Synthesis, anticancer activity and molecular modeling studies of 1,2,4-triazole derivatives as EGFR inhibitors

Hany A.M. El-Sherief, Bahaa G.M. Youssif, Syed Nasir Abbas Bukhari, Ahmed H. Abdelazeem, Mohamed Abdel-Aziz, Hamdy M. Abdel-Rahman

PII: S0223-5234(18)30580-4
DOI: 10.1016/j.ejmech.2018.07.024
Reference: EJMECH 10559

To appear in: European Journal of Medicinal Chemistry

Received Date: 17 May 2018
Revised Date: 5 July 2018
Accepted Date: 9 July 2018


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
<table>
<thead>
<tr>
<th>One dose NCI results</th>
<th>Renal Cancer-786-0 (GI at 10 μM)</th>
<th>-75.81 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiproliferative activity</td>
<td>H-460 (IC$_{50}$ ± SEM)</td>
<td>1.6±0.5 μM</td>
</tr>
<tr>
<td>Mechanistic study</td>
<td>EGFR inhibition (IC$_{50}$ ± SEM)</td>
<td>1.52±0.6 μM</td>
</tr>
</tbody>
</table>
Synthesis, anticancer activity and molecular modeling studies of 1,2,4-triazole derivatives as EGFR inhibitors

Hany A. M. El-Sherief¹,²,³, Bahaa G. M. Youssif²,³, Syed Nasir Abbas Bukhari³, Ahmed H. Abdelazeem⁴, Mohamed Abdel-Aziz⁵, *, Hamdy M. Abdel-Rahman¹,⁶

¹Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Nahda University, Beni-suef, Egypt; ²Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt; ³Department of Pharmaceutical Chemistry, College of Pharmacy, Jouf University, Aljouf, Sakaka 2014, Saudi Arabia; ⁴Department of Medicinal Chemistry, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt; ⁵Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, 61519-Minia, Egypt; ⁶Department of Medicinal Chemistry, Faculty of Pharmacy, Assiut University, 71526-Assiut, Egypt.

Short running title: Synthesis, anticancer activity and mechanistic study of new 1,2,4-triazole derivatives

*To whom correspondence should be addressed.

Mohamed Abdel-Aziz, Ph.D. Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, 61519-Minia, Egypt.
Tel.: (002)-01003311327
E-mail address: Abulnil@hotmail.com

Hany A. M. El-Sherif, M.Sc. Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt
Tel.: (+966)-598031838
E-mail address: drhanyabdeltawab@yahoo.com
Abstract

A series of novel compounds carrying 1,2,4-triazole scaffold were prepared and evaluated for their antiproliferative activities against NCI 60 cell line. Compounds 10 (a, c), 11 (a-d), and 14 (a-e) were selected for evaluation at single concentration of 10 µM towards panel of sixty cancer cell lines. Some of nitric oxide (NO) donating triazole/oxime hybrids 11a-d showed antiproliferative activity better than their corresponding ketones. On the other hand, the thiazolo[3,2-b][1,2,4]-triazoles 14a-e showed remarkable antiproliferative activities against the same cell lines. Compound 14d was selected for five dose testing against the full panel of 60 human tumor cell lines. Compound 14d showed high selectivity against renal subpanel with selectivity ratio of 6.99 at GI50 level. Compounds 11a-d, 10a-d and 14a-e were tested against four cell lines using MTT assay then compounds of the least IC50 were evaluated against three known anticancer targets including EGFR, BRAF and Tubulin. The results revealed that compound 14d showed promising EGFR inhibitory activity of cancer cell proliferation and were also observed to be moderate BRAF and tubulin inhibitors. Moreover, cell cycle analysis and apoptosis assay were finished for compounds 14d and 14f. Finally molecular modeling studies were performed to explore the binding mode of the most active compounds to the target enzymes.

Keywords: 1,2,4-Triazoles, thiazolo[3,2-b][1,2,4]-triazoles, antiproliferative, cell cycle, apoptosis and EGFR inhibitors.
1. Introduction

Cancer is a life threatening disease and remains a major health problem around the globe. It is the second most occurring disease after cardiovascular diseases. Thus, the development of potent and effective novel antineoplastic drugs is one of the most intensely persuaded goals of contemporary medicinal chemistry [1]. On the other hand, epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase (RTK) of the ErbB family [2]. EGFR is known to be a key regulator of many complex biological processes, including cell motility, adhesion, and cell cycle regulation, as well as angiogenesis, apoptosis, and metastasis [3, 4]. Aberrant activity of EGFR has been observed in a wide variety of human cancers [5], such as non-small cell lung cancer (NSCLC) [6], head and neck cancer [7], breast cancer [8], and colorectal cancer [9], and the dysregulation of EGFR signaling is invariably associated with poor clinical outcomes. Based on its critical role in the tumor progression, many antibodies and small molecule inhibitors that target EGFR have been developed over the last three decades. Several of them (e.g., Gefitinib and erlotinib) [10-13] have been approved by the US Food and Drug Administration (FDA).

Furthermore, the chemistry of 1,2,4-triazoles and their fused heterocyclic derivatives have received considerable attention owing to their synthetic and effective biological importance. For example, a large number of 1,2,4-triazoles have been incorporated into a wide variety of therapeutically interesting drug candidates possessing antimicrobial [14], anti-inflammatory [15], analgesic [16] and anticancer activities [17, 18]. We already reported the synthesis of a series of acylated 1,2,4-triazole derivatives with potent anticancer and EGFR inhibitory activities [19].
Based on these findings, and in continuation of our search, we designed the synthesis of two series of 1,2,4-triazoles – Nitric oxide (NO) hybrids to be evaluated for their anticancer activity and EGFR inhibition. According to our published research [19], acylation on N-1 of 5-amino-1,2,4-triazoles enhanced their EGFR activity. Thus this work was designed to make alkylation of 5-amino-1,2,4-triazoles at ring N-1 incorporation of NO donating moiety to further increase the anticancer activity. The target compounds will incorporate both 1,2,4-triazole acetamide structure as apoptosis inducers [20] connected to the NO donor moiety (Fig. 1). Already 1,2,4-triazoles/nitric oxide (NO) hybrids were obtained by reaction of triazolylaminoacetophenones with hydroxylamine hydrochloride (Fig. 1).
During the course of our synthesis of the target compounds, the reaction of 1,2,4-triazole-3-thiones with aromatic ketones in boiling acetic acid and catalytic amount of conc. H$_2$SO$_4$ was expected to afford the 1,2,4-triazolylthioacetophenones, however, the obtained products were identified as substituted thiazolo[3,2-b][1,2,4]triazoles (Fig. 2). Furthermore, the cell cycle activity will be determined for the most active compounds, to give an idea about the possible phase in which those new compounds halt the growth of cancer cells. Finally, molecular docking of the synthesized compounds inside the active site of EGFR will be performed in order to confirm their possible mode with enzyme.

![Compounds 10a-d; X= O](image1)

Compounds 10a-d; X= O

Compounds 11a-d; X= N-OH

10, 11a: R= CF$_3$
10, 11b: R= C$_6$H$_5$
10, 11c: R= 4-Cl-C$_6$H$_4$
10, 11d: R= 3,4,5tri(OCH$_3$)-C$_6$H$_2$

![Compounds 14a-f](image2)

Figure 2: The final synthesized compounds

2. Result and discussion

2.1 Chemistry

The target compounds 11a-d were prepared according to Scheme 1 and 2. The key intermediates 3 [21], 7a-c [22] [23] and compound 9 were prepared according to reported literatures and their structures were confirmed by matching their physical characters with the reported ones.

Compounds 10a-d were prepared in good yields by alkylation of compounds 3 and 7a-c with compound 9 in acetonitrile using triethylamine as a base.
IR, $^1$H NMR and $^{13}$C NMR studies as well as high resolution mass provide a confirm support for the structural elucidation of compounds 10a-d. IR spectra of compounds 10a-d showed significant bands at 3450-3470 cm$^{-1}$ (NH), 1645-1650 cm$^{-1}$ (CO-NH), 1710-1720 cm$^{-1}$ (COCH$_3$) and forked peak at 3150-3200 cm$^{-1}$ corresponding to NH$_2$ group indicating that alkylation of amino triazole occurs on acidic NH not on NH$_2$. $^1$H NMR spectra showed singlet signals at 2.13 ppm (CH$_3$), 4.89-4.93 ppm (CH$_2$), 6.43-6.84 ppm (NH$_2$) and 10.66-10.67 ppm (NH). In addition to a doublet at 7.72-7.98 ppm (J = 8.4-8.8 Hz) corresponding to aromatic protons of N-acetylphenylacetamide which confirm cis configuration. $^{13}$C NMR spectra of compounds 10a-d showed different significant peaks at δ 56 and 60 ppm related to triOCH$_3$ gp, 158, 166 and 199 ppm, representative for C=N, carbonyl of CONH and COCH$_3$, respectively. Moreover, aliphatic groups CH$_3$ and CH$_2$ were detected in regions of 26 and 56 ppm, respectively.

Refluxing compounds 10a-d with hydroxylamine hydrochloride in ethanol to give the oximes 11a-d in good yields (Scheme 2). The structure assignment of oxime derivatives 11a-d is based on IR, $^1$H NMR, $^{13}$C NMR spectral data and high resolution mass. IR spectra of compounds 11a-d showed intense broad bands at 3000-3480 cm$^{-1}$ due to OH, NH and NH$_2$ groups, strong absorption bands at 1645-1655 cm$^{-1}$ corresponding to (CO-NH) and disappearance of the ketone peaks at 1710-1720 cm$^{-1}$ (COCH$_3$) which confirm oxime formation. The $^1$H NMR spectra of compounds 11a-d showed a characteristic singlet signal at 10.40-10.42 ppm due to NH group. The resonance of OH, CH$_3$ and CH$_2$ were observed as four singlets in the expected regions at 11.08-11.09 ppm, 2.13 ppm and 4.84-4.92 ppm, respectively. $^{13}$C NMR spectra of compounds 11a-d showed peaks at δ 56 and 60 ppm corresponding to triOCH$_3$ gp, 158 and 166 ppm signifying for C=N, and carbonyl of CONH, respectively. Moreover, detection of aliphatic groups CH$_3$ and CH$_2$ at chemical shift 26 and 56 ppm with disappearance of the characteristic peak of carbonyl (COCH$_3$) indicate the formation of oxime.
Scheme 1: Synthesis of the key intermediates $3$, $7a$-$c$ and $9$.

**Reagents and reaction conditions** (i) $210^\circ C$, 2 h; (ii) Conc $\text{H}_2\text{SO}_4$ / $\text{CH}_3\text{OH}$, reflux, 4-8 h; (iii) $\text{NH}_2\text{NH}_2\cdot\text{H}_2\text{O}$, EtOH, reflux, 2-4 h; (iv) S-methylthiourea, $\text{NaOH}$, RT, 72 h; (v) $\text{H}_2\text{O}$, reflux, 4 h; (vi) Bromoacetyl bromide, $\text{CH}_3\text{OH}$, $\text{K}_2\text{CO}_3$, RT, 4 h.
Scheme 2: Synthesis of the target compounds 11a-d.

Reagents and reaction conditions (i) CH₃CN, triethylamine, reflux; (ii) NH₂OH.HCl, EtOH, reflux.

Compounds 14a-f were prepared according to Scheme 3. Reaction of compounds 5a-c with NH₄SCN under basic condition followed by cyclization with NaOH afforded 5-substituted-4H-1,2,4-triazole-3-thiones 12a-c [24]. Reaction of 12a-c with aromatic ketones 13a-b in boiling acetic acid and catalytic amount of conc. H₂SO₄ was expected to afford the 1,2,4-triazolythioacetophenones as reported [25] and afforded the cyclized products identified as the 2,5-di(substitutedphenyl)thiazolo[3,2-b][1,2,4]triazoles 14a-f in one step with 63-84% yields (Scheme 3). The chemical structures of compounds 14a-f were elucidated on the basis of their IR, ¹H NMR, C¹³NMR spectra and high resolution mass. IR spectra of compounds 14a-f showed no peaks corresponding to NH₂, NH, CO and CONH groups. ¹H NMR spectra of compounds 14a-f showed singlet signal at δ 7.91- 8.01 ppm (1H) corresponding for CH of thiazole. On the other hand, only compounds 14c-d derivatives exhibited doublet at 7.08 and 8.07 ppm for p-methoxyphenyl moiety. Compounds 14e-f showed singlet signal at δ 7.42 ppm (2H) of trimethoxyphenyl moiety. Appropriate signals for other aryl substituents were detected.
in the $^1$H NMR spectra of compounds 14a-f. $^{13}$C NMR spectra of compound 14a-f showed different significant peaks at δ 56 and 60 ppm related to triOCH$_3$ gp, 111, 134 and 166 ppm, corresponding for, C-S, C-Cl and C=N, respectively.

![Scheme 3](image)

**Scheme 3**: Synthesis of the final target compounds 14a-f.

**Reagents and reaction conditions** (i) NH$_4$SCN, dil. HCl, reflux; (ii) NaOH reflux, then 5M HCl; (iii) CH$_3$COOH, Conc. H$_2$SO$_4$, Reflux.

### 2.2 Biology

#### 2.2.1 Evaluation of *in vitro* antiproliferative activity for compounds selected by NCI

The newly synthesized compounds 10a-d, 11a-d and 14a-f were submitted to National Cancer Institute (NCI, Bethesda, ML, USA; http://www.dtp.nci.nih.gov.), and twelve compounds 10 (a, c), 11 (a-d), and 14 (a-e) were selected for evaluation at single concentration of 10 µM on the basis of their structural variation towards panel of sixty cancer cell lines taken from nine different tissues (lung, blood, CNS, colon, skin, kidney, ovary, prostate and breast). The results for each compound were reported as the percent growth of treated cells compared to untreated control cells. Compounds 11a-d possessing oxime group were more active than their parent ketones 10a-c. This directly reflects the influences of such moieties on the antiproliferative activity of the title scaffold. Compound 10c exhibited moderate cell growth inhibition activity in opposition to all types of cancer cell lines, wherever it inhibited Non-Small Cell Lung Cancer NCI-H522, Non-Small Cell Lung Cancer A549/ATCC and Ovarian Cancer SK-OV-3 with GI
values of 28, 22 and 22 % respectively (Table 1, Appendix A). Compound 11d showed a moderate cell growth inhibition activity against all types of cancer cell lines. It inhibited Melanoma Cancer v and Non-Small Cell Lung Cancer NCI-H522 activity with GI value 36 and 29 %, respectively. Compounds 11a, 11c and 11d showed higher antiproliferative activity than the corresponding phenyl analogues 11b. Compound 11d with electron donating trimethoxy moiety has superior activity than the corresponding electron withdrawing chloro moiety against most of all cell lines.

On the other hand, compounds 14a-e showed highly promising activities of all tested compounds against all cancer cell lines. By comparing the effect of substituent attached to two phenyl moieties on activity, it was found that compounds 14c and 14d possessing p-methoxy and p-chloro substituent were more active than compounds 14a, 14b and 14e. Compound 14a showed potent cell growth inhibition activity against all types of cancer cell lines with. It inhibited Breast Cancer T-47D, Breast Cancer MCF7 and Renal Cancer UO-31 with (GI) values of 63, 61 and 53 %, respectively (Table 2, Appendix A). Compound 14b showed powerful cell growth inhibition activity against all types of cancer cell lines. It inhibited Breast Cancer T-47D, Ovarian Cancer SK-OV-3 and Ovarian Cancer OVCAR-4 with (GI) values of 74, 62 and 60 %, respectively (Table 2, Appendix A). P-methoxy derivatives 14c showed extremely persuasive cell growth inhibition activity against all types of cancer cell lines. It has inhibitory effect on Breast Cancer MDA-MB-468, Leukemia K-562, Breast Cancer MCF7, Melanoma M14, Colon Cancer KM12, Colon Cancer HT29, Non-Small Cell Lung Cancer NCI-H522 and Colon Cancer HCT-15 with (GI) values of 81, 76, 74, 71, 70, 70, 70 and 69 %, respectively. P-methoxy derivatives 14c showed weak lethal effect on Melanoma MDA-MB-435 (Table 2, Appendix A). Compound 14d showed the most potent cell growth inhibition activity of all tested compounds. It has inhibitory effect on Renal Cancer SN12C, Ovarian cancer IGROV1, Breast Cancer MDA-MB-468, CNS Cancer SF-539, Non-Small Cell Lung Cancer NCI-
H322M, Colon Cancer HT29 and Non-Small Cell Lung Cancer HOP-92 with (GI) values of 95, 93, 87, 85, 83, 83 and 79 %, respectively. P-methoxy derivatives 14d showed weak lethal effect on Colon Cancer HCT-116, CNS Cancer SNB-19, CNS Cancer SNB-75, Renal Cancer UO-31 and Renal Cancer CAKI-1. Moreover, compound 14d showed moderate lethal effect on Non-Small Cell Lung Cancer A549/ATCC, Non-Small Cell Lung Cancer NCI-H460, Renal Cancer A498 and Renal Cancer TK-10. Also it exhibited potent lethal effect on CNS Cancer U251, Ovarian cancer OVCAR-4, Renal Cancer 786-0 and Renal Cancer ACHN (Table 2, Appendix A). Compound 14d was selected for five dose screening as promising compound. Compound 14e showed forceful cell growth inhibition activity against all types of cancer cell lines. It inhibited Leukemia SR, Non-Small Cell Lung Cancer EKVX and Breast Cancer T-47D with (GI) values of 65, 63 and 55 %, respectively (Table 2, Appendix A). According to the above, the following can be deduced, compounds 14a-e showed potent cytotoxic activity especially in presence of electron withdrawing group (like chloro), if compared to compounds containing electron donating groups. Among all target compounds, compound 14c and 14d showed the most promising results. They exerted broad-spectrum antiproliferative activity against different cell lines of different cancer types. Therefore, these compounds could be potential lead compounds for the future development of broad spectrum anticancer agents.

2.2.2 In vitro five dose full NCI 60 cell panel assay

Compound 14d was selected for the advanced five dose testing against the full panel of 60 human tumor cell lines. All the 60 cell lines representing nine tumor subpanels were incubated at five different concentrations (0.01, 0.1, 1, 10, and 100 µM). The outcomes were used to form log concentration versus % growth inhibition curves and three response parameters (GI50, TGI, and LC50) were calculated for each cell line. The GI50 value (growth inhibitory activity) corresponds to the concentration of the compound causing 50% decrease in net cell growth, the TGI value (cytostatic activity) is the concentration of the compound resulting in total growth
inhibition (TGI) and LC₅₀ value (cytotoxic activity) is the concentration of the compound causing net 50% loss of initial cells at the end of the incubation period of 48 h [26]. Compound 14d exhibited noteworthy antiproliferative activity against the majority of the tested cell lines representing nine different subpanels. Compound 14d showed high activities alongside most of the tested cell lines with GI₅₀ ranging from 0.766 to >50 μM (Table 3, Appendix A). The principle for selectivity of compound depends upon the ratio obtained by dividing the full panel MID (the average sensitivity of all cell lines toward the test agent) (μM) by their individual subpanel MID (μM). Ratios between 3 and 6 refer to moderate selectivity; ratios >6 indicate high selectivity toward the corresponding cell line, whilst compounds not meeting either of these criterion rated nonselective. In this case, compound 14d was found to have potent cell growth inhibition activity against most of the tumor subpanels tested with selectivity ratios ranging between 0.30 and 6.99 at the GI₅₀ level. Compound 14d was found to be broad-spectrum cell growth inhibition activity against most of the tested tumor subpanels cell lines with high selectivity against renal subpanel with selectivity ratio of 6.88 μM at GI₅₀ level (Table 3, Appendix A)

\[
\text{Selectivity index} = \frac{\text{MID}_a}{\text{MID}_b}
\]
2.2.3 Effect of synthetic compounds on cancer cell growth

In this study, propidium iodide (PI) fluorescence assay was performed to evaluate the effect of new synthetic compounds on Panc-1 (pancreas cancer cell line), PaCa-2 (pancreatic carcinoma cell line), HT-29 (colon cancer cell line) and H-460 (lung cancer cell line), while Erlotinib hydrochloride was used as a positive control.

The anticancer activity results are summarized in Table 4 and are presented as IC₅₀ values. It is obvious from the results, that the synthesized compounds demonstrated promising anticancer activity almost against all the cell lines tested to a reasonable extent. Among the first series (10a-d and 11a-d) there were two types of compounds, first class (10a-d) were ketone intermediates containing 1,2,4 triazole linked with phenyl acetyl through an acetamide bridge...
while on the other side second group (11a-d) obtained from ketones were also 1,2,4 triazole but linked to phenyl ethanone oxime through acetamide bridge. Substitution pattern was same on both groups of compounds for position three of triazole moiety and position five was containing amino group in both groups of compounds. Compound 11a of oxime class showed highly potent anticancer activity (IC\(_{50}\) 3.5 µM) against pancreas cancer cell line. Significantly, compound 11a exhibited highest activity against Panc-1 cell line, which is 2 fold higher activity than that of other compounds in same group of oximes. The other potent molecule in this series is 10a with IC\(_{50}\) 3.7 µM for lung cancer cell line.

Interestingly, in this series two compounds 10b and 11b, with an unsubstituted phenyl moiety at position of triazole core showed weak cytotoxicity against almost all the cell lines tested. On a careful observation an interesting structure activity relationship can be observed between all these tested compounds of these two groups. First of all on the basis of results included in table 4 a general comparison between compounds clearly shows that all compound oxime compound 11a bearing trifluoromethyl at position 3 of triazole was more active than compound 10a bearing same substitution on same position but without oxime core ketone intermediates for only pancreas cancer cell line while for other tested cancer cell lines results are more promising for non oxime group of compounds. Some oxime triazole hybrids were found more active than ketone triazole hybrids. It’s also proven recently that NO-donating oximes hybrids showed pronounced gastroprotective activity better than their corresponding ketone intermediates that may be attributed to the release of NO [27].

The substitution variations at position 3 of triazole can be correlated with the activity of compounds. Presence of trifluoromethyl (10a & 11a) showed best activities as compared to other substitution groups while presence of unsubstituted phenyl ring at same position (10b & 11b) exhibited lowest effects against the growth of cancer cell lines. Chloro substituted phenyl group exhibited better activities as compared to unsubstituted phenyl ring and 3,4,5-tri methoxy
substituted phenyl expressed more improved results as shown by compound 11d compared to other phenyl substituted triazole oxime hybrids.

Previously reported similar class of compounds were found considerable anticancer agents [20] as a series of compounds bearing 2-(4H-1,2,4-triazole-3-ylthio) acetamide structure have been synthesized and were evaluated for their anti-HIV activity and against the full panel of 60 human cancer cell lines derived from nine different cancer types. Antiproliferative effects of the selected compounds were demonstrated in human tumor cell lines K-562, A549 and PC-3. These compounds inhibited cell growth assessed by MTT assay. Selected compounds exhibited anticancer activity and showed a noteworthy boost in caspase-3 activity in a dose-dependent manner. Our synthesized compounds were found more effective as compared to these reported compounds [20].

Table 4: Antiproliferative activities of synthetic compounds 10a-d and 11a-d

<table>
<thead>
<tr>
<th>Code</th>
<th>Panc-1 (µM)</th>
<th>PaCa-2 (µM)</th>
<th>HT-29 (µM)</th>
<th>H-460 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10a</td>
<td>4.5±1.4</td>
<td>4.7±1.5</td>
<td>3.8±2.0</td>
<td>3.7±2.1</td>
</tr>
<tr>
<td>10b</td>
<td>9.5±3.1</td>
<td>8.5±1.3</td>
<td>8.4±1.7</td>
<td>9.8±1.0</td>
</tr>
<tr>
<td>10c</td>
<td>7.4±2.4</td>
<td>8.1±2.4</td>
<td>6.9±2.1</td>
<td>6.8±7.4</td>
</tr>
<tr>
<td>11a</td>
<td>3.5±1.1</td>
<td>4.9±1.2</td>
<td>5.4±0.8</td>
<td>5.2±2.3</td>
</tr>
<tr>
<td>11b</td>
<td>8.7±2.7</td>
<td>5.7±1.7</td>
<td>4.7±2.5</td>
<td>4.4±1.9</td>
</tr>
<tr>
<td>11c</td>
<td>6.6±1.5</td>
<td>8.4±1.8</td>
<td>5.8±1.4</td>
<td>7.1±2.4</td>
</tr>
<tr>
<td>11d</td>
<td>5.2±1.4</td>
<td>5.7±2.9</td>
<td>7.9±0.5</td>
<td>7.9±1.2</td>
</tr>
</tbody>
</table>


Other six compounds (14a-f) synthesized in this study were also evaluated for same cancer cell lines by same method mentioned above and results are shown in Table 5. These all compounds were bearing thiazolo-triazole moiety and on the both sides of main core, substituted phenyls
made the difference for anticancer activities. This class of compounds was found more effective as compared to other series of compounds having ketone and oxime groups. Three compounds \( 14 (a, c \& e) \) were bearing unsubstituted phenyl ring on thiazole moiety while other three were bearing 4-chloro substituted phenyl ring. A compound 14a bearing unsubstituted phenyl ring on both sides of thiazolo-triazole core was found least effective against cancer cell lines. Attachment of 4-chloro substituted benzene increased the considerable effect of compound 14b. Other four derivatives 14c-f were bearing substituted phenyl at triazole moiety and strongly increased the antiproliferative effects of these new compounds. It was observed that when only methoxy substituted phenyl ring was attached to compounds (14c), the activity was increased almost double as compared to compounds without any substituted phenyls 14a. Methoxy substitution at position 4 of phenyl attached to triazole was even more potent than 4-chloro substituted phenyl at thiazole core. Trimethoxy availability at phenyl linked to triazole also showed noteworthy effects but most potent and best antiproliferative effects were exhibited by compound 14d bearing combination of substituted phenyls as 4-chloro phenyl on thiazole and 4-methoxy phenyl on triazole ring. Compound 14d lowest IC\(_{50}\) (1.60 µM) for H-460 cell line.

The mechanistic effects of most active antiproliferative compounds from thiazolo-triazole series were investigated on tubulin polymerization, EGFR TK kinases and BRAF\(^{V600E}\). Therefore, compounds 14d and 14f were subjected to further investigations to explore the possible mechanism of action on A549 cell line.
Table 5: Antiproliferative activities of synthetic compounds 14a-f

<table>
<thead>
<tr>
<th>Code</th>
<th>Panc-1 (µM)</th>
<th>PaCa-2 (µM)</th>
<th>HT-29 (µM)</th>
<th>H-460 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14a</td>
<td>7.2±1.9</td>
<td>9.2±2.2</td>
<td>8.4±1.8</td>
<td>8.3±2.7</td>
</tr>
<tr>
<td>14b</td>
<td>5.2±2.9</td>
<td>6.5±0.2</td>
<td>5.9±1.8</td>
<td>5.1±1.7</td>
</tr>
<tr>
<td>14c</td>
<td>4.5±1.5</td>
<td>4.9±1.7</td>
<td>6.6±2.7</td>
<td>6.9±2.2</td>
</tr>
<tr>
<td>14d</td>
<td>2.8±1.2</td>
<td>2.6±0.5</td>
<td>2.4±0.9</td>
<td>1.6±0.5</td>
</tr>
<tr>
<td>14e</td>
<td>4.9±1.5</td>
<td>4.7±1.2</td>
<td>4.9±0.7</td>
<td>4.8±1.4</td>
</tr>
<tr>
<td>14f</td>
<td>3.5±1.7</td>
<td>3.9±1.2</td>
<td>3.5±1.8</td>
<td>3.0±1.8</td>
</tr>
</tbody>
</table>


2.2.4 Cell cycle analysis and apoptosis assay.

Compounds 14d and 14f were subjected to cell cycle analysis and apoptotic assay at confirmatory diagnostic unit, VACSERA-EGYPT.

2.2.4.1 Cell cycle analysis and apoptosis detection.

Once a cell goes through the cell cycle, it replicates two new indistinguishable cells. Each of the two cells made from the first cell can go through this cell cycle again when new cells are needed. The cell cycle includes four phases: G1 phase, S phase (synthesis), G2 phase and M phase. During G1, cell enlargement and preparation of DNA duplication occurs. The S phase is the stage of DNA replication and chromatid duplication. During G2, repairing of new DNA and more growth occurs. In the M stage nuclear division takes place. Studies on the effect of compounds 14d and 14f on cell cycle development and induction of apoptosis in the A549 was done. A549 was incubated with IC$_{50}$ concentration of compound 14d and 14f for 24 h. The cell line was stained with PI/Annexin V and analyzed by flow cytometry using BD FASCALibur. Investigation of the results (Fig. 4) exposed that percentage of pre G1 apoptosis induced by
compound 14d on A549 after 24 h incubation was 10.19%. A high percent of cell accumulation was observed in G2/M phase in A549 treated with compound 14d after 24 h incubation indicating arrest of cell cycle at G2/M phase. Compound 14f on A549 induced pre G1 apoptosis by 14.87% after 24 h incubation. Also, A549 treated with compound 14f showed cell accumulation at G1 phase and G2/M phase after 24 h incubation but more at G2/M. This indicated that it arrested cell cycle also at G2/M phase.

![Cell cycle analysis](image)

**Figure 4:** Cell Cycle analysis results for compounds 14d and 14f

2.2.4.2 Apoptosis assay.

Cell cycle analysis of A549 after treatment with compound 14d and 14f, respectively showed presence of pre-G1 peak which is an indication of apoptosis. To confirm the ability of both compounds to induce apoptosis, cells were stained with Annexin V/PI, incubated for 24 h and analyzed. Analysis of early and late apoptosis showed that, compounds 14d and 14f were certainly able to induce significant levels of apoptosis with necrosis percent 1.34 and 1.84, respectively (Fig. 5). On the other hand, compounds 14d and 14f were able to induce late apoptosis with a percent more than early apoptosis which making recovery of apoptic cell to be healthy is more difficult.
Figure 5: Apoptosis induction analysis using Annexin V/PI for compounds 14d and 14f.

Figure 6: Cell cycle analysis and Apoptosis induction analysis of compound 14d and 14f on A549.
2.2.5 Tubulin Inhibitory Activity

*In vitro* tubulin polymerization inhibitory activity of most potent compound **14d** selected was evaluated, and it inhibited the assembly of tubulin. On comparison to model drugs docetaxel and vincristine, compound **14d** was found inhibitor as it decreased the arbitrary units as compared to control but not more than vincristine. Previously new cis-restricted Combretastatin A4 analogues containing 1,2,4-triazole in place of the olefinic bond were designed and synthesized. The synthesized compounds were evaluated for their *in vitro* antiproliferative activity and most potent compounds were selected for *in vitro* tubulin polymerization inhibitory activity. The assay exposed that some 1,2,4-triazole derivatives showed a remarkable tubulin inhibition [28].

2.2.6 EGFR Inhibitory Activity

To evaluate the EGFR inhibitory potential of compounds, EGFR-TK assay was performed. The findings of the cancer cell-based assays have been strongly complemented by the results from this assay. All compounds showed potent EGFR inhibition with IC₅₀ in the range of 1.5±0.6 to 9.8±2.4 µM. The compound **14d** displayed most potent activity (IC₅₀ = 1.5±0.6 µM) among all compounds. After **14d**, compound **14f** was also found to be more effective. Similar structure activity relationship was observed for thiazolo-triazole compounds as observed for their antiproliferative effects. This study demonstrates the compounds to be strong inhibitors of EGFR and can probably be used as anticancer agents.

2.2.7 BRAF^{V600E} inhibitory activity

The RAS-RAF-MEK-ERK pathway is hyper-activated in roughly 30% of human cancers, where it stimulates cell growth and survival. This hyper activation is motive in fraction by mutations in receptor tyrosine kinases, the small G-proteins of the RAS family, and the serine or threonine specific protein kinase BRAF [29]. A broad cancer genome mutation mapping has exposed BRAF mutation in 7% of human cancers, and this verdict indicates BRAF as a prime
oncogene, mainly in melanomas. These combined reasons render BRAF kinase as a potential target for the development of anticancer drugs. In this study, most potent antiproliferative compound 14d bearing combination of substituted phenyls as 4-chloro phenyl on thiazole and 4-methoxy phenyl on triazole ring was subjected to *in vitro* BRAF\(^{V600E}\) inhibitory assay and it exhibited IC\(_{50}\) 4.7 µM.

**Table 6**: Effects of compounds 14d on Tubulin Polymerization, EGFR and BRAF\(^{V600E}\).

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Tubulin inhibition (arbitrary units)</th>
<th>BRAF inhibition IC(_{50}) ± SEM (µM)</th>
<th>EGFR inhibition IC(_{50}) ± SEM (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14a</td>
<td>-</td>
<td>-</td>
<td>9.8±2.4</td>
</tr>
<tr>
<td>14b</td>
<td>-</td>
<td>-</td>
<td>7.2±2.9</td>
</tr>
<tr>
<td>14c</td>
<td>-</td>
<td>-</td>
<td>5.6±1.9</td>
</tr>
<tr>
<td>14d</td>
<td>1506±298</td>
<td>4.7±1.9</td>
<td>1.5±0.6</td>
</tr>
<tr>
<td>14e</td>
<td>-</td>
<td>-</td>
<td>2.8±1.0</td>
</tr>
<tr>
<td>14f</td>
<td>-</td>
<td>-</td>
<td>1.7±0.5</td>
</tr>
<tr>
<td>Erlotinib</td>
<td>-</td>
<td>0.04±0.03</td>
<td>0.07±0.03</td>
</tr>
<tr>
<td>DPBS</td>
<td>2880±237</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vincristine</td>
<td>722±249</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>4735±217</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### 2.3 Molecular docking

The present docking simulation study was carried out in order to correlate the observed potency and SAR of our newly synthesized thiazolotriazole fused system derivatives with their behavior, binding modes and the possible interactions within the active site of EGFR kinase. This study was performed using LIGDOCK, the hotspots-based docking protocol, embedded in the Discovery Studio software 2.5 (San Diego, USA). The thiazolotriazole series 14a-f were
docked into the ATP-active sites of EGFR using the 3D protein structure (PDB ID: 1M17) retrieved from protein data bank for EGFR [30, 31]. The superimposition of the top docking poses was depicted in Fig. 7 where these poses demonstrated good shape complementarities with the ATP-binding sites of EGFR kinase with various binding patterns and interactions.

Figure 7: (A) Superimposition of the top docked poses 14a-f into the EGFR kinase binding site (PDB code: 1M17) where the protein is represented as a secondary structure; (B) Superimposition of the top docked poses 14a-f into the EGFR kinase binding site (PDB code: 1M17) where the protein is represented as a solid surface colored according to atom charges. The binding sites are depicted as transparent green solid surface. From the analysis of the docking results, it was clear that compounds 14d and 14f with the highest EGFR inhibitory activities of 1.5 and 1.7 µM, respectively adopted the best and same binding mode among the tested compounds confirming their great potency with the highest docking scores (LibDockScore) of 90.41 and 98.69, respectively, (Table 7). The thiazolotriazolole core in both compounds was leaned in the middle of the binding cavity forming one H-bond interaction with Met769 amino acid residue. Meanwhile, the monomethoxy or
trimethoxyphenyl moieties was directed near the DFG motif (Asp831, Phe832 and Gly833) forming one more H-bond with Thr830 residue in case of 14d while, the trimethoxyphenyl of 14f was engaged in a network of H-bonds with Thr830 and Lys721 amino acids, Fig.8 (A and B). Phenyl ring in both compounds also formed some hydrophobic interactions near the DFG motif with Ala719, Thr766 and Leu764 amino acids. Moreover, compound 14d formed π-cation interaction with Lys721 residue explaining the higher activity of 14d than 14f. However, the p-chlorophenyl attached to the thiazole ring was extended towards the hinge region making some hydrophobic interactions with the surrounding amino acids including Leu694, Leu768, Phe771, Cys773 and Leu820 residues. Additionally, the p-chlorophenyl moiety formed a favorable π-stacking interaction with phenyl ring of Phe771 amino acids.

Figure 8: (A) Docking and binding pattern of compound 14d (orange) into ATP-active pocket of EGFR kinase (PDB code: 1M17); (B) Docking and binding pattern of compound 14f (yellow) into ATP-active pocket of EGFR kinase; The poses were rendered as green line models. H-bond interactions were represented as dashed blue lines. All hydrogens were removed for the purposes of clarity.

Despite compound 14e showed similar disposition to that of compounds 14d and 14f, it has an intermediate activity comparing with 14d and 14f with IC₅₀ = 2.8 µM. This decrease in the potency could be attributed to the absence of the chloride atom and the important H-bond with Met769 residue, Fig. 9(A and B).
**Figure 9:** (A) Docking and binding pattern of compound 14e (green) into ATP-active pocket of EGFR kinase (PDB code: 1M17); (B) Superimposition of 14d-f docked poses within the ATP-active site of EGFR protein; The poses were rendered as green line models. H-bond interactions were represented as dashed blue lines. All hydrogens were removed for the purposes of clarity.

On the contrary, the rest of the tested compounds against EGFR kinase 14a-c with the weak activity ranging 5.6-9.8 µM showed an inverse orientation to that of compounds 14d-f where the thiazole ring was directed inside towards DFG motif. The opposite orientation and the loss of the significant H-bonds and other interactions in case of compound 14a due to the absence of the methoxy and chloride substituents might be the reason behind the least inhibitory activity (IC$_{50}$ = 9.8 µM) exhibited by this compound against EGFR, Fig.10(A). Interestingly, the potency was slightly enhanced in compound 14b (IC$_{50}$ = 7.2 µM) by engaging the nitrogen of the triazole ring in the important H-bond with Met769 residue, Fig.10(B). It was observed that compound 14c incorporating a methoxy group adopted similar binding pattern to that of active compounds 14d-f and forming H-bond between the thiazole ring and Met-769 amino acids owing to much improvement in the activity (IC$_{50}$ = 5.6 µM) comparing with compound 14a, Fig.10(C).
**Figure 10:** (A) Docking and binding pattern of compound 14a (magenta red) into ATP-active site of EGFR kinase (PDB code: 1M17); (B) Docking and binding pattern of compound 14b (cyan) into ATP-active site of EGFR kinase; (C) Docking and binding pattern of compound 14c (blue) into ATP-active site of EGFR kinase; (D) Superimposition of 14d-f docked poses within the ATP-active site of EGFR protein. The poses were rendered as green line models. H-bond interactions were represented as dashed blue lines. All hydrogens were removed for the purposes of clarity.

Interestingly, the docking score in table 7 of compounds 14a-f was in consistency with their EGFR inhibitory activities. Overall, the docking study, along with EGFR *in vitro* results, confirmed that the presence of methoxy and chloride substitutions greatly contribute to the enhancement of the inhibitory activities of these thiazolotriazole-based compounds through contributing in formation of the significant H-bonds and other interactions inside the ATP-binding cavity.
Table 7: The docking score and interactions of the newly synthesized compounds with the amino acid residues in the ATP-active pocket of EGFR kinase.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>LibDockS core</th>
<th>Number of H-Bonds</th>
<th>Amino Acid involved</th>
<th>Other interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>14a</td>
<td>83.32</td>
<td>0</td>
<td>None</td>
<td>Hydrophobic, $\pi$-stacking with Phe771</td>
</tr>
<tr>
<td>14b</td>
<td>84.93</td>
<td>1</td>
<td>Met769</td>
<td>Hydrophobic, $\pi$-stacking with Phe771</td>
</tr>
<tr>
<td>14c</td>
<td>87.52</td>
<td>1</td>
<td>Met769</td>
<td>Hydrophobic, $\pi$-stacking with Phe771</td>
</tr>
<tr>
<td>14d</td>
<td>90.41</td>
<td>2</td>
<td>Met769, Thr830</td>
<td>and $\pi$-cation with Lys721</td>
</tr>
<tr>
<td>14e</td>
<td>90.08</td>
<td>3</td>
<td>Met769, Thr830</td>
<td>Hydrophobic, $\pi$-stacking with Phe771</td>
</tr>
<tr>
<td>14f</td>
<td>98.69</td>
<td>3</td>
<td>Met769, Thr830</td>
<td>Hydrophobic, $\pi$-stacking with Phe771</td>
</tr>
</tbody>
</table>
3. Conclusion

In conclusion, a new class of novel compounds carrying 1,2,4-triazole scaffold were synthesized and screened for anticancer activity. Compound 14d displayed highest activity among all new compounds against cancer cell growth and also exhibited a significant inhibitory activity against EGFR. Compound 14d was selected for five dose test by NCI showing selectivity ratios ranging between 0.30 and 6.99 at the GI₅₀ level. All the thiazolo[3,2-b][1,2,4]-triazoles 14a-e backbone derivatives were found better inhibitors of cancer cell growth as compared to triazole/oxime hybrids 11a-d derivatives. Compounds 14d and 14f showed high cell accumulation at G2/M phase, confirming their ability to cause cell cycle arrest at G2/M phase. Compounds 14d and 14f were certainly able to induce significant levels of apoptosis with necrosis percent 1.34 and 1.84, respectively.

4. Experimental

4.1. Chemistry

Reactions were monitored by TLC analysis using Merck 9385 pre-coated aluminum plate silica gel (Kieselgel 60) with F₂₅₄ indicator thin layer plates. Melting points were determined on Stuart electrothermal melting point apparatus and were uncorrected. IR spectra were recorded as KBr disks on a Shimadzu S8400 IR spectrophotometer. ¹H NMR spectra were carried out on 400 MHz Brucker spectrometer, using TMS as an internal reference. Chemical shift (δ) values are given in parts per million (ppm) relative to CDCl₃ (7.29) or DMSO-d₆ (2.5) and coupling constants (J) in Hertz. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; m, multiplet. High resolution mass spectrometric data were obtained using MicroTOF-Q mass spectrometer (Bruker), Malaysia. Compounds 3, 7a-c, 9 and 12a-c were prepared according to reported procedures [14, 23].
4.1.1 \(N\)-(4-acetylphenyl)-2-(5-amino-3-alkyl or aroyl-1H-1,2,4-triazol-1-yl) acetamide. (10a-d)

General procedure
An equimolar mixture of compound 3, 7a–c and \(N\)-(4-acetylphenyl)-2-bromoacetamide 9 (0.25 g, 1 mmol) in acetonitrile (50 mL) and TEA (0.10 g, 1.2 mmol) was heated at reflux for 4–8 h. The reaction mixture was evaporated to dryness. The residue was crystallized from aqueous ethanol affording products 10a-d.

4.1.1.1 \(N\)-(4-Acetylphenyl)-2-(5-amino-3-(trifluoromethyl)-1H-1,2,4-triazol-1-yl)acetamide. (10a)
White powder (0.25 g, 75 % yield), m.p. 240-242°C, IR (KBr) \(\nu_{\text{max}}\) (cm\(^{-1}\)) 3180- 3470 (NH, NH\(_2\)), 1715 (COCH\(_3\)), 1650 (CONH); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 2.54 (s, 3H, CH\(_3\)), 4.89 (s, 2H, CH\(_2\)), 6.84 (s, 2H, NH\(_2\)), 7.67 (d, 2H, \(J = 8.8\) Hz, Ar-H), 7.93 (d, 2H, \(J = 8.8\) Hz, Ar-H), 10.67 (s, 1H, NH); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) = 26.91, 50.21, 118.91, 121.35, 123.20, 125.46, 130.08, 132.50, 143.24, 149.40, 149.78, 158.34, 165.43, 197.06; HRMS (ESI) \(m/z\): [M-H]\(^-\) calc = 326.0943; found 326.0876 for C\(_{13}\)H\(_{12}\)F\(_3\)N\(_5\)O\(_2\).

4.1.1.2 \(N\)-(4-Acetylphenyl)-2-(5-amino-3-phenyl-1H-1,2,4-triazol-1-yl)acetamide. (10b)
Whitish brown powder (0.22 g, 67 % yield), m.p. 199-201°C, IR (KBr) \(\nu_{\text{max}}\) (cm\(^{-1}\)) 3200- 3450 (NH, NH\(_2\)), 1720 (COCH\(_3\)), 1645 (CONH); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \(\delta\) (ppm): 2.53 (s, 3H, CH\(_3\)), 4.89 (s, 2H, CH\(_2\)), 6.44 (s, 2H, NH\(_2\)), 7.33-7.42 (m, 3H, Ar-H), 7.74 (d, 2H, \(J = 8.8\) Hz, Ar-H), 7.88 (d, 2H, \(J = 6.8\) Hz, Ar-H), 7.98 (d, 2H, \(J = 8.8\) Hz, Ar-H), 10.66 (s, 1H, NH); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \(\delta\) = 26.91, 50.01, 118.88, 119.27, 125.78, 128.88, 129.81, 130.07, 132.43, 143.44, 157.74, 157.94, 166.27, 196.99; HRMS (ESI) \(m/z\): [M-H]\(^-\) calc = 334.1304; found 334.1307 for C\(_{18}\)H\(_{17}\)N\(_5\)O\(_2\).
4.1.1.3 \( \text{N-(4-Acetylphenyl)-2-(5-amino-3-(4-chlorophenyl)-1H-1,2,4-triazol-1-yl)acetamide. (10c)} \)

Yellowish white powder (0.26 g, 70 % yield), m.p. 234-236 °C, IR (KBr) \( v_{\text{max}} \) (cm\(^{-1}\)) 3150- 3450 (NH, NH\(_2\)), 1710 (CO\(_3\)), 1650 (CONH); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 2.53 (s, 3H, CH\(_3\)), 4.89 (s, 2H, CH\(_2\)), 6.48 (s, 2H, NH\(_2\)), 7.46 (d, 2H, \( J = 8.4 \) Hz, Ar-H), 7.74 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 7.87 (d, 2H, \( J = 8.4 \) Hz, Ar-H), 7.98 (d, 2H, \( J = 8 \) Hz, Ar-H), 10.66 (s, 1H, NH); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) = 26.91, 50.03, 118.89, 123.19, 125.46, 127.47, 129.95, 130.07, 131.13, 133.42, 143.40, 157.02, 157.86, 166.17, 197.01; HRMS (ESI) \( m/z \): [M-H] \(^-\) calc = 368.0914; found 368.0922 for C\(_{18}\)H\(_{16}\)ClN\(_5\)O\(_2\).

4.1.1.4 \( \text{N-(4-Acetylphenyl)-2-(5-amino-3-(3,4,5-trimethoxyphenyl)-1H-1,2,4-triazol-1-yl)acetamide. (10d)} \)

White powder (0.29 g, 68 % yield), m.p. 197-199 °C, IR (KBr) \( v_{\text{max}} \) (cm\(^{-1}\)) 3190- 3470 (NH, NH\(_2\)), 1720 (CO\(_3\)), 1650 (CONH); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 2.37 (s, 3H, CH\(_3\)), 3.69 (s, 3H, OCH\(_3\)), 3.80 (s, 6H, 2OCH\(_3\)), 4.37 (s, 2H, CH\(_2\)), 6.18 (s, 2H, NH\(_2\)), 6.59 (d, 2H, \( J = 8.4 \) Hz, Ar-H), 7.16 (s, 2H, Ar-H), 7.66 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 9.96 (s, 1H, NH); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) = 27, 50, 56, 60, 108, 119, 126, 129, 132, 138, 143, 154, 157, 158, 166, 198.

4.1.2 Synthesis of \( \text{N-(4-(1-hydroxyimino)-ethyl)-phenyl)-2-(5-amino-3-alkyl or aroyl-1H-1,2,4-triazol-1-yl)acetamide. (11a-d)} \)

**General procedure**

A mixture of equimolar amounts of the appropriate ketones 10a-d (1 mmol) and hydroxylamine hydrochloride (0.2 g, 3 mmol) in absolute ethanol (30 ml) was heated under reflux for 8-12 h then cooled. The separated solid was filtered off, washed with dil. ammonia solution (10%) and distilled water, dried, and crystallized from aqueous ethanol affording the pure products 11a-d.
4.1.2.1  \( N-(4-(1-(\text{Hydroxyimino})\text{ethylphenyl})-2-(5\text{-amino-3-(trifluoromethyl)}-1H-1,2,4\text{-triazol-1-yl})\text{acetamide. (11a)} \)

Green powder (0.23 g, 71 % yield), m.p. 248-250 °C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 3100- 3480 (OH, NH and NH\(_2\)), 1645 (CONH); \( ^1\text{H} \text{NMR (400 MHz, DMSO-}\text{d}_6 \) \( \delta \) (ppm): 2.13 (s, 3H, CH\(_3\)), 4.90 (s, 2H, CH\(_2\)), 6.82 (s, 2H, NH\(_2\)), 7.58- 7.65 (m, 4H, Ar-H), 10.42 (s, 1H, NH), 11.08 (s, 1H, N-OH); \( ^{13}\text{C} \text{NMR (100 MHz, DMSO-}\text{d}_6 \) \( \delta = \) 11.80, 49.06, 119.27, 121.37, 126.60, 132.63, 139.32, 150.60, 152.82, 158.33, 164.88; HRMS (ESI) \( m/z \): [M-H]\(^-\) calc = 341.0974; found 341.0991 for C\(_{13}\)H\(_{13}\)F\(_3\)N\(_6\)O\(_2\).

4.1.2.2  \( N-(4-(1-(\text{Hydroxyimino})\text{ethylphenyl})-2-(5\text{-amino-3-phenyl-1H-1,2,4-triazol-1-} \)\text{-yl})acetamide. (11b) \)

Yellow powder (0.23 g, 67 % yield), m.p. 230-232 °C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 3000- 3470 (OH, NH and NH\(_2\)), 1650 (CONH); \( ^1\text{H} \text{NMR (400 MHz, DMSO-}\text{d}_6 \) \( \delta \) (ppm): 2.13 (s, 3H, CH\(_3\)), 4.86 (s, 2H, CH\(_2\)), 6.45 (s, 2H, NH\(_2\)), 7.33-7.42 (m, 3H, Ar-H), 7.2 (d, 4H, \( J = 8.8 \) Hz, Ar-H), 7.88 (d, 2H, \( J = 7.2 \) Hz, Ar-H), 10.41 (s, 1H, NH), 11.09 (s, 1H, N-OH); \( ^{13}\text{C} \text{NMR (100 MHz, DMSO-}\text{d}_6 \) \( \delta = \) 11.81, 49.93, 119.26, 125.79, 126.59, 128.89, 128.92, 132.20, 132.54, 139.47, 152.85, 157.64, 157.76, 165.69; HRMS (ESI) \( m/z \): [M-H]\(^-\) calc = 349.1413; found 349.1429 for C\(_{18}\)H\(_{18}\)N\(_6\)O\(_2\).

4.1.2.3  \( N-(4-(1-(\text{Hydroxyimino})\text{ethylphenyl})-2-(5\text{-amino-3-(4-chlorophenyl)-1H-1,2,4-triazol-1-yl})\text{acetamide. (11c)} \)

Yellow powder (0.25 g, 64 % yield), m.p. 258-260 °C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 3150- 3460 (OH, NH and NH\(_2\)), 1655 (CONH); \( ^1\text{H} \text{NMR (400 MHz, DMSO-}\text{d}_6 \) \( \delta \) (ppm): 2.12 (s, 3H, CH\(_3\)), 4.85 (s, 2H, CH\(_2\)), 6.46 (s, 2H, NH\(_2\)), 7.46 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 7.61 (d, 4H, \( J = 8.8 \) Hz, Ar-H), 7.87 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 10.39 (s, 1H, NH), 11.08 (s, 1H, N-OH); \( ^{13}\text{C} \text{NMR (100 MHz, DMSO-}\text{d}_6 \) \( \delta = \) 11.81, 49.94, 119.25, 126.59, 129.01, 131.17, 132.54, 133.40, 136.70, 139.54,
4.1.2.4 \( N-(4-(1-(\text{Hydroxyimino})\text{ethylphenyl})-2-(5\text{-amino}-3-(3,4,5\text{-trimethoxyphenyl})-1H-1,2,4\text{-triazol-1-yl})\text{ acetamide. (11d)}\)

Yellow powder (0.23 g, 53 % yield), m.p. 216-218°C, IR (KBr) \( \nu_{\text{max}} (\text{cm}^{-1}) \) 3000-3480 (OH, NH and NH\(_2\)), 1650 (CONH); \(^1\)HNMR (400 MHz, DMSO-\(\text{d}_6\)) \( \delta \) (ppm): 2.13 (s, 3H, CH\(_3\)), 3.69 (s, 3H, OCH\(_3\)), 3.83 (s, 6H, 2OCH\(_3\)), 4.87 (s, 2H, CH\(_2\)), 6.53 (s, 2H, NH\(_2\)), 7.16 (s, 2H, Ar-H), 7.54-7.60 (m, 4H, Ar-H), 10.43 (s, 1H, NH), 11.08 (s, 1H, N-OH); \(^{13}\)C NMR (100 MHz, DMSO-\(\text{d}_6\)) \( \delta = 11.87, 49.81, 56.26, 60.62, 103.05, 119.90, 126.54, 126.93, 132.60, 138.43, 139.26, 153.29, 153.36, 157.13, 157.27, 165.51; HRMS (ESI) \( m/z : [M+H]^+ \) calc = 441.1886; found 441.1893 for C\(_{21}\)H\(_{24}\)N\(_6\)O\(_5\).

4.1.3 Synthesis of 2,5-di (substitutedphenyl)thiazolo[3,2-b][1,2,4]triazole. (14a-f)

**General method**

A mixture of mercaptotriazole 12a-c (10 mmol) and substituted acetophenones 13a-b (15 mmol) was refluxed in acetic acid (15 ml) containing a few drops of conc. sulphuric for 2-3h. The reaction mixture was cooled and neutralized with NH\(_4\)OH solution. The resulting precipitate was collected by filtration, washed several times with water, dried well and crystallized from ethanol or methanol to give the corresponding 14a-f.

**2,5-Diphenylthiazolo[3,2-b][1,2,4]triazole. (14a)**

White powder (0.17 g, 63 % yield), m.p. 187-189°C, IR (KBr) \( \nu_{\text{max}} (\text{cm}^{-1}) \) 1650 (C=N); \(^1\)HNMR (400 MHz, DMSO-\(\text{d}_6\)) \( \delta \) (ppm): 7.47-7.55 (m, 3H, Ar-H), 7.61 (t, 2H, \( J = 7.6 \) Hz, Ar-H), 7.95 (s, 1H, Ar-H), 8.16 (d, 1H, \( J = 7.2 \) Hz, Ar-H), 8.29 (d, 1H, \( J = 8.4 \) Hz, Ar-H); \(^{13}\)C NMR (100 MHz, DMSO-\(\text{d}_6\)) \( \delta = 111.03, 126.76, 126.79, 128.13, 129.39, 129.48, 130.18, 130.38, 131.18, 132.11, 158.13, 166.21; HRMS (ESI) \( m/z : [M+H]^+ \) calc = 278.0752; found 278.0756 for C\(_{16}\)H\(_{11}\)N\(_3\)S.
5-(4-Chlorophenyl)-2-phenylthiazolo[3,2-b][1,2,4]triazole, (14b)

White powder (0.26 g, 83 % yield), m.p. 181-183°C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1650 (C=N); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 7.48-7.56 (m, 3H, Ar-H), 7.68 (d, 2H, \( J = 8.4 \) Hz, Ar-H), 8.01 (s, 1H, Ar-H), 8.15 (d, 2H, \( J = 6 \) Hz, Ar-H), 8.34 (d, 2H, \( J = 8.4 \) Hz, Ar-H); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta = 111.78, 126.78, 126.95, 128.50, 129.42, 129.55, 130.47, 130.96, 131.05, 134.74, 158.15, 166.25; HRMS (ESI) \( m/z: [M+H]^+ \) calc = 312.0362; found 312.0362 for C\(_{16}\)H\(_{10}\)ClN\(_3\)S.

2-(4-Methoxyphenyl)-5-phenylthiazolo[3,2-b][1,2,4]triazole. (14c)

White powder (0.23 g, 74 % yield), m.p. 201-203°C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1632 (C=N); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 3.83 (s, 3H, OCH\(_3\)), 7.08 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 7.51-7.54 (m, 1H, Ar-H), 7.60 (d, 2H, \( J = 8 \) Hz, Ar-H), 7.91 (s, 1H, Ar-H), 8.07 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 8.28 (d, 2H, \( J = 8 \) Hz, Ar-H); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta = 55.72, 110.50, 114.75, 123.71, 126.76, 128.31, 128.60, 129.45, 130.12, 132.08, 157.98, 161.04, 166.22; HRMS (ESI) \( m/z: [M+H]^+ \) calc = 308.0858; found 308.0860 for C\(_{17}\)H\(_{13}\)N\(_3\)OS.

5-(4-Chlorophenyl)-2-(4-methoxyphenyl)thiazolo[3,2-b][1,2,4]triazole. (14d)

White powder (0.26 g, 77 % yield), m.p. 179-181°C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1650 (C=N); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 3.83 (s, 3H, OCH\(_3\)), 7.08 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 7.67 (d, 2H, \( J = 8.4 \) Hz, Ar-H), 7.97 (s, 1H, Ar-H), 8.07 (d, 2H, \( J = 8.8 \) Hz, Ar-H), 8.33 (d, 2H, \( J = 8.4 \) Hz, Ar-H); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta = 55.75, 111.29, 114.76, 123.68, 127.08, 128.32, 128.46, 129.52, 130.90, 134.64, 157.98, 161.08, 166.28; HRMS (ESI) \( m/z: [M+H]^+ \) calc = 342.0468; found 342.0468 for C\(_{17}\)H\(_{12}\)ClN\(_3\)OS.

2-(3,4,5-Trimethoxyphenyl)-5-phenylthiazolo[3,2-b][1,2,4]triazole. (14e)
White powder (0.25 g, 67 % yield), m.p. 165-167°C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1651 (C=N); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 3.74 (s, 3H, OCH\(_3\)), 3.89 (s, 6H, 2OCH\(_3\)), 7.42 (s, 2H, Ar-H), 7.53 (t, 1H, \( J = 7.2 \) Hz, Ar-H), 7.61 (t, 2H, \( J = 7.2 \) Hz, Ar-H), 7.91 (s, 1H, Het-H), 8.28 (d, 2H, \( J = 8 \) Hz, Ar-H); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) = 56.46, 60.63, 104.11, 110.96, 126.70, 126.87, 128.17, 129.50, 130.15, 132.18, 139.49, 153.68, 158.01, 166.09; HRMS (ESI) \( m/z \): \([\text{M+H}]^+\) \text{calc} = 368.1069; found 368.1079 for C\(_{19}\)H\(_{17}\)N\(_3\)O\(_3\)S.

5-(4-Chlorophenyl)-2-(3,4,5-trimethoxyphenyl)thiazolo[3,2-b][1,2,4]triazole. (14f)

White powder (0.28 g, 69 % yield), m.p. 169-171°C, IR (KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)) 1616 (C=N); \(^1\)HNMR (400 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 3.74 (s, 3H, OCH\(_3\)), 3.89 (s, 6H, 2OCH\(_3\)), 7.42 (s, 2H, Ar-H), 7.66 (d, 2H, \( J = 8.4 \) Hz, Ar-H), 7.97 (s, 1H, Ar-H), 8.31 (d, 2H, \( J = 8.4 \) Hz, Ar-H); \(^13\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) = 56.57, 60.62, 104.14, 111.69, 126.61, 127.08, 126.61, 127.08, 128.56, 129.53, 130.98, 134.67, 139.54, 153.68, 157.98, 166.11; HRMS (ESI) \( m/z \): \([\text{M+H}]^+\) \text{calc} = 402.0679; found 402.0680 for C\(_{19}\)H\(_{16}\)ClN\(_3\)O\(_3\)S.

4.2. Biology

4.2.1 Evaluation of \textit{in vitro} antiproliferative activity for compounds selected by NCI

The methodology of the NCI anticancer screening has been described in detail elsewhere (http://www.dtp.nci.nih.gov). Primary anticancer assay was performed at approximately 60 human tumor cell lines panel derived from nine neoplastic diseases, in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda, USA. Detailed methods are described in supplementary material related to this article.

4.2.2 Cell cycle analysis and apoptosis assay

4.2.2.1. Cell cycle analysis and apoptosis detection.
The A549 cell line was used for Cell cycle analysis and apoptosis detection. Assay was performed as reported previously [32]. (Appendix A)

4.2.2.2. Apoptosis assay.

The A549 cell line was used for apoptosis assay as performed previously using A549 cell line [31]. Final analysis was done by flow cytometry using FACScalibur (Becton Dickinson). The cell cycle distributions were calculated using Phoenix Flow Systems and Verity Software House. Detailed methodology is provided in appendix A [32].

4.2.3 MTT assay

To investigate the effect of synthesized compounds on mammary epithelial cells (MCF-10A), MTT assay was performed (Appendix A) [1, 33].

4.2.4 Assay for antiproliferative effect

For evaluation of antiproliferative effects Propidium iodide fluorescence assay was carried out on Panc-1 (pancreas cancer), PaCa-2 (pancreatic carcinoma), MCF-7 (breast cancer): A-549 (epithelial): HT-29 (colon cancer), H-460 (lung cancer) and PC-3 (prostate cancer) cell lines as reported previously by established method (Appendix A) [1, 33].

4.2.5 Tubulin polymerization assay

The activity of compounds on tubulin polymerization was investigated by Tubulin Polymerization Assay Kit (Cytoskeleton Inc., Denver, CO, USA) as per instructions of supplier and details are summarized in appendix A [1, 33].

4.2.6 EGFR inhibitory assay

EGFR assay was performed by established reported method for selected synthetic compounds and details are summarized in appendix A [1, 33].
4.2.7 BRAF kinase assay

Each compound in this study was subjected to V600E mutant BRAF kinase assay in triplicate as reported previously (Appendix A) [1, 33].

Appendix A

Detail methodology regarding titles Evaluation of in vitro antiproliferative activity for compounds selected by NCI, Cell cycle analysis and apoptosis assay, MTT assay, Assay for antiproliferative effect, Tubulin polymerization assay, EGFR inhibitory assay and BRAF kinase assay provided in appendix A. Appendix A related to this article can be found at http://dx........

Acknowledgment

The authors of this work are so grateful to the members of Drug and Herbal Research Centre, Faculty of pharmacy, University Kebangsaan Malaysia (UKM) for helping in performing in vitro antiproliferative, kinase and tubulin assays.

Reference


Appendix A

Synthesis, anticancer activity and molecular modeling studies of 1,2,4-triazole derivatives as EGFR inhibitors

Hany A. M. El-Sherief\textsuperscript{1,3*}, Bahaa G. M. Youssif\textsuperscript{2,3}, Syed Nasir Abbas Bukhari\textsuperscript{3}, Ahmed H. Abdelazeem\textsuperscript{3}, Mohamed Abdel-Aziz\textsuperscript{5*}, Hamdy M. Abdel-Rahman\textsuperscript{1,6}

\textsuperscript{1}Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Nahda University, Beni-Suef, Egypt

\textsuperscript{2}Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Assiut University, Assiut 71526, Egypt

\textsuperscript{3}Department of Pharmaceutical Chemistry, College of Pharmacy, Jouf University, Aljouf, Sakaka 2014, Saudi Arabia

\textsuperscript{4}Department of Medicinal Chemistry, Faculty of Pharmacy, Beni-Suef University, Beni-Suef 62514, Egypt

\textsuperscript{5}Department of Medicinal Chemistry, Faculty of Pharmacy, Minia University, 61519-Minia, Egypt

\textsuperscript{6}Department of Medicinal Chemistry, Faculty of Pharmacy, Assiut University, 71526-Assiut, Egypt.
Contents:

Table 1: Single concentration mean graph growth inhibition of nine different cancer cell types for compounds 10 (a,c) and 11 (a-d).

Table 2: Single concentration mean graph growth inhibition of nine different cancer cell types for compounds 14a-e.

Table 3: NCI in vitro testing results of compound 14d at five dose level in µM.

4.2.1 Evaluation of in vitro antiproliferative activity for compounds selected by NCI.

4.2.2 Cell cycle analysis and apoptosis assay

4.2.2.1. Cell cycle analysis and apoptosis detection.

4.2.2.2. Apoptosis assay.

4.2.3 MTT assay

4.2.4 Assay for antiproliferative effect

4.2.5 Tubulin polymerization assay

4.2.6 EGFR inhibitory assay

4.2.7 BRAF kinase assay
### Table 1: Single concentration mean graph growth inhibition of nine different cancer cell types for compounds 10 (a-c) and 11 (a-d)

<table>
<thead>
<tr>
<th>Panel</th>
<th>Cell line</th>
<th>Growth inhibition percent % at 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10a</td>
</tr>
<tr>
<td>Leukemia</td>
<td>CCRF-CEM</td>
<td>6.78</td>
</tr>
<tr>
<td></td>
<td>HL-60(TB)</td>
<td>15.42</td>
</tr>
<tr>
<td></td>
<td>K-562</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MOLT-4</td>
<td>7.46</td>
</tr>
<tr>
<td></td>
<td>RPMI-8226</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>-</td>
</tr>
<tr>
<td>Non-Small Cell Lung Cancer</td>
<td>A549/ATCC</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>EKVX</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HOP-62</td>
<td>9.94</td>
</tr>
<tr>
<td></td>
<td>HOP-92</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NCI-H226</td>
<td>8.52</td>
</tr>
<tr>
<td></td>
<td>NCI-H23</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NCI-H322M</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NCI-H460</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NCI-H522</td>
<td>21</td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>COLO 205</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HCCC-2998</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HCT-116</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HCT-15</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>15.96</td>
</tr>
<tr>
<td></td>
<td>KM12</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SW-620</td>
<td>-</td>
</tr>
<tr>
<td>CNS Cancer</td>
<td>SF-268</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF-295</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF-539</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SNB-19</td>
<td>4.37</td>
</tr>
<tr>
<td></td>
<td>SNB-75</td>
<td>12.54</td>
</tr>
<tr>
<td></td>
<td>U251</td>
<td>-</td>
</tr>
<tr>
<td>Melanoma</td>
<td>LOX IMVI</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MALME-3M</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M14</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-435</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-2</td>
<td>4.03</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>UACC-257</td>
<td>14.63</td>
</tr>
<tr>
<td></td>
<td>UACC-62</td>
<td>-</td>
</tr>
<tr>
<td>Ovarian Cancer</td>
<td>IGROVI</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OVCAR-3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OVCAR-4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OVCAR-5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OVCAR-8</td>
<td>13.31</td>
</tr>
<tr>
<td></td>
<td>NCI/ADR-RES</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK-OV-3</td>
<td>7.02</td>
</tr>
<tr>
<td>Renal Cancer</td>
<td>786-0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A498</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ACHN</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>RXF 393</td>
<td>SN12C</td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Single concentration mean graph growth inhibition of nine different cancer cell types for compounds 14a-e

<table>
<thead>
<tr>
<th>Panel</th>
<th>Cell line</th>
<th>Growth inhibition percent % at 10 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>14a</td>
</tr>
<tr>
<td>Leukemia</td>
<td>CCRF-CEM</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>HL-60(TB)</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>K-562</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>MOLT-4</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>RPMI-8226</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>9</td>
</tr>
<tr>
<td>Non-Small Cell Lung Cancer</td>
<td>A549/ATCC</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>EKVX</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>HOP-62</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>HOP-92</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>NCI-H226</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>NCI-H23</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>NCI-H322M</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>NCI-H460</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>NCI-H522</td>
<td>19</td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>COLO 205</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HCC-2998</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HCT-116</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>HCT-15</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>KM12</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>SW-620</td>
<td>5</td>
</tr>
<tr>
<td>CNS Cancer</td>
<td>SF-268</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>SF-295</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF-539</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SNB-19</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>SNB-75</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>U251</td>
<td>6</td>
</tr>
<tr>
<td>Melanoma</td>
<td>LOX IMVI</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>MALME-3M</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>M14</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-435</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-2</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-28</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>UACC-257</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>UACC-62</td>
<td>33</td>
</tr>
<tr>
<td>Ovarian Cancer</td>
<td>IGROV1</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>OVCAR-3</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>OVCAR-4</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>OVCAR-5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OVCAR-8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NCI/ADR-RES</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>SK-OV-3</td>
<td>5</td>
</tr>
<tr>
<td>Renal Cancer</td>
<td>786-0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A498</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>ACHN</td>
<td>RXF 393</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>---------</td>
</tr>
<tr>
<td><strong>Prostate Cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>14</td>
<td>29.12</td>
</tr>
</tbody>
</table>
Table 3: NCI in vitro testing results of compound 14d at five dose level in µM.

<table>
<thead>
<tr>
<th>Panel</th>
<th>subpanel</th>
<th>Conc. per cell line</th>
<th>Conc. per cell line</th>
<th>Conc. per cell line</th>
<th>Conc. per cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GI&lt;sub&gt;50&lt;/sub&gt;</td>
<td>TGI</td>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subpanel</td>
<td>Selectivity ratio</td>
<td>MID&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>MID&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(MID&lt;sup&gt;a&lt;/sup&gt;/MI</td>
<td>D&lt;sup&gt;b&lt;/sup&gt;)</td>
<td></td>
</tr>
<tr>
<td>Leukemia</td>
<td>CCRF-CEM</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HL-60(TB)</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>K-562</td>
<td>29.4</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MOLT-4</td>
<td>11.3</td>
<td>24.82</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RPMI-8226</td>
<td>3.67</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SR</td>
<td>4.53</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Small Cell Lung</td>
<td>A549/ATCC</td>
<td>1.59</td>
<td>5.89</td>
<td>46.9</td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>EKVX</td>
<td>4.82</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HOP-62</td>
<td>2.36</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HOP-92</td>
<td>0.91</td>
<td>5.048</td>
<td>2.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCI-H226</td>
<td>11.1</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCI-H23</td>
<td>6.66</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCI-H322M</td>
<td>1.76</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCI-H460</td>
<td>1.33</td>
<td>4.00</td>
<td>18.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCI-H522</td>
<td>14.9</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td>Colon Cancer</td>
<td>COLO 205</td>
<td>2.56</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCC-2998</td>
<td>≥50</td>
<td>39.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT-116</td>
<td>1.21</td>
<td>3.48</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCT-15</td>
<td>24.5</td>
<td>14.15</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>2.96</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>KM12</td>
<td>12.2</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SW-620</td>
<td>5.51</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td>CNS Cancer</td>
<td>SF-268</td>
<td>48.00</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SF-295</td>
<td>2.81</td>
<td>31.8</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SF-539</td>
<td>1.85</td>
<td>21.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SNB-19</td>
<td>2.47</td>
<td>9.63</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>Cancer Type</td>
<td>Cell Line</td>
<td>LOX IMVI</td>
<td>IMVI</td>
<td>VAF</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------</td>
<td>----------</td>
<td>--------</td>
<td>-------</td>
<td></td>
</tr>
<tr>
<td>Melanoma</td>
<td>SNB-75</td>
<td>1.61</td>
<td>6.85</td>
<td>23.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U251</td>
<td>1.03</td>
<td>2.29</td>
<td>5.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LOX IMVI</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MALME-3M</td>
<td>2.71</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>M14</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MDA-MB-435</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-MEL-2</td>
<td>≥50</td>
<td>36.10</td>
<td>0.409</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-MEL-28</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-MEL-5</td>
<td>14.00</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UACC-257</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UACC-62</td>
<td>8.24</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td>Ovarian Cancer</td>
<td>IGROV1</td>
<td>1.15</td>
<td>8.13</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVCAR-3</td>
<td>1.72</td>
<td>7.20</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVCAR-4</td>
<td>0.766</td>
<td>1.63</td>
<td>3.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVCAR-5</td>
<td>2.78</td>
<td>3.49</td>
<td>4.23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVCAR-8</td>
<td>2.60</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NCI/ADR-RES</td>
<td>3.75</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SK-OV-3</td>
<td>11.70</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td>Renal Cancer</td>
<td>786-0</td>
<td>1.06</td>
<td>2.41</td>
<td>6.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACHN</td>
<td>0.927</td>
<td>2.02</td>
<td>4.41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAKI-1</td>
<td>1.79</td>
<td>8.92</td>
<td>48.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RXF 393</td>
<td>5.99</td>
<td>2.11</td>
<td>6.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SN12C</td>
<td>1.84</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TK-10</td>
<td>2.01</td>
<td>5.62</td>
<td>34.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UO-31</td>
<td>1.19</td>
<td>4.51</td>
<td>30.60</td>
<td></td>
</tr>
<tr>
<td>Prostate Cancer</td>
<td>PC-3</td>
<td>≥50</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DU-145</td>
<td>≥50</td>
<td>50</td>
<td>0.295</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥50</td>
<td>50</td>
<td>0.295</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MCF7</td>
<td>2.62</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td>Breast Cancer</td>
<td>MDA-MB-231/ATCC</td>
<td>2.06</td>
<td>16.7</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HS 578T</td>
<td>2.31</td>
<td>9.30</td>
<td>1.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥50</td>
<td>50</td>
<td>≥50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BT-549</td>
<td>43.2</td>
<td>≥50</td>
<td>≥50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T-47D</td>
<td>3.54</td>
<td>$50</td>
<td>$50</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------</td>
<td>------</td>
<td>-----</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>2.08</td>
<td>$50</td>
<td>$50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MID*</td>
<td></td>
<td>14.76</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.2. Biology

4.2.1 Evaluation of \textit{in vitro} antiproliferative activity for compounds selected by NCI

The methodology of the NCI anticancer screening has been described in detail elsewhere (http://www.dtp.nci.nih.gov). Briefly, the primary anticancer assay was performed at approximately 60 human tumor cell lines panel derived from nine neoplastic diseases, in accordance with the protocol of the Drug Evaluation Branch, National Cancer Institute, Bethesda, USA. Tested compounds were added to the culture at a single concentration ($10^{-5}$ M) and the cultures were incubated for 48 h. End point determinations were made with a protein binding dye, SRB. Results for each tested compound were reported as the percent of growth of the treated cells when compared to the untreated control cells. The percentage growth was evaluated spectrophotometrically versus controls not treated with test agents. The cytotoxic and/or growth inhibitory effects of the most active selected compound were tested \textit{in vitro} against the full panel of about 60 human tumor cell lines at 10-fold dilutions of five concentrations ranging from $10^{-4}$ to $10^{-8}$ M. A 48-h continuous drug exposure protocol was followed and an SRB protein assay was used to estimate cell viability or growth. Using the seven absorbance measurements [time zero (Tz), control growth in the absence of drug (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage growth was calculated at each of the drug concentrations levels. Percentage growth inhibition was calculated as: $\{(Ti - Tz)/(C - Tz)\} \times 100$ for concentrations for which Ti > Tz, and $\{(Ti - Tz)/Tz\} \times 100$ for concentrations for which Ti < Tz.

Three-dose response parameters were calculated for each compound. Growth inhibition of 50% (GI\textsubscript{50}) was calculated from $\{(Ti - Tz)/(C - Tz)\} \times 100 = 50$, which is
the drug concentration resulting in a 50% lower net protein increase in the treated cells (measured by SRB staining) as compared to the net protein increase seen in the control cells. The drug concentration resulting in TGI was calculated from Ti = Tz. The LC$_{50}$ (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated from [(Ti - Tz)/Tz] × 100 = -50. Values were calculated for each of these three parameters if the level of activity is reached; however, if the effect was not reached or was exceeded, the value for that parameter was expressed as more or less than the maximum or minimum concentration tested. The log GI$_{50}$, log TGI, and log LC$_{50}$ were then determined, defined as the mean of the logs of the individual GI$_{50}$, TGI, and LC$_{50}$ values. The lowest values are obtained with the most sensitive cell lines. Compound having log GI$_{50}$ values -4 and <-4 was declared to be active.

4.2.2 Cell cycle analysis and apoptosis assay

4.2.2.1. Cell cycle analysis and apoptosis detection.

The A549 cell line was treated with 1.4 µM of compound 14d and 0.4 µM of compound 14f, for 24 h. After treatment, the cells were suspended in 0.5 mL of PBS, collected by centrifugation, and fixed in ice-cold 70% (v/v) ethanol washed with PBS, resuspended with 0.1 mg/mL RNase, stained with 40 mg/ml PI, and analyzed by flow cytometry using FACScalibur (Becton Dickinson). The cell cycle distributions were calculated using Phoenix Flow Systems and Verity Software House [31].

4.2.2.2. Apoptosis assay.

The A549 was treated with 1.4 µM of compound 14d and 0.4 µM of compound 14f for 24 h. After treatment, the cells were suspended in 0.5 mL of PBS, collected by centrifugation, and fixed in ice-cold 70% (v/v) ethanol, centrifuged the ethanol-
suspended cells for 5 min, suspended in 5 mL PBS and centrifuged for 5 min, re-
suspended with 1 mL PI staining solution (0.1 mg/ml RNase) + PE Annexin V
(component no. 51-65875X) and kept in dark at 37 °C for 10 min, finally analyzed by
flow cytometry using FACScalibur (Becton Dickinson). The cell cycle distributions
were calculated using Phoenix Flow Systems and Verity Software House [31].

4.2.3 MTT assay
To investigate the effect of synthesized compounds on mammary epithelial cells
(MCF-10A), MTT assay was performed. The cells were propagated in medium
consisting of Ham’s F-12 medium/ Dulbecco’s modified Eagle’s medium (DMEM)
(1:1) supplemented with 10% foetal calf serum, 2 μM glutamine, insulin (10 μg/mL),
hydrocortisone (500 ng/mL) and epidermal growth factor (20 ng/mL). Trypsin
ethylenediamine tetra acetic acid (EDTA) was used to passage the cells after every 2-3
days. 96-well flat-bottomed cell culture plates were used to seed the cells at a
density of 10^4 cells mL^-1. The medium was aspirated from all the wells of culture
plates after 24 h followed by the addition of synthesized compounds (in 200 μL
medium to yield a final concentration of 0.1% (v/v) dimethylsulfoxide) into individual
wells of the plates. Four wells were designated to a single compound. The plates were
allowed to incubate at 37°C for 96 h. Afterwards, the medium was aspirated and 3-
[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (0.4 mg/mL) in
medium was added to each well and subsequently incubated for 3 h. The medium was
aspirated and 150 μL dimethyl sulfoxide (DMSO) was added to each well. The plates
were vortexed followed by the measurement of absorbance at 540 nm on a microplate
reader. The results were presented as inhibition (%) of proliferation in contrast to
controls comprising 0.1% DMSO [1, 32].

4.2.4 Assay for antiproliferative effect
Propidium iodide fluorescence assay was carried out on Panc-1 (pancreas cancer), PaCa-2 (pancreatic carcinoma), MCF-7 (breast cancer): A-549 (epithelial): HT-29 (colon cancer), H-460 (lung cancer) and PC-3 (prostate cancer) cell lines to investigate the antiproliferative activity of compounds. Propidium iodide is a fluorescence dye which possesses ability to attach with DNA, therefore providing a precise and quick method for the calculation of total nuclear DNA. PI is incapable of crossing the plasma membrane and its fluorescence signal intensity is directly proportional to the amount of cellular DNA. Thus, cells with damaged plasma membranes or altered permeability are totaled as dead ones. To perform the assay, cells were seeded in 96-well flat-bottomed culture plates at a density of 3000-7500 cells/well in 200 µl medium and incubated at 37 °C for 24 h in humidified 5% CO₂/95% air atmosphere. Later, the compounds at 10 µM concentrations (in 0.1% DMSO) were added in triplicate wells while 0.1% DMSO served as control, followed by a 48 h incubation of plates. The medium was removed and 25 µl PI (50 µg/mL in water/medium) was added in each well. The plates were then frozen at -80 °C for 24 h, followed by thawing and equilibration to 25°C. The readings were recorded at excitation and emission wavelengths of 530 and 620 nm using a fluorometer (Polar-Star BMG Tech). Following formula was used to calculate the cytotoxicity (%) of compounds:

\[
\% \text{ Cytotoxicity} = \frac{A_c - A_{TC}}{A_c} \times 100
\]

Where \(A_c\) = Absorbance of control and \(A_{TC}\) = Absorbance of treated cells. To equate the results, erlotinib was used as positive control [1, 32].

4.2.5 Tubulin polymerization assay

The activity of compounds on tubulin polymerization was investigated by Tubulin Polymerization Assay Kit (Cytoskeleton Inc., Denver, CO, USA), which works via
fluorescent reporter enhancement. The fluorescence of compounds (dissolved in DMSO at 5 and 25 µM concentration) was recorded in triplicates using FLUO star OPTIMA. Docetaxel and vincristine (Apoteket AB, Sweden) served as positive stabilizing and destabilizing controls. Both were used at 3 µM concentration in PBS [1, 32].

**4.2.6 EGFR inhibitory assay**

Baculoviral expression vectors such as pFASTBacHTc and pBlueBacHis2B were separately used to clone 1.6 kb cDNA encoding for EGFR cytoplasmic domain (EGFR-CD, amino acids 645–1186) were cloned into, separately. 5′ upstream to the EGFR sequence contained a sequence that encodes (His)$_6$. For protein expression, Sf-9 cells were infected for 3 days. Sf-9 cell pellets were solubilized at pH 7.4 at 0° C for 20 min in a buffer comprising 16 µg/mL benzamidine HCl, 10 µg/mL pepstatin, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 100 µM sodium vanadate, 10 µM ammonium molybdate, 1% Triton, 10 mM NaCl and 50 mM HEPES, followed by 20 min centrifugation. Using an equilibrated Ni-NTA superflow packed column, the crude extract supernatant was passed through and washed first with 10 mM and later with 100 mM imidazole for the elimination of nonspecifically bound material. Histidine-tagged proteins were eluted first with 250 and later with 500 mM imidazole followed by dialysis against 10% glycerol, 20 mM HEPES, 50 mM NaCl, and 1 µg/mL each of pepstatin, leupeptin and aprotinin for 2 h. This purification was carried out either on ice or at 4 °C. On the basis of DELFIA/Time-Resolved Fluorometry, EGFR kinase assay was performed to measure the level of autophosphorylation. DMSO (100%) was used to dissolve the compounds, followed by dilution to suitable concentrations using 25 mM HEPES at pH 7.4. In every well, 10 µL (5 ng for EGFR) recombinant
enzyme (1:80 dilution in 100 mM HEPES) was incubated with 10 µL compound at 25°C for 10 min, followed by addition of 10 µL 5X buffer (containing 1 mM DTT, 100 µM Na₃VO₄, 2 mM MnCl₂ and 20 mM HEPES) and 20 µL 0.1 mM ATP-50 mM MgCl₂ for 1 h. By incubating the enzyme with or without ATP-MgCl₂, positive and negative controls were included in every plate. After incubation, liquid was removed, and wash buffer was used to wash the plates thrice. Each well of plate was added with 75 µL (400 ng) europium-labeled antiphosphotyrosine antibody for another 1 h, followed by washing. After adding the enhancement solution, the signal was detected (with excitation and emission at 340 at 615 nm, respectively) using Victor (Wallac Inc.). Following equation was used to calculate the autophosphorylation inhibition (%) by the compounds:

$$100\% - \frac{[\text{negative control}]}{[\text{positive control}]} - \frac{\text{negative control}}{\text{positive control}}$$

The IC₅₀ was calculated using the curves of inhibition (%) with eight concentrations of compound. Most of the signal detected by antiphosphotyrosine antibody is from EGFR, as the impurities in the enzyme preparation are quite low [1, 32].

4.2.7 BRAF kinase assay

Each compound in this study was subjected to V600E mutant BRAF kinase assay in triplicate. 1 µL drug and 4 µL assay dilution buffer were pre-incubated with 7.5 ng mouse full-length GST-tagged BRAF₉⁶⁰E (Invitrogen, PV3849) at 25°C for 1 h. The assay was started by adding 5 µL solution comprising 200 ng recombinant human full length, N-terminal His-tagged MEK1 (Invitrogen), 30 mM MgCl₂ and 200 µM ATP in assay dilution buffer, followed by continuation at 25°C for 25 min. Using 5X protein denaturing buffer (LDS) solution (5 µL), the assay was quenched. Further denaturing of protein was performed by heating at 70°C for 5 min. Electrophoresis was
performed at 200 V by loading 10 µL of each reaction into a 15-well 4-12% precast NuPage gel plate (Invitrogen). Once the electrophoresis was finished, the front (containing additional hot ATP) was cut from the gel and subsequently discarded. A phosphor screen was used to develop the dried gel. A reaction containing no inhibitor was used as positive control whereas a reaction without active enzyme served as negative control. ELISA kits (Invitrogen) were used as per manufacturer’s protocol to investigate the effect of compounds on cell based pERK1/2 activity in cancer cells [1, 32].
Highlights

- A series of novel compounds 10a-d, 11a-d and thiazolo[3,2-b][1,2,4]-triazoles 14a-f was designed and synthesized.

- Compounds 10a, c, 11a-d, and 14a-e were selected for anticancer evaluation against the NCI 60 cell line assay with 14d for the *In vitro* five dose assay.

- Compounds 10a, 11a, 14d and 14f showed potential activities in MTT assay.

- Compounds 14d and 14f were selected for cell cycle analysis, apoptosis assay and for their inhibitory activity against EGFR, BRAF and tubulin anticancer targets.

- Molecular modeling for compounds 14d and 14f were performed in the 3D crystal structures of EGFR (PDB ID:1M17).
学霸图书馆

www.xuebalib.com

本文献由“学霸图书馆-文献云下载”收集自网络，仅供学习交流使用。

学霸图书馆（www.xuebalib.com）是一个“整合众多图书馆数据库资源，
提供一站式文献检索和下载服务”的24小时在线不限IP图书馆。

图书馆致力于便利、促进学习与科研，提供最强文献下载服务。

图书馆导航：

图书馆首页    文献云下载    图书馆入口    外文数据库大全    疑难文献辅助工具