>{19}$N$_2$OS$_2$</td>
<td>431 (7)</td>
<td>10.0</td>
</tr>
<tr>
<td>66</td>
<td>Ph</td>
<td>3,4-(OCH$_3$)$_2$</td>
<td>65</td>
<td>148–50</td>
<td>CHCl$_3$/EtOH</td>
<td>C$<em>{24}$H$</em>{20}$N$_2$OS$_2$</td>
<td>447 (2)</td>
<td>40.0</td>
</tr>
<tr>
<td>67</td>
<td>Bn</td>
<td>3,4-(OCH$_3$)$_2$</td>
<td>78</td>
<td>202–3</td>
<td>CHCl$_3$/EtOH</td>
<td>C$<em>{25}$H$</em>{21}$N$_2$OS$_2$</td>
<td>461 (3)</td>
<td>8.0</td>
</tr>
<tr>
<td>68</td>
<td>Ph</td>
<td>3,4,5-(OCH$_3$)$_3$</td>
<td>82</td>
<td>238–40</td>
<td>CHCl$_3$/EtOH</td>
<td>C$<em>{26}$H$</em>{22}$N$_2$OS$_2$</td>
<td>477 (6)</td>
<td>15.0</td>
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<tr>
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<td>Bn</td>
<td>3,4,5-(OCH$_3$)$_3$</td>
<td>87</td>
<td>115–17</td>
<td>CHCl$_3$/EtOH</td>
<td>C$<em>{27}$H$</em>{23}$N$_2$OS$_2$</td>
<td>491 (5)</td>
<td>12.0</td>
</tr>
</tbody>
</table>

a Analyzed for C, H, N; results are within ± 0.4% of the theoretical values for the formulae given.
4.3 Biological Investigations

The synthesized compounds were evaluated for their in vitro DHFR inhibition activity at the Department of Pharmacology, College of Pharmacy, King Saud University and for their in vitro antitumor activity at the National Cancer Institute (NCI), Bethesda, Maryland, USA.

4.3.1 Dihydrofolate Reductase (DHFR) Inhibition Assay

The potency of the synthesized compounds (17–69) as inhibitors of bovine liver DHFR enzyme was evaluated using reported procedure. Bovine liver DHFR is 70% homologous to hDHFR. The used assay was carried out in Tris-HCl buffer (pH 7.4) and NADPH. Test compounds were dissolved in DMSO, and bovine liver DHFR was added. The mixture was incubated at room temperature, then FH2, the substrate of the enzyme, was also added. The change in absorbance at 340 nm was followed using MTX (3) as a positive control (IC50 0.006 μM, Figure 10). Results are reported as IC50 values (see Tables 7–10).

Compounds 28, 40–44, 46, 48 proved to be the most active inhibitors of DHFR in this investigation with IC50 values of 2.0, 1.0, 0.5, 0.4, 0.4, 1.0, 2.0, and 2.0 μM, respectively. Compounds 49, 51, 54, 57, 61, 63, 67 showed moderate inhibition activity with IC50 values of 5.0, 7.0, 5.0, 8.0, 8.0, 7.0 and 8.0 μM, respectively; while compounds 29, 35, 45, 47, 50, 55, 56, and 62 showed an equal IC50 value of 10 μM. Compounds 25, 30, 52, 60, and 64 proved to be the least effective members in this investigation with IC50 values of 50–70 μM.

Structure activity correlation of the obtained results revealed that, in the 6-nitro series (17–24), the 2-SH or 2-SCH3 moieties do not really affect the magnitude of activity as evidenced by the IC50 values of the SH containing compound 17–20 and their SCH3 counterpart 21–24. All showed IC50 values range of 20.0–30.0 μM. Reduction of
the 6-nitro function into the 6-amino analogues resulted in controversial figures of inhibition. In the 3-phenyl analogues, the activity decreased by 2-fold \((22, 25.0 \, \mu M \textit{versus} \ 25, 50.0 \, \mu M)\) upon reduction of the 6-nitro function; while in the 3-Benzyl series, the activity increased by 2.5-fold \((23, 25.0 \, \mu M \textit{versus} \ 26, 10.0 \, \mu M)\), in accordance with the molecular modeling predictions. In most cases, moving the 6-nitro group into position 7- did not markedly affect the magnitude of activity. While the translocation of the 6-amino moiety to position 7- reduced the activity noticeably \((26, 10.0 \, \mu M \textit{versus} \ 27, 50.0 \, \mu M)\).

In the 6-benzylidinamino series \((28\textendash33)\), the combination of bearing a phenyl group at position 3- and 4-methoxy-benzylidinamino at position 6- proved to remarkably enhance the activity; as shown in compound 28 with IC\(_{50}\) value of 2.0 \, \mu M. Replacing the 3-phenyl group by 3-benzyl moiety decreased the activity by 5-fold \((29, 10.0 \, \mu M)\). The introduction of 3,4-dimethoxy-phenyl replacing the 4-methoxy-phenyl
moiety decreased the activity by 25-fold (28, 2.0 μM versus 30, 50.0 μM), while the 3,4,5-trimethoxy-phenyl moiety restored some of the DHFR inhibitory potency as shown in compounds 32 and 33 (IC₅₀ 13.0 and 20.0 μM, respectively).

In general, reduction of the 6-benzylideneamino function into the 6-benzylamino analogues (34–39), showed a slight improvement in DHFR inhibition activity. Upon methylation, using formaldehyde and NaCNBH₃ to produce the methotrexate (MTX, 3) like derivatives (40–45), the activity increased dramatically producing the most active members in this study (compounds 40–44), which showed IC₅₀ values of 1.0, 0.5, 0.4, 0.4, and 1.0 μM, respectively in accordance with the molecular modeling predictions. In this group, the 3-phenyl substitution proved to favor the activity, rather than the 3-benzyl, as shown in compound 44 (IC₅₀, 1.0 μM) and the 10-fold decrease in potency in compound 45 (IC₅₀, 10.0 μM).

In the 4-substituted benzenesulphonamide series (46–51), unsubstitution or the presence of 4-bromo moiety favored the activity producing compounds 46 and 48 with IC₅₀ value of 2.0 μM for both compounds. The existence of 4-methyl group decreased the activity by 5-fold as shown in compound 50 (IC₅₀ value of 10.0 μM). In the 6-thioureido series (52–57), aromatic substitution on the thioureido moiety favored the activity rather than the aliphatic substitution. The N-ethyl thioureido substitution proved to be 10-fold less active than the N-phenyl analogue, as seen in compounds 52 (50.0 μM) and 54 (5.0 μM).

In the 6-benzamido series (58–69), the 3-benzyl substitution as in 61, 65, and 67 with IC₅₀ values 8.0, 10.0 and 8.0 μM, respectively, favored the activity rather than the 3-phenyl analogues as in 60, 64, and 66 with IC₅₀ values of 50.0, 70.0, and 40.0 μM.
Table 11: *In vitro* three cell lines primary antitumor assay\(^a\) result of the synthesized compounds.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Lung NCI-H460</th>
<th>Breast MCF7</th>
<th>CNS SF-268</th>
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<tbody>
<tr>
<td>26</td>
<td>61</td>
<td>54</td>
<td>64</td>
</tr>
<tr>
<td>27(^b)</td>
<td>22</td>
<td>62</td>
<td>74</td>
</tr>
<tr>
<td>29</td>
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<td>103</td>
</tr>
<tr>
<td>31(^b)</td>
<td>7</td>
<td>46</td>
<td>74</td>
</tr>
<tr>
<td>33</td>
<td>41</td>
<td>62</td>
<td>84</td>
</tr>
<tr>
<td>35</td>
<td>72</td>
<td>109</td>
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</tr>
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<td>99</td>
</tr>
<tr>
<td>43(^b)</td>
<td>7</td>
<td>60</td>
<td>84</td>
</tr>
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<td>65</td>
<td>82</td>
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<tr>
<td>49</td>
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<td>110</td>
</tr>
<tr>
<td>53(^b)</td>
<td>11</td>
<td>50</td>
<td>74</td>
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<td>55</td>
<td>52</td>
<td>55</td>
<td>72</td>
</tr>
<tr>
<td>59(^b)</td>
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<td>18</td>
</tr>
<tr>
<td>60</td>
<td>85</td>
<td>91</td>
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</tr>
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<tr>
<td>69</td>
<td>75</td>
<td>70</td>
<td>91</td>
</tr>
</tbody>
</table>

\(^a\)Compounds which reduce the growth of the three selected cell lines to 32% or less are passed on for evaluation in the full panel of 60 cell lines. \(^b\)Active compounds.

### 4.3.2 Antitumor Testing

The synthesized compounds were subjected to the NCI’s *in vitro*, one dose primary anticancer assay, using a 3-cell line panel consisting of MCF-7 (breast), NCI-H460 (lung), and SF-268 (CNS) cancers. Compounds which reduce the growth of any one of the cell lines to 32% or less are passed on for evaluation in the full panel of 60 cell lines over a 5-log dose range.\(^{116–118}\) Three response parameters, median growth inhibition (\(\text{GI}_{50}\)), total growth inhibition (\(\text{TGI}\)), and median lethal concentration (\(\text{LC}_{50}\)) were calculated for each cell line,\(^{119}\) using the known drug Melphalan as a positive control. The NCI antitumor drug discovery screen has been designed to distinguish between broad-spectrum antitumor and tumor or subpanel-selective compounds.\(^{119}\)
In the present study, compounds 27, 31, 43, 53, and 59 passed primary anticancer assay at an arbitrary concentration of 100 μM (Table 11). Consequently, those active compounds were carried over and tested against a panel of 60 different tumor cell lines. The tested quinazoline analogues showed a distinctive potential pattern of selectivity as well as a broad-spectrum antitumor activity.

With regard to sensitivity against individual cell lines, compound 31 showed GI₅₀ effectiveness against leukemia CCRF-CEM and SR; melanoma SK-MEL-5 cell lines at concentrations of 1.0, 0.2, and 2.0 μM, respectively. Compound 43 showed GI₅₀ activity against leukemia RPMI-8226, SR; melanoma SK-MEL-5 cell lines at concentrations of 2.9, 1.6, and 2.9 μM, respectively. Compound 59 proved to be active at GI₅₀ level against colon HCT-116, ovarian OVCAR-3, and renal SN12C cell lines at concentrations of 3.3, 4.3, and 2.4 μM, respectively (Table 12).

With regard to broad spectrum antitumor activity, the tested compounds showed GI₅₀, TGI, and LC₅₀ (MG-MID) values < 100 μM, against leukemia, non-small cell lung, colon, CNS, melanoma, ovarian, renal, prostate, and breast cancer subpanel cell lines. Compounds 27 and 43 showed (MG-MID) values < 100 μM at only the GI₅₀ and TGI levels, while compounds 31, 53 and 59 showed potency at the three levels of activity GI₅₀, TGI and LC₅₀ (Tables 13 and 14). Compound 59 proved to be the most active member in this study with GI₅₀, TGI and LC₅₀ values of 9.1, 39.5, 78.1 μM, respectively.

Structure activity correlation of the obtained results revealed that the 7-amino group of 27 (GI₅₀, TGI – 34.4, 90.4 μM, respectively) favored the antitumor activity rather than the 6-amino analogue 26. Also, compound 31 (GI₅₀, TGI, LC₅₀ – 20.01,
### Table 12: Growth inhibitory and lethal concentrations (GI\textsubscript{50}, TGI\textsubscript{50}, LC\textsubscript{50}) of some selected \textit{in vitro} cell lines (\textmu{}M).\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cell line</th>
<th>27</th>
<th>31</th>
<th>43</th>
<th>53</th>
<th>59</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>GI\textsubscript{50}</td>
<td>TGI</td>
<td>LC\textsubscript{50}</td>
<td>GI\textsubscript{50}</td>
<td>TGI</td>
</tr>
<tr>
<td>Leukemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>31.3</td>
<td>76.0</td>
<td>b</td>
<td>1.0</td>
<td>5.3</td>
</tr>
<tr>
<td>RPMI-8226</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>17.8</td>
<td>37.2</td>
</tr>
<tr>
<td>SR</td>
<td>nt</td>
<td>nt</td>
<td>nt</td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Non-small cell lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H322M</td>
<td>36.6</td>
<td>b</td>
<td>b</td>
<td>36.7</td>
<td>b</td>
</tr>
<tr>
<td>HOP-92</td>
<td>27.4</td>
<td>b</td>
<td>b</td>
<td>7.7</td>
<td>44.1</td>
</tr>
<tr>
<td>Colon</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCT-116</td>
<td>b</td>
<td>b</td>
<td>b</td>
<td>31.5</td>
<td>b</td>
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<td>Melanoma</td>
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<tr>
<td>SK-MEL-5</td>
<td>19.8</td>
<td>54.0</td>
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<td>2.0</td>
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<tr>
<td>IGORV1</td>
<td>79.3</td>
<td>b</td>
<td>b</td>
<td>12.4</td>
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<td>OVCAR-3</td>
<td>40.1</td>
<td>b</td>
<td>b</td>
<td>26.2</td>
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<tr>
<td>Renal</td>
<td></td>
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<tr>
<td>786-0</td>
<td>53.9</td>
<td>b</td>
<td>b</td>
<td>25.9</td>
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<td>ACHN</td>
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<td>b</td>
<td>15.3</td>
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<tr>
<td>RXF-393</td>
<td>38.6</td>
<td>73.7</td>
<td>b</td>
<td>26.8</td>
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<tr>
<td>SN12C</td>
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<td>b</td>
<td>b</td>
<td>20.3</td>
<td>90.4</td>
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<tr>
<td>TK10</td>
<td>29.2</td>
<td>97.8</td>
<td>b</td>
<td>39.6</td>
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<tr>
<td>Breast</td>
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<td>MCF7</td>
<td>29.0</td>
<td>b</td>
<td>b</td>
<td>35.6</td>
<td>b</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>44.8</td>
<td>b</td>
<td>b</td>
<td>7.5</td>
<td>50.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Data obtained from NCI’s \textit{in vitro} disease oriented human tumor cell screen. \textsuperscript{b}Value > 100 \textmu{}M. nt, compound not tested.
Table 13: Median growth inhibitory concentration (GI₅₀, μM) of in vitro subpanel tumor cell lines.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Subpanel tumor cell lines</th>
<th>MG-MID&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>27</td>
<td>27.7</td>
<td>44.6</td>
</tr>
<tr>
<td>31</td>
<td>12.5</td>
<td>26.4</td>
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<tr>
<td>43</td>
<td>8.7</td>
<td>48.2</td>
</tr>
<tr>
<td>53</td>
<td>15.8</td>
<td>29.5</td>
</tr>
<tr>
<td>59</td>
<td>6.7</td>
<td>18.4</td>
</tr>
<tr>
<td>Melphalan</td>
<td>20.1</td>
<td>38.5</td>
</tr>
</tbody>
</table>

<sup>a</sup>I, leukemia; II, non-small cell lung cancer; III, colon cancer; IV, CNS cancer; V, melanoma; VI, ovarian cancer; VII, renal cancer; VIII, prostate cancer; IX, breast cancer. <sup>b</sup>GI₅₀ full panel mean-graph midpoint (μM).

Table 14: Total growth inhibitory concentration (TGI, μM) of in vitro subpanel tumor cell lines.

<table>
<thead>
<tr>
<th>Compd</th>
<th>Subpanel tumor cell lines&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MG-MID&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>27</td>
<td>76.2</td>
<td>92.3</td>
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<td>31</td>
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<td>80.5</td>
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<tr>
<td>43</td>
<td>c</td>
<td>89.7</td>
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<td>53</td>
<td>59.2</td>
<td>90.5</td>
</tr>
<tr>
<td>59</td>
<td>c</td>
<td>50.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>For subpanel tumor cell lines, see footnote a of Table 13. <sup>b</sup>TGI full panel mean-graph midpoint (μM). <sup>c</sup>Compound showed values > 100 μM. <sup>d</sup>Median lethal concentrations (LC₅₀, μM) are shown in parentheses, Melphalan TGI and LC₅₀ values are 35.3 and 65.5 μM, respectively.

68.0, 97.8 μM, respectively) with 3,4-dimethoxyphenyl fragment in which the number and setting of the methoxy groups proved crucial for activity. The 4-methoxy 29 and the 3,4,5-tri-methoxy 33 counterpart proved inactive. Compound 53 (GI₅₀, TGI, and LC₅₀ – 26.7 74.7, 97.8 μM, respectively) with its 6-ethylthioureido function reversed what have been discussed earlier (see Section 4.3.1) about the essentiality of aliphatic substitution rather than aromatic substitution on the thioureido function. Aromatic substitution produced inactive compounds as seen in case of 55 and 57. The 6-benzamido analogue 59 (GI₅₀, TGI, LC₅₀ – 9.1, 39.5, 78.1 μM, respectively) represents another example of the effect of substituent on biological activity in this study. Introduction of the electron
withdrawing bromine atom to 59 or electron donating methyl function produced compounds 61 and 63, respectively with total loss of activity.

5. Conclusion

As can be concluded from the obtained results, compounds 2-methylthio-3-benzyl-6-[N-(4-methoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (41), 2-methylthio-3-phenyl-6-[N-(3, 4-dimethoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (42), and 2-methylthio-3-benzyl-6-[N-(3, 4-dimethoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (43) are the most active members of this study as DHFR inhibitors with IC50 values of 0.5, 0.4, and 0.4 uM, respectively, Figure 11.

Compounds 2-methylthio-3-benzyl-6-(3, 4-dimethoxy-benzylidineamino)-3H-quinazolin-4-one (31), with GI50, TGI, LC50 values of 20.1, 68.0, 97.8 uM,

![Molecules](image)

**Figure 11:** Structures of the active DHFR inhibitors.
respectively; 2-methylthio-3-benzyl-6-[N-(3, 4-dimethoxy-benzyl)-N-methyl-amino]-3H-quinazolin-4-one (43), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 23.5, 83.1, \textless 100.0 \text{ uM}, respectively; N-ethyl-N’-(2-methylthio-3-benzyl-3H-quinazolin-4-one-6-yl)thiourea (53), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 26.7, 74.7, 97.8 \text{ uM}, respectively; and 2-methylthio-3-benzyl-6-(phenylcarbonylamino)-3H-quinazolin-4-one (59), with GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 9.1, 39.5, 78.1 \text{ uM}, respectively; are the most active antitumor agents in this study (Figure 12), as compared with the known antitumor drug ‘Melphalan’ (GI\textsubscript{50}, TGI, LC\textsubscript{50} values of 27.1, 35.3, 65.5 \text{ uM}, respectively). It seemed that compound 43 exerts its antitumor potency through DHFR inhibition mode of action, while the other active compounds, namely 31, 53, and 59, might exert their antitumor potency through DHFR inhibition and/or some other mechanism of action.

Figure 12: Structures of the active antitumor agents.

These studied quinazoline analogues could be considered as useful templates for future development and further derivatization or modification to obtain more potent DHFR inhibitors.
6. Experimental

All modeling experiments were conducted with Hyperchem 6.03 package from Hypercube and PowerFit1.0 program from “Micro Simulations” running on a PC computer.\textsuperscript{113, 120} The docking of the candidates into hDHFR pocket was performed with PowerFit software. Starting coordinate of hDHFR enzyme in tertiary complex with reduced-nicotinamide adenine dinucleotide phosphate (NADPH) and MTX (3), code ID 1DLS, was obtained from the Protein Data Bank of Brookhaven National Laboratory (Figure 5).\textsuperscript{121}

Melting points (°C) were determined on Mettler FP80 melting point apparatus and are uncorrected. Microanalyses were performed on a Perkin Elmer 240 elemental analyzer at the Central Research Laboratory, College of Pharmacy, King Saud University. All of the new compounds were analyzed for C, H, and N and agreed with the proposed structures within ±0.4% of the theoretical values. \textsuperscript{1}H and \textsuperscript{13}C-NMR spectra were recorded on a Varian XL 500 MHz FT spectrometer, chemical shifts are expressed in δ ppm with reference to TMS. Mass spectral (MS) data were obtained on a Shimadzu GC/MS QP 5000 apparatus. Thin layer chromatography was performed on precoated (0.25 mm) silica gel GF\textsubscript{254} plates (E. Merck, Germany), compounds were detected with 254 nm UV lamp. Silica gel (60–230 mesh) was employed for routine column chromatography separations. DHFR inhibition activity experiments were performed at Pharmacology Department, College of Pharmacy, King Saud University. Bovine liver DHFR enzyme, and methotrexate (MIX, 3) were used in the assay (Sigma Chemical Co, USA). Antitumor activity was performed at the National Cancer Institute (NCI), Bethesda, Maryland, USA. Compounds 19 and 23 were previously reported.\textsuperscript{114}
6.1 Molecular Dynamic Calculations

6.1.1 Molecular Structure of the Synthesized Quinazolines

The quinazoline analogues 17-69 were constructed from fragment libraries in the Hyperchem program followed by energy minimization using the "Amber force field". The partial atomic charges for each analogue were assigned with the semiempirical mechanical calculation method “AM1” implemented in Hyperchem 6.03. Conformational search was performed around all the rotatable bonds with an increment of $10^0$ using conformational search module as implemented in HyperChem 6.03 All the conformers were minimized until the RMS deviation was 0.01 Kcal/mol Å.

6.1.2 Docking and Molecular Dynamic Simulations

Lowest energy conformer of each new analogue “global-minima” was docked into the hDHFR enzyme-binding domain. For each of the quinazoline analogues, energy minimizations (EM) were performed using 1000 steps of steepest descent, followed by conjugate gradient minimization to a RMS energy gradient of 0.01 Kcal/mol Å. The active site of the enzyme was defined using a radius of 8.0 Å around MTX. MD simulations were carried out for each ligand, with the rest of the enzyme kept fixed. The cofactor (NADPH) as a part of the enzyme structure was not fixed during the simulations. MD simulation was performed using time steps of 0.001 pico-second (ps), a distance dependant dielectric of 4.00, and a non-bonded cut-off distance of 8.0 Å, at 300 K. Complexes were first equilibrated for 10 ps and then simulated for 40 ps. Trajectory frames were collected every 200 steps for detailed analysis on the basis of potential energy and hydrogen bond interactions. These selected frames were minimized until RMS deviation values of 0.01 kcal/mol Å were reached for the active-site residues. Energy of binding was calculated as the difference between the energy of the complex and individual energies of the enzyme and ligand:
$E_{\text{binding}} = E_{\text{Complex}} - (E_{\text{Ligand}} + E_{\text{enzyme}})$.

where $E_{\text{Complex}}$ is the energy of the ligand-enzyme complex, $E_{\text{Ligand}}$ is the energy of the ligand corresponding to the binding conformation and $E_{\text{enzyme}}$ is the energy of the enzyme.\textsuperscript{122}

6.2 Synthesis

6.2.1 2-Thioxo-3-substituted-6 or 7-nitro-3$H$-quinazolin-4-ones (17,18,20).

A mixture of 4- or 5-nitroanthranilic acid (16a,b, 1.82 g, 0.01 mol), the appropriate isothiocyanate derivative (0.012 mol), and TEA (2 mL) in ethanol (50 mL) was heated under reflux for 4 h. The reaction mixture was then cooled and the solvent was evaporated under vacuum. The obtained residue was washed with petroleum ether 40:60, filtered, dried and recrystallized to give 17, 18 and 20 (Table 1). $^1$H NMR, 17 (CDCl$_3$): $\delta$ 1.24 (t, 3H, $J = 7.0$ Hz, CH$_3$CH$_2$), 3.35 (brrs, 1H, SH), 4.41–4.45 (q, 2H, $J = 7.0$ Hz, CH$_3$CCH$_2$), 7.49 (d, 1H, $J = 9.0$ Hz, ArH), 8.50–8.53 (dd, 1H, $J = 9.0$, 3.0 Hz, ArH), 8.60 (d, 1H, $J = 3.0$ Hz, ArH), 18 (DMSO-d$_6$): $\delta$ 3.07 (brrs, 1H, SH), 6.80 (d, 1H, $J = 9.0$ Hz, ArH), 7.30–7.58 (m, 5H, ArH), 7.98–7.99 (dd, 1H, $J = 9.0$, 3.0 Hz, ArH), 8.62 (d, 1H, $J = 3.0$ Hz, ArH). 20 (DMSO-d$_6$): $\delta$ 3.35 (s, 1H, SH), 5.34 (s, 2H, CH$_2$Ph), 8.15–8.17 (dd, 1H, $J = 9.0$, 2.0 Hz, ArH), 8.24 (d, 1H, $J = 2.0$ Hz, ArH), 8.31 (d, 1H, $J = 9.0$ Hz, ArH).

6.2.2 2-Methylthio-3-substituted-6 or 7-nitro-3$H$-quinazolin-4-ones (21, 22, 24).

A mixture of the 2-thioxo-quinazoline analogues 17, 18, or 20 (0.01 mol), methyl iodide (5 mL) and anhydrous potassium carbonate (2 g) in acetone (50 mL) was heated under reflux for 8 h. The separated solid was filtered while hot, washed with acetone, the filtrate was then evaporated and the obtained residue was dried and recrystallized to give
21, 22 and 24 (Table 1). $^1$H NMR (DMSO), 21: $\delta$ 1.29 (t, 3H, $J = 7.0$ Hz, CH$_3$CH$_2$), 2.67 (s, 3H, SCH$_3$), 4.08–4.12 (q, 2H, $J = 7.0$ Hz, CH$_3$CH$_2$), 7.64 (d, 1H, $J = 9.0$ Hz, ArH), 8.46 (d, 1H, $J = 9.0$ Hz, ArH), 8.68 (s, 1H, ArH). 22: $\delta$ 2.51 (s, 3H, SCH$_3$), 7.49–7.60 (m, 5H, ArH), 7.61 (d, 1H, $J = 9.0$ Hz, ArH), 8.52–8.55 (m, 1H, ArH), 8.71 (s, 1H, ArH). 24: $\delta$ 2.64 (s, 3H, SCH$_3$), 5.36 (s, 2H, CH$_2$Ph), 7.29–7.36 (m, 5H, ArH), 8.19 (d, 1H, $J = 9.0$ Hz, ArH), 8.29 (s, 1H, ArH), 8.33 (d, 1H, $J = 9.0$ Hz, ArH).

6.2.3 2-Methylthio-3-(phenyl or benzyl)-6 or 7-amino-3$H$-quinazolin-4-ones (25–27).

A mixture of the 6 or 7-nitro-quinazoline derivatives 22, 23 or 24 (0.01 mol), Fe powder prewashed with dilute HCl and water (0.5 g), concentrated HCl (10 mL), in ethanol (50 mL), was heated under reflux for 2 h. Concentrated ammonia solution (5 mL) was added to precipitate Fe salts. The resulting mixture was filtered while hot through celite. Filtrate was concentrated to give the crude products which were recrystallized to yield compounds 25–27 (Table 1). $^1$H NMR (CDCl$_3$), 25: $\delta$ 2.50 (s, 3H, SCH$_3$), 5.60 (brs, 2H, NH$_2$), 7.1–7.17 (m, 2H, ArH), 7.35–7.38 (m, 3H, ArH), 7.39–7.53 (m, 3H, ArH). 26: $\delta$ 2.52 (s, 3H, SCH$_3$), 5.30 (s, 2H, CH$_2$Ph), 5.61 (brs, 2H, NH$_2$), 7.18–7.32 (m, 8H, ArH). 27: $\delta$ 2.52 (s, 3H, SCH$_3$), 5.12 (s, 2H, CH$_2$Ph), 6.17 (brs, 2H, NH$_2$), 6.57 (s 1H, ArH), 6.68 (d, 1H, $J = 8.5$ Hz, ArH), 7.21–7.34 (m, 5H, ArH), 7.77 (d, 1H, $J = 8.5$ Hz, ArH).

6.2.4 2-Methylthio-3-(phenyl or benzyl)-6-[(substituted)benzylimineamino]-3$H$-quinazolin-4-ones (28–33).

An equimolar amounts (0.01 mol) of the 6-aminoquinazoline analogues 25 or 26 and the appropriate methoxybenzaldehyde were dissolved in DMF (30 mL) and heated under reflux for 4h. Solvent was then removed under reduced pressure. The obtained
residue was filtered, washed with water, dried and recrystallized to afford 28–33 (Table 2). $^1$H NMR (TFA), 28: $\delta$ 2.51 (s, 3H, SCH$_3$), 3.86 (s, 3H, OCH$_3$), 7.01 (d, 1H, $J$ = 8.5 Hz, ArH), 7.48–7.59 (m, 5H, ArH), 7.60–7.78 (dd, 4H, $J$ = 8.5, 2.5 Hz, ArH), 7.82 (s, 1H, ArH), 7.94 (d, 1H, $J$ = 8.5 Hz, ArH), 8.69 (s, 1H, CH=N). 29: $\delta$ 2.53 (s, 3H, CH$_3$), 3.85 (s, 3H, OCH$_3$), 5.30 (s, 2H, CH$_2$Ph), 7.09–7.11 (m, 1H, ArH), 7.21–7.35 (m, 6H, ArH), 7.62–7.95 (m, 5H, ArH), 8.71 (s, 1H, CH=N). 30: $\delta$ 2.51 (s, 3H, SCH$_3$), 3.86 (s, 3H, OCH$_3$), 7.11 (d, 1H, $J$ = 8.5 Hz, ArH), 7.47–7.78 (m, 10H, ArH), 8.67 (s, 1H, CH=N). 31: $\delta$ 2.52 (s, 3H, SCH$_3$), 3.87 (s, 6H, OCH$_3$), 5.32 (s, 2H, CH$_2$Ph), 7.13 (d, 1H, $J$ = 8.5 Hz, ArH), 7.45–7.81 (m, 9H, ArH), 8.71 (s, 1H, ArH), 8.69 (s, 1H, CH=N). 32: 2.51 (s, 3H, SCH$_3$), 3.75 (s, 3H, OCH$_3$), 3.87 (s, 6H, OCH$_3$), 7.35 (s, 2H, ArH), 7.43–7.52 (m, 2H, ArH), 7.56–7.62 (m, 3H, ArH), 7.69 (d, 1H, $J$ = 8.5 Hz, ArH), 7.78–7.82 (dd, 1H, $J$ = 8.5, 2.5 Hz, ArH), 7.88 (d, 1H, $J$ = 2.5 Hz, ArH), 8.71 (s, 1H, CH=N). 33: 2.50 (s, 3H, SCH$_3$), 3.76 (s, 3H, OCH$_3$), 3.87 (s, 6H, OCH$_3$), 5.30 (s, 2H, CH$_2$Ph), 7.32 (s, 2H, ArH), 7.41–7.53 (m, 2H, ArH), 7.54–7.60 (m, 3H, ArH), 7.65–7.82 (m, 2H, ArH), 7.88 (s, 1H, ArH), 8.70 (s, 1H, CH=N).

6.2.5 2-Methylthio-3-(phenyl or benzyl)-6-(substituted benzylamino-3H-quinazolin-4-ones (34–39).

The 2-methylthio-3-(phenyl or benzyl)-6-(substituted-benzylidine)amino-3H-quinazolin-4-ones (28–33, 0.01 mol) were dissolved in methanol (50 mL) and stirred. NaBH$_4$ (1.0 g) was added portionwise over a period of 0.5 h and stirred at room temperature for another 2 h. Solvent was removed under reduced pressure. Water (20 mL) was then added, and the undissolved solid was filtered and recrystallized from the appropriate solvent to afford 34–39 (Table 3). $^1$H NMR (CDCl$_3$), 34: $\delta$ 2.42 (s, 3H, SCH$_3$), 3.72 (s, 3H, OCH$_3$), 4.27 (d, 2H, $J$ = 5.2 Hz, CH$_2$Ph), 6.71 (t, 1H, $J$ = 5.2 Hz,
NH(H), 6.89 (d, 1H, J = 8.0, Hz, ArH), 7.03 (s, 2H, ArH), 7.28–7.53 (m, 9H, ArH), 35: δ 2.50 (s, 3H, SCH₃), 3.72 (s, 3H, OCH₃), 4.26 (d, 2H, J = 5.8 Hz, CH₂Ph), 5.27 (s, 2H, CH₂Ph), 6.88 (t, 1H, J = 5.8 Hz, NH), 6.92 (d, 1H, J = 8.0 Hz, ArH), 7.20 (s, 1H, ArH), 7.28–7.31 (m, 10H, ArH).

36: δ 2.50 (s, 3H, SCH₃), 3.72 (s, 6H, OCH₃), 4.26 (s, 2H, CH₂Ph), 6.90 (s, 1H, NH), 6.88 (d, 1H, J = 8.0 Hz, ArH), 7.22–7.35 (m, 10H, ArH).

37: δ 2.50 (s, 3H, SCH₃), 3.71 (s, 6H, OCH₃), 4.25 (s, 2H, CH₂Ph), 5.28 (m, 3H, CH₂Ph&NH), 6.89–7.38 (m, 11H, ArH).

38: δ 2.42 (s, 3H, SCH₃), 3.63 (s, 3H, OCH₃), 3.74 (s, 6H, OCH₃), 4.26 (d, 2H, J = 5.5 Hz, CH₂Ph), 6.72–6.75 (m, 3H, NH&ArH), 7.08 (s, 1H, ArH), 7.21–7.23 (m, 1H, ArH), 7.37–7.43 (m, 3H, ArH), 7.52–7.53 (m, 3H, ArH).

39: 2.41 (s, 3H, SCH₃), 3.62 (s, 3H, OCH₃), 3.75 (s, 6H, OCH₃), 4.27 (d, 2H, J = 5.6 Hz, CH₂Ph), 5.29 (s, 2H, CH₂Ph), 6.71–6.76 (m, 3H, NH&ArH), 7.08–7.23 (m, 2H, ArH), 7.38–7.54 (m, 6H, ArH).

6.2.6 2-Methylthio-3-(phenyl or benzyl)-6-[N-(substituted benzyl)-N-methylamino]-3H-quinazolin-4-ones (40–45).

To a solution of 2-methylthio-3-(phenyl or benzyl)-6-(substituted benzylamino)-3H-quinazoline-4-ones (34–39, 0.05 mol) in 50 mL of acetonitrile, formaldehyde (4 g, 0.11 mol) was added with constant stirring. To this suspension, NaCNBH₃ (5 g, 0.08 mol) was added, and the pH of the mixture was adjusted to 2–3 using concentrated HCl. Five min later a bright yellow precipitate was obtained. The acetonitrile was evaporated under reduced pressure and the obtained residue was suspended in 10 mL of water, and neutralized using NH₄OH to afford 40–45 which were filtered, dried and recrystallized (Table 3). ¹H NMR (DMSO–d₆), 40: δ 2.43 (s, 3H, SCH₃), 3.09 (s, 3H, NCH₃), 3.72 (s, 3H, OCH₃), 4.61 (s, 2H, CH₂Ph), 6.88 (d, 2H, J = 8.5 Hz, ArH), 7.13–7.17 (m, 3H, ArH), 7.34–7.38 (m, 3H, ArH), 7.48–7.54 (m, 4H, ArH). 41: δ 2.40 (s, 3H, SCH₃), 2.98
(s, 3H, NCH₃), 3.71 (s, 3H, OCH₃), 4.51 (s, 2H, CH₂Ph), 5.08 (s, 2H, CH₂Ph), 6.87 (d, 2H, \( J = 8.5 \) Hz, ArH), 7.07–7.13 (m, 4H, ArH), 7.22–7.32 (m, 6H, ArH). 42: \( \delta \) 2.43 (s, 3H, S.CH₃), 3.11 (s, 3H, NCH₃), 3.71 (s, 6H, OCH₃), 4.60 (s, 2H, CH₂Ph), 6.68 (d, 1H, \( J = 8.0 \) Hz, ArH), 6.85–6.88 (m, 2H, ArH), 7.16 (d, 1H, \( J = 3.0 \) Hz, ArH), 7.36–7.40 (m, 3H, ArH), 7.48–7.56 (m, 4H, ArH). 43: \( \delta \) 2.55 (s, 3H, S.CH₃), 3.11 (s, 3H, NCH₃), 3.71 (s, 6H, OCH₃), 4.62 (s, 2H, CH₂Ph), 5.33 (s, 2H, CH₂Ph), 6.71 (s, 1H, ArH), 6.89–6.91 (m, 2H, ArH), 7.19 (s, 1H, ArH), 7.41–7.45 (m, 3H, ArH), 7.51–7.57 (m, 4H, ArH). 44: \( \delta \) 2.43 (s, 3H, S.CH₃), 3.13 (s, 3H, NCH₃), 3.62 (s, 3H, OCH₃), 3.70 (s, 6H, OCH₃), 4.60 (s, 2H, CH₂Ph), 6.52 (s, 2H, ArH), 7.18 (d, 1H, \( J = 2.5 \) Hz, ArH), 7.37–7.40 (s, 3H, ArH), 7.50–7.55 (s, 4H, ArH). 45: \( \delta \) 2.56 (s, 3H, S.CH₃), 3.13 (s, 3H, NCH₃), 3.63 (s, 3H, OCH₃), 3.70 (s, 6H, OCH₃), 4.60 (s, 2H, CH₂Ph), 5.31 (s, 2H, CH₂Ph), 6.54 (s, 2H ArH), 7.22–7.46 (m, 8H, ArH).

6.2.7 2-Methylthio-3-(phenyl or benzyl)-6-[(substituted phenyl)sulphonyl]amino-3H-quinazolin-4-ones (46–51).

A solution of the 6-aminoquinazoline analogue 25 or 26 (0.002 mol) and the appropriate phenylsulphonyl chloride derivative (0.003 mol) in pyridine (10 mL) was heated under reflux for 1 h. Solvent was evaporated under reduced pressure and the remaining residue was then triturated with water and filtered. The obtained solid was dried and recrystallized to give 46–51 (Table 4). \(^1\)H NMR (DMSO–d₆), 46: \( \delta \) 2.43 (s, 3H, S.CH₃), 7.28–7.98 (m, 13H, ArH), 10.66 (s, 1H, NH). 47: \( \delta \) 2.54 (s, 3H, S.CH₃), 5.32 (s, 2H, CH₂Ph), 7.19–7.98 (m, 13H, ArH), 10.69 (brs, 1H, NH). 48: \( \delta \) 2.43 (s, 3H, S.CH₃), 7.29–7.80 (m, 12H, ArH), 10.72 (brs, 1H, NH). 49: \( \delta \) 2.54 (s, 3H, S.CH₃), 5.31 (s, 2H, CH₂Ph), 7.19–7.79 (m, 13H, ArH&NH). 50: \( \delta \) 2.33 (s, 3H, CH₃Ar), 2.44 (s, 3H,
SCH$_3$), 7.28–7.86 (m, 12H, ArH), 10.54 (brs, 1H, NH). **51**: $\delta$ 2.32 (s, 3H, CH$_3$Ar), 2.54 (s, 3H, SCH$_3$), 5.28 (s, 2H, CH$_2$Ph), 5.61 (brs, 1H, NH), 7.07–7.78 (m, 12H, ArH).

6.2.8 N-Substituted-N'-[2-methylthio-3-(phenyl or benzyl)-4-oxo-3H-quinazolin-6-yl]thioureas (52–57).

A solution of the 6-aminoquinazoline derivative **25** or **26** (0.002 mol), and the appropriate isothiocyanate (0.0022 mol) in ethanol (10 mL) was heated under reflux for 6 h. The separated solid was filtered, dried and recrystallized to yield **52–57** (Table 4).

$^1$H NMR (DMSO-d$_6$), **52**: $\delta$ 1.15 (t, 3H, CH$_2$CH$_3$), 2.43 (s, 3H, SCH$_3$), 3.45–3.51 (m, 2H, CH$_2$CH$_3$), 4.34 (brs, 1H, NH), 5.58 (brs, 1H, NH), 7.18–8.17 (m, 8H, ArH). **53**: $\delta$ 1.19 (t, 3H, CH$_2$CH$_3$), 2.43 (s, 3H, SCH$_3$), 3.46–3.49 (m, 2H, CH$_2$CH$_3$), 5.37 (s, 2H, CH$_2$Ph), 6.31 (brs, 1H, NH), 7.28–8.03 (m, 8H, ArH), 8.18 (brs, 1H, NH). **54**: $\delta$ 2.49 (s, 3H, SCH$_3$), 7.27–7.60 (m, 11H, ArH), 7.94 (s, 1H, ArH), 8.22 (s, 1H, ArH); 8.35 (brs, 1H, NH), 9.89 (brs, 1H, NH). **55**: $\delta$ 2.59 (s, 3H, SCH$_3$), 5.34 (s, 2H, CH$_2$Ph), 7.15 (t, 1H, ArH), 7.24–7.28 (m, 3H, ArH), 7.33–7.37 (m, 4H, ArH), 7.49 (d, 2H, $J = 7.5$ Hz, ArH), 7.57 (d, 1H, $J = 9.0$ Hz, ArH), 7.94–7.96 (dd, 1H, $J = 9.0$, 2.5 Hz, ArH), 8.26 (d, 1H, $J = 2.5$ Hz, ArH), 10.00 (brs, 1H, NH), 10.08 (brs, 1H, NH). **56**: $\delta$ 2.49 (s, 3H, SCH$_3$), 4.78 (s, 2H, CH$_2$Ph), 7.27–7.60 (m, 11H, ArH), 7.94 (d, 1H, $J = 9.0$ Hz, ArH), 8.22 (s, 1H, ArH), 8.35 (brs, 1H, NH), 8.89 (brs, 1H, NH). **57**: $\delta$ 2.53 (s, 3H, SCH$_3$), 5.30 (s, 2H, CH$_2$Ph), 5.61 (s, 2H, CH$_2$Ph), 7.06–7.11 (dd, 2H, $J = 9.0$, 2.5 Hz, ArH), 7.19–7.37 (m, 13H, ArH&NH).
6.2.9 2-Methylthio-3-(phenyl or benzyl)-6-[(substituted phenyl)carbonylamino]-3H-quinazolin-4-ones (58–69).

A solution of the 6-amino-quinazoline derivatives 25 or 26 (0.022 mol) and the appropriate benzoyl chloride analogue (0.003 mol) in pyridine (10 mL) was heated under reflux for 1h, and continued as mentioned under 6.2.7 to produce the N-acetylated compounds 58–69 (Table 4). $^1$H NMR (DMSO–d$_6$), 58: $\delta$ 2.50 (s, 3H, SCH$_3$), 7.31–7.72 (m, 10H, ArH), 8.01 (d, 1H, $J$ = 7.0 Hz, ArH), 8.28 (d, 1H, $J$ = 7.5 Hz, ArH), 8.58 (s, 1H, ArH), 10.59 (brs, 1H, NH). 59: $\delta$ 2.50 (s, 3H, SCH$_3$), 5.36 (s, 2H, CH$_2$Ph), 7.22–7.36 (m, 5H, ArH), 7.51–7.65 (m, 5H, ArH), 7.98–8.01 (m, 1H, ArH), 8.20 (dd, 1H, $J$ = 9.0, 2.5 Hz, ArH), 8.64 (d, 1H, $J$ = 2.5 Hz, ArH), 10.59 (s, 1H, NH). 60: $\delta$ 2.52 (s, 3H, SCH$_3$), 5.31 (brs, 1H, NH), 7.05–7.36 (m, 12H, ArH). 61: $\delta$ 2.51 (s, 3H, SCH$_3$), 5.41 (s, 1H, NH), 7.10–7.30 (m, 12H, ArH). 62: $\delta$ 2.40 (s, 3H, CH$_3$Ph), 2.51 (s, 3H, SCH$_3$), 7.32–7.69 (m, 9H, ArH), 7.88–7.97 (m, 1H, ArH), 8.23–8.32 (m, 1H, ArH), 8.56 (s, 1H, ArH), 10.46 (s, 1H, NH). 63: $\delta$ 2.40 (s, 3H, CH$_3$Ph), 2.51 (s, 3H, SCH$_3$), 5.36 (s, 2H, CH$_2$Ph), 7.22–7.31 (m, 3H, ArH), 7.31–7.39 (m, 4H, ArH), 7.62 (d, 1H, $J$ = 8.5 Hz, ArH), 7.92 (d, 2H, $J$ = 8.5 Hz, ArH), 8.19–8.25 (dd, 1H, $J$ = 8.5, 2.5 Hz, ArH), 8.65 (d, 1H, $J$ = 2.5 Hz, ArH), 10.50 (brs, 1H, NH). 64: $\delta$ 2.49 (s, 3H, SCH$_3$), 3.89 (s, 3H, OCH$_3$), 6.82–7.18 (m, 2H, ArH), 7.40–7.68 (m, 6H, ArH), 7.86–8.12 (m, 3H, ArH), 8.27 (d, 1H, $J$ = 8.5 Hz, ArH), 8.56 (s, 1H, ArH), 10.40 (brs, 1H, ArH). 65: $\delta$ 2.50 (s, 3H, SCH$_3$), 3.86 (s, 3H, OCH$_3$), 5.36 (s, 2H, CH$_2$Ph), 7.09 (d, 2H, $J$ = 9.0 Hz, ArH), 7.24–7.37 (m, 5H, ArH), 7.61 (d, 1H, $J$ = 9.0 Hz, ArH), 8.01 (d, 2H, $J$ = 8.5 Hz, ArH), 8.21–8.23 (dd, 1H, $J$ = 8.5, 2.5 Hz, ArH), 8.62 (d, 1H, $J$ = 2.5 Hz, ArH), 10.43 (s, 1H, NH). 66: $\delta$ 2.49 (s, 3H, SCH$_3$), 3.86 (s, 6H, OCH$_3$), 7.10–7.12 (m, 2H, ArH), 7.45–7.69 (m, 7H, ArH), 8.27–8.29 (m, 1H, ArH), 8.53–8.54 (m, 1H, ArH), 10.37 (brs, 1H, NH). 67: $\delta$ 2.50 (s, 3H, SCH$_3$), 3.86 (s, 6H, OCH$_3$), 5.36 (s, 2H,
CH₂Ph), 7.11 (d, 1H, J = 8.5 Hz, ArH), 7.25–7.34 (m, 3H, ArH), 7.58–7.68 (m, 5H, ArH), 8.23 (d, 1H, J = 8.5 Hz, ArH), 8.60 (s, 1H, ArH), 10.40 (s, 1H, NH). 68: δ 2.49 (s, 3H, SCH₃), 3.75 (s, 3H, OCH₃), 3.89 (s, 6H, OCH₃), 7.35 (s, 2H, ArH), 7.46–7.49 (m, 2H, ArH), 7.56–7.59 (m, 3H, ArH), 7.67 (d, 1H, J = 9.0 Hz, ArH), 8.23–8.31 (m, 1H, ArH), 8.52 (d, 1H, J = 2.5 Hz, ArH), 10.42 (s, 1H, NH). 69: δ 2.50 (s, 3H, SCH₃), 3.75 (s, 3H, OCH₃), 3.89 (s, 6H, OCH₃), 5.35 (s, 2H, CH₂Ph), 7.22–7.31 (m, 2H, ArH), 7.33–7.41 (m, 5H, ArH), 7.51 (d, 1H, J = 9.0 Hz, ArH), 8.17–8.25 (dd, 1H, J = 9.0, 2.5 Hz, ArH), 8.53 (d, 1H, J = 2.5 Hz, ArH), 10.45 (s, 1H, NH).

6.3 Dihydrofolate Reductase (DHFR) Inhibition Assay

The assay mixture contained 50 mM Tirs-HCl buffer (pH 7.4), 50 μM NADPH, 20 μL DMSO or the same volume of DMSO solution containing the test compounds to a final concentration of 10⁻¹¹ to 10⁻⁵ M, and 0.02 units of bovine liver DHFR, in a final volume of 2.0 mL. After addition of the enzyme, the mixture was incubated at room temperature for 2.0 min, and the reaction was initiated by adding 25 μM FH₂, the change in absorbance (ΔA/min) was measured at 340 nm. The activity under these conditions was linear for 10 min.¹¹⁵a-c Results are reported as % inhibition of enzymatic activity calculated using the following formula:

\[
\text{% Inhibition} = \left(1 - \frac{\Delta A/\text{min}_{\text{test}}}{\Delta A/\text{min}_{\text{DMSO}}} \right) \times 100
\]

The % inhibition values were plotted versus drug concentration (log scale). The 50% inhibitory concentration (IC₅₀) of each compound was obtained. Inhibition plot of MTX (3) is shown in Figure 10 (IC₅₀ 0.006 μM).
6.4 Antitumor Screening

Under a sterile condition, cell lines were grown in RPMI 1640 media (Gibco, NY, USA) supplemented with 10% fetal bovine serum (Biocell, CA, USA). $5 \times 10^5$ cell/ml was used to test the growth inhibition activity of the synthesized compounds. The concentrations of the compounds ranging from 0.01–100 μM were prepared in phosphate buffer saline. Each compound was initially solubilized in dimethyl sulfoxide (DMSO), however, each final dilution contained less than 1% DMSO. Solutions of different concentrations (0.2 ml) were pipetted into separate well of a microtiter tray in duplicate. Cell culture (1.8 ml) containing a cell population of $6 \times 10^4$ cells/ml was pipetted into each well. Controls, containing only phosphate buffer saline and DMSO at identical dilutions, were also prepared in the same manner. These cultures were incubated in a humidified incubator at 37°C. The incubator was supplied with 5% CO$_2$ atmosphere. After 48 h, cells in each well were diluted 10 times with saline and counted by using a coulter counter. The counts were corrected for the dilution.\textsuperscript{116–119}
7. References


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وبعد ذلك تم اخضاع مجموعة 6 - 7 نباتات إلى 6 - 7 أمينو واستخدامها في تشديد المركبات المطلوبة. فقد تم أيضا إثبات التركيب الانتقائي للمركبات باستخدام التحليل الكمي للعناصر والرنين النووي المغناطيسي ومطياف الكتلة. وتتم التقييم الحيوي للمركبات بقسم علم الأدوية بكلية الصيدلة جامعة الملك سعود والمعهد القومي للسرطان بمريلاند بأمريكا.

ويبدو أن المركب 43 يعطي فعاليته المضادة للسرطان من خلال ميكانيكية تثبيط إنزيم ثنائي هيدروفولات المختلط وأما المركبات الأخرى 31، 59 فإنها تعطي فعاليتها المضادة للسرطان من خلال ميكانيكية أخرى.
الملخص العربي

يفحص إنزيم ثنائي هيدروفرولات المختزل عملية اختزال حمض الفوليك وحمض ثنائي هيدروفرولات إلى حمض رباعي هيدروفرولات والذي يتحول بالتالي إلى N5، N10-ميثيلين رباعي هيدروفرولات. هذا المركب الأخير يعتبر مصدراً لمجموعة الميثيل، كمجموعة وحيدة ذرة الكاربون، والتي تستخدم حيوياً لتحويل النيكوتين أحادي الفوسفات المنزوع منها ذرة الأوكسجين إلى الميثيدين أحادي الفوسفات المنزوع منها ذرة الأوكسجين. عملية تثبيط إنزيم ثنائي هيدروفرولات المختزل أصبحت ومنذ فترة طويلة هدفاً للبحث العلمي، أمّا في التوصل إلى عقار فعال ضد العدوى البكتيرية والطفيلية وكذلك ضد أمراض السرطان.

يهدف هذا المقال البحثي إلى إيجاد مركب أو مجموعة من مركبات تثبيطية رائدة، تستعمل مستقبلاً لتطوير مثبطات إنزيم ثنائي الهيدروفرولات المختزل.

ثبت من خلال البحوث العلمية المنشورة أن لنواة الكينازولين وكذلك العديد من المركبات الحلقية غير المجانسة فعالية مثبطة لحمض الفوليك من خلال تثبيط إنزيم ثنائي هيدروفرولات المختزل. وأن مثبطات هذا الأنزيم لها فعالية مضادة للفطرات المسببة للالتهاب الرئوي المصاحب لداء الإيدز وهي فطر نيوموستس كارينيهي والتوكوسبرازما جوندياي، إلا أنها ذات سمية عالية وأعراض جانبية كثيرة.

لقد تم في هذه الأطروحة تصميم العديد من مركبات نواة أنترولاين استرشاداً بتقنية دراسة النماذج الجزيئية للمركبات التي تكون مثبطة جيدة لإنزيم المذكور. وقد عين هذا التصميم بإضافة مجموعات وظيفية كيميائية معروفة بقدرتها على تشجيع الفعالية المؤدية إلى تثبيط هذا الأنزيم، مع وضع العقار البديل "ميثورتركسات" وتركيبته البinary في الاعتبار.

وقد تم بعد الحدث تثبيط ثمان وأربعون مركباً جديداً ومركباً وسطي، جميعها ينتمي إلى نواة إنترولاين وذلك من خلال تفاعل المادة N4- أو N5-تينزرو حمض الأمينوتيزيلك مع الفينيل أو البنزيل أيزو نيبوسيات، لإنتاج المواد البديعة N5-ميثيل ثيو-3-فينيل أو بنزيل-6 أو N7-تينزرو-3-هـ-كينازولين-4-أون. أن.
أتقدم بالشكر لمدينة الملك عبد العزيز للعلوم والتقنية

لدعمها لهذا البحث بالمنحى رقم أط- 12- 18
تشييد وتقييم بعض مركبات 2 ميثيل ثيو - 6 - أمينو (3 هـ)
كينازولينون كمثبطات إنزيم ثنائي هيدروفولات المختزل.

رسالة مقدمة من:
سارة بنت طارق بن عبد المحسن الرشود
"بكلوريوس علم صيدلية"

استكمالاً لمتطلبات درجة الماجستير في الكيمياء الصيدلية
قسم الكيمياء الصيدلية ـ كلية الصيدلة
جامعة الملك سعود

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