MedChemComm

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: C. Bagul, G. K. Rao, V. K. K. Makani, J. R. Tamboli, M. P. Bhadra and A. Kamal, *Med. Chem. Commun.*, 2017, DOI: 10.1039/C7MD00193B.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/medchemcomm

View Article Online
DOI: 10.1039/C7MD00193B

Journal Name

ARTICLE

Synthesis and biological evaluation of Chalcone-linked pyrazolo[1,5-*a*]pyrimidines as potential anticancer agents

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Chandrakant Bagul,[a,†] Garikapati Koteswara Rao,[c,†] Venkata Krishna Kanth Makani,[c] Jaki R. Tamboli,[b] Manika Pal-Bhadra,*[,c] and Ahmed Kamal*[,a,b]

A series of pyrazolo[1,5-*a*]pyrimidines substituted at C5 with 1-phenylprop-2-en-1-one (**6a-q**) and 3-phenylprop-2-en-1-one (**7a-k**) was synthesized and evaluated for its antiproliferative activity. Among them, **6h** was found to be the most active compound with IC_{50} 2.6 µM against the MDA-MB-231 cell line. Antiproliferative activity of this series of compounds ranged from 2.6 – 34.9 µM against A549 (lung cancer), MDA-MB-231 (breast cancer) and DU-145 (prostate cancer) cell lines. FACS analysis revealed that these hybrids arrest cell cycle at subG1 phase. Western blot analysis and immu nofluorescence assay showed the inhibition of EGFR and STAT3 axis which plays an important role in the cell survival and apoptosis. Western blot and RT-PCR analysis that displayed an increase in apoptotic proteins such as p53, p21 and Bax, and decrease in the antiapoptotic protein Bcl-2 and procaspase-9, confirmed the ability of these hybrids to trigger cell death by apoptosis. Molecular docking studies described the binding of these hybrids to the ATP binding site of EGFR.

Introduction

Cancer has become a leading cause of mortality which solely accounted for 8.5 million deaths (17% of total deaths) in 2012 and is estimated to cause 13.1 million deaths in 2030.[1,2] Despite the advance in diagnosis and treatment the burden of cancer is increasing every year. Despite the fact that, enormous efforts are being carried out for developing novel molecules and newer chemotherapeutic strategies to treat different types of cancers, this remains a key concern across the globe.[3,4]

Protein tyrosine kinase inhibitors are of tremendous interest due to their potential as therapeutic agents for the treatment of a variety of diseases, particularly cancer.[5-7] Among the protein tyrosine kinases, epidermal growth factor receptor (EGFR) has emerged as a key and validated target for the development of anticancer agents.[8-10] Over ten drugs including erlotinib[11] **1**, gefitinib, lapatinib, icotinib, vandetanib etc., which act on EGFR have been approved by the United States Food and Drug Administration (US FDA) in the past two decades and several molecules, such as BMS-690514[12,13] are currently in clinical trials. EGFR transduces the signals across the membrane important for cellular functions such as proliferation,[10,14] angiogenesis, invasiveness, decreased apoptosis and differentiation,[15] through which it regulates the development and progression of several types of cancers. EGFR is overexpressed in many solid tumors and has been associated with poor prognosis.[16-18] It plays a central role in the development of non-small-cell lung cancer (NSCLC),[19,20] as well as breast cancer.[21,22] Therefore the molecules which are inhibiting EGFR are effective in the treatment of cancer. Since, EGFR has become a fascinating and validated target for developing new anticancer agents, exploration of EGFR with newly synthesized hybrids is likely to be a fruitful research area.

Chalcones (1,3-diarylprop-2-en-1-one) represents one of the largest class of plant metabolites important precursors in the biosynthesis of flavonoids and related compounds.[23] It remain an attractive scaffold for researchers due to their abundance in plants, ease of synthesis, availability of various ways to introduce diversity in the core structure and ability to confer drug-like properties to the synthesized compounds. Derivatives of chalcone are known to possess broad spectrum of biological activities, such as antidiabetic, antihypertensive, antihistaminic, antiretroviral, anti-inflammatory, antioxidant, antibacterial, antituberculosis, and anticancer.[24-26] Moreover, chalcones are successfully employed as tubulin inhibitors (**2**)[27] and EGFR inhibitors (**3, 4**)[28,29] towards the development of anticancer agents, which highlights the importance of the chalcone pharmacophore in the development of therapeutic agents. Similarly, pyrazolo[1,5-*a*]pyrimidine derivatives demonstrate a wide variety of promising biological activities, such as antitumor,[30, 31] anxiolytic,[32,33] antimicrobial,[34,35] antifungal, antiobesity and anti-inflammatory.[36] They are effectively employed in the development of tyrosine kinase inhibitors (CDK).[30,37,38] Compound SCH727965 (**5**)[39] with pyrazolo[1,5-*a*]pyrimidine scaffold in it, was found to be a

[a] Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research (NIPER), Hyderabad-500 037, India
[b] Medicinal Chemistry & Pharmacology, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India; Email: ahmedkamal@iict.res.in
[c] Chemical Biology, CSIR-Indian Institute of Chemical Technology, Hyderabad 500 007, India; Email: manika@iict.res.in
[†] These authors contributed equally to this work

View Article Online
DOI: 10.1039/C7MD00193B

selective CDK inhibitor and is undergoing clinical trials. Also, our previous efforts towards the synthesis of pyrazolo[1,5-*a*]pyrimidine scaffold coupled with anthranilamide,[40] aminobenzothiazole[37] led to several compounds with promising anticancer activity, thus substantiating the potential of pyrazolo[1,5-*a*]pyrimidine scaffold further.

The amalgamation of two pharmacophores into a single chemical entity is an effective and commonly used strategy for finding novel potential molecules, the resulting molecules being known as hybrids or chimeric molecules.[41,42] The selection of two pharmacophores is based on their anticipated additive or synergistic effect. Hybrids are designed to synergise biological activity, minimizing resistance, effecting multiple targets and/or reducing known side effects.[43-45] Thus, owing to the importance of chalcone, pyrazolo[1,5-*a*]pyrimidine pharmacophores and our interest in the synthesis of biologically active molecules, herein we have combined both pharmacophores into a single chemical entity by anticipating their better pharmacological activity. The resulting series of chalcone-linked pyrazolo[1,5-*a*]pyrimidines were evaluated for their antiproliferative activity.

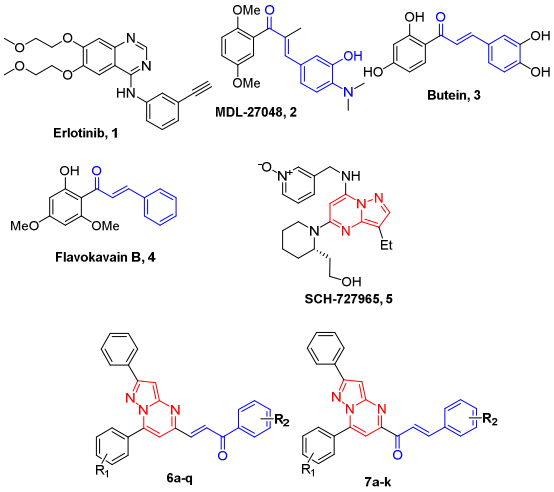


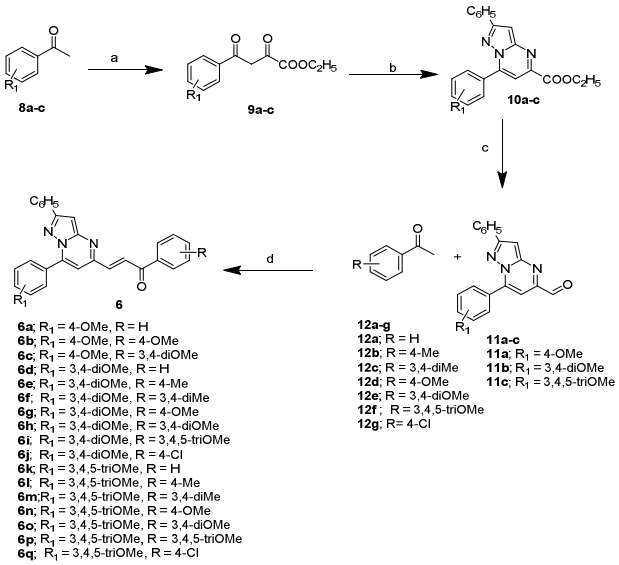
Fig. 1 Chemical structures of EGFR inhibitors: 1 (erlotinib), chalcone scaffold containing compounds **2** (MDL-27048), **3** (butein), **4** (flavokavain B), pyrazolo[1,5-*a*]pyrimidine containing scaffold compound **5** (SCH-727965) and designed chalcone-linked pyrazolo[1,5-*a*]pyrimidines (**6a-q**, **7a-k**).

Results and discussion

Chemistry

Synthesis of 6a-q hybrids

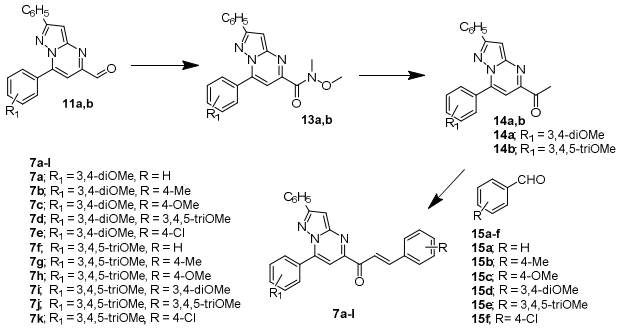
6a-q were synthesized as shown in Scheme **1**. Different acetophenones **8a-c** were oxalylated by diethyl oxalate in presence of sodium ethoxide in ethanol to give **9a-c**. These were condensed with 3-amino-5-phenyl-1*H*-pyrazole in presence of a catalytic amount of conc. HCl in ethanol to yield ethyl pyrazolo[1,5-*a*]pyrimidine-5-carboxylates **10a-c**. The ester functionality of ethyl pyrazolo[1,5-*a*]pyrimidine-5-carboxylate was then reduced with DIBAL-H in dichloromethane to give the corresponding pyrazolo[1,5-*a*]pyrimidine-5-carbaldehydes **11a-c**. Different acetophenones **12a-g** were condensed with pyrazolo[1,5-*a*]pyrimidine-5-carbaldehydes to afford the corresponding chalcone-linked pyrazolo[1,5-*a*]pyrimidines (**6a-q**).



Scheme 1 Synthesis of pyrazolo[1,5-*a*]pyrimidines (**6a-q**); Reagents and conditions: a) a) diethyl oxalate, NaOEt, EtOH, rt, 12 h, (yield 80-85%); b) 3-amino-5-phenyl-pyrazole, conc. HCl (cat.), EtOH, reflux, 2-4 h, (yield 90-95%); c) DIBAL-H, CH_2Cl_2, -78 °C, 2 h, (yield 78-80%); d) $Ba(OH)_2$, MeOH, rt, 4-6 h, (yield 71-91%).

Synthesis of 7a-k hybrids

7a-k were synthesized as shown in Scheme **2**. Substituted pyrazolo[1,5-*a*]pyrimidine-5-carbaldehydes **11a, b** subjected with Weinreb amide in presence of trimethylaluminum gives corresponding amides (**13a, b)** which were further subjected to Grignard reaction with methylmagnesium bromide to give the corresponding 1-(pyrazolo[1,5-*a*]pyrimidin-5-yl)ethan-1-ones (**14a, b)**. Different aldehydes (**15a-f)** were condensed with 1-(pyrazolo[1,5-*a*]pyrimidin-5-yl)ethan-1-one to give chalcone-linked pyrazolo[1,5-*a*]pyrimidines (**7a-k)**.



Scheme 2 Synthesis of pyrazolo[1,5-*a*]pyrimidines (**7a-k**); Reagents and conditions: a) *N,O*-dimethylhydroxylamine hydrochloride, $Al(CH_3)_3$, dry dichloromethane, 0-25 °C, 2 h, (yield 77-79%); b) methylmagnesium bromide, dry tetrahydrofuran, 0 °C, 1 h, (yield 85-92%); c) barium hydroxide, methanol, rt, 6 h, (yield 79-92%).

View Article Online
DOI: 10.1039/C7MD00193B

Biology

Anti-proliferative activity MTT assay was performed to evaluate the cytotoxic effect of newly synthesized chalcone-linked pyrazolo[1,5-*a*]pyrimidine hybrids against the selected cell lines viz A549 (lung cancer), MDA-MB-231 (breast cancer) and DU-145 (prostate cancer) using erlotinib as a reference standard, the results obtained are summarized in Table 1. All the hybrids in this series possess moderate to good antiproliferative potential against selected cell lines. Hybrid **6h** with 3,4-dimethoxy substitutions on C7 phenyl ring and phenylprop-2-en-1-one on C5 of pyrazolo[1,5-*a*]pyrimidine scaffold was found to be the most active against MDA-MB-231 (IC_{50} 2.6 µM) cell line. The same hybrid showed effective potential against A549 and DU-145 cell lines with IC_{50} values 3.9 µM and 7.2 µM respectively. In a SAR study, it was very interesting to evaluate the effect of substitutions on C5 phenylprop-2-en-1-one and C7 phenyl ring. It was observed that the series **6a-q** having superior antiproliferative activity than the series **7a-k** except for DU-145 cell line. In both series, the only difference is in the orientation of the enone bond. In the case of **6a-q** series, the double bond is near to the pyrazolo[1,5-*a*]pyrimidine scaffold whereas in the case of **7a-k** series the carbonyl group is near to pyrazolo[1,5-*a*]pyrimidine scaffold. The probable reason for this activity difference might be due to the extra hydrogen bonding formed by the carbonyl group of the hybrids of **6a-q** series with Cys773 amino acid of EGFR, similar hydrogen bond was not possible for the hybrids in **7a-k** series (explained with docking poses in molecular modeling section). Further structure-activity relationship is discussed for **6a-q** series. Hybrids with 4-methoxy and 3,4-dimethoxy substitutions on C7 phenyl ring displayed superior antiproliferative potential with IC_{50} ranging from 2.6 to 19.8 µM against MDA-MB-231 cell line. Hybrids with 3,4,5-trimethoxy substitution on C7 phenyl ring showed lower antiproliferative potential with IC_{50} ranging from 13.2-32.5 µM against MDA-MB-231 cell line. The activity order for C7 phenyl ring substitutions were 3,4-dimethoxy ≥ 4-methoxy > 3,4,5-trimethoxy. Furthermore, the hybrids with electron withdrawing substitution on C5 phenylprop-2-en-1-one showed lower cytotoxic potential than the hybrids with electron donating substituent. Hybrids with *meta-*, *para-* disubstitution has more cytotoxic potential than *meta-*, *para-*, *meta-* trisubstitution followed by hybrids with no substitution. However hybrids with only *para-* substitution exhibit least cytotoxic potential except for **6b**. The activity order for C5 phenylprop-2-en-1-one substitutions was 3,4-dimethoxy > 3,4,5-trimethoxy > 3,4-dimethyl > no substitution > 4-methoxy > 4-chloro > 4-methyl. Thus it could be concluded that 3,4-dimethoxy and 4-methoxy substitutions on C7 phenyl ring were optimum for antiproliferative potential and 3,4-di substitution on C5 phenylprop-2-en-1-one was enhancing the activity. In contrast, 3,4,5-trimethoxy substitution on C7 phenyl ring was detrimental for activity. Based on the results obtained with the MTT assay, four of the most potent hybrids (**6b**, **6h**, and **6i)** were considered for further mechanistic studies.

Table 1 Cytotoxicity (IC_{50} values in µM)[a] of chalcone-linked pyrazolo[1,5-*a*]pyrimidines **6a-q** and **7a-k** on selected cell lines

	[b]A549	[c]MDA-MB-231	[d]DU-145	[e]HEK293
6a	8.6 ± 0.4	9.9 ± 0.2	13.7 ± 0.3	53.2 ± 1.9
6b	2.9 ± 0.3	6.3 ± 0.3	8.5 ± 0.4	36.1 ± 0.9
6c	7.4 ± 0.2	8.7 ± 0.4	16.4 ± 0.4	48.5 ± 0.5
6d	10.7 ± 0.3	11.8 ± 0.4	10 ± 0.3	51.2 ± 0.6
6e	17.2 ± 0.4	19.8 ± 0.3	26.8 ± 0.4	62.6 ± 0.4
6f	9.3 ± 0.6	11.5 ± 0.2	12.1 ± 0.6	46.2 ± 0.7
6g	11.9 ± 0.3	13.9 ± 0.5	14.6 ± 0.5	40.0 ± 0.3
6h	3.9 ± 0.4	2.6 ± 0.6	7.2 ± 0.4	32.5 ± 0.7
6i	7.2 ± 0.4	4.7 ± 0.3	8.3 ± 0.3	35 ± 1.2
6j	15.4 ± 0.5	17.1 ± 0.4	21.3 ± 0.4	44.1 ± 0.8
6k	18.2 ± 0.4	15.8 ± 0.4	34.3 ± 0.5	≥ 100
6l	19.4 ± 0.3	23.9 ± 0.6	27.1 ± 0.3	50.4 ± 1.9
6m	10.6 ± 0.4	13.5 ± 0.6	15.4 ± 0.6	72.5 ± 1.2
6n	14.6 ± 0.5	13.2 ± 0.4	25.2 ± 0.6	79.8 ± 0.7
6o	17.8 ± 0.3	18.3 ± 0.4	29.9 ± 0.4	81.7 ± 1.6
6p	20.5 ± 0.6	24.3 ± 0.5	34.9 ± 0.4	≥ 100
6q	15.5 ± 0.4	32.5 ± 0.4	29.3 ± 0.2	≥ 100
7a	18.62±1.04	23.32±0.8	15.57±1.65	64.1 ± 1.4
7b	22.19±2.86	22.59±1.87	19.49±1.31	57.4 ± 0.9
7c	19.27±1.91	21.98±0.92	14.93±0.78	50.7 ± 1.2
7d	15.44±2.11	16.73±0.67	14.74±1.41	46.8 ± 1
7e	22.01±2.15	23.74±0.91	22.4±1.87	39.1 ± 0.7
7f	17.97±0.57	19.15±1.11	16.31±1.41	44.8 ± 1.3
7g	20.03±2.71	18.64±1.12	21.89±1.03	56.1 ± 0.8
7h	21.23±1.91	16.5±1.46	16.91±0.53	46.6 ± 1
7i	20.47±1.87	14.19±2.16	15.33±0.9	41.0 ± 1.9
7j	25.86±2.8	18.37±1.8	17.91±1.2	41.0 ± 0.3
7k	23.83±2.4	21.62±.92	19.51±2.4	53.9 ± 1.6
Erlo	10.39±1.6	14.74±2.5	18.4±1.4	30.3 ± 2.1

Note: [a]Cell lines were treated with different concentrations of compounds. Cell viability was measured by employing the MTT assay. The concentration required for 50% inhibition of cell growth was calculated and the values represent means ± S.D. of three different experiments performed in triplicates, [b]lung cancer, [c]breast cancer, [d]prostate cancer, and [e]Human embryonic kidney cells.

Effect on Cell cycle distribution Anticancer molecules exert their action either by arresting the cell cycle at a checkpoint or by induction of apoptosis or a combined effect of both cycle block and apoptosis. Furthermore, regulation of the cell cycle and apoptosis are considered to be effective strategies in the development of cancer therapeutics. To shed light on the effects of active hybrids **6b**, **6h**, and **6i** on cell cycle progression, cell cycle analysis was performed on A549 cells. These cells were treated with 2 µM concentration of the test compounds for 24 h. Cell cycle analysis performed with PI staining showed accumulation of cells at G2/M phase (Fig. 2). Untreated cells showed 20.4% cells in G2/M phase. In contrast, compounds **6b**, **6h**, and **6i**-treated cells showed accumulation of 29.6%, 29.5%, and 33.11% respectively. Wherein erlotinib was used aspositive control, it showed 24.5% in G2/M population. Thus, compounds of this investigation showed a significant effect in restricting A549 cells to G2/M phase, which in itself is a strong evidence of apoptotic cell death. Also, the effect of the compounds was validated in a non-cancerous HEK

View Article Online
DOI: 10.1039/C7MD00193B

cell line, which revealed the distribution of treated cells similar to the untreated cells (Supplementary Fig 1).

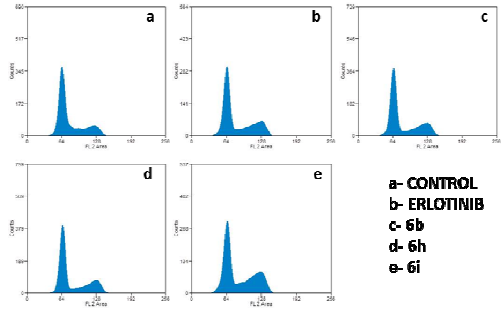


Fig. 2. Flow cytometric analysis displaying G2/M arrest in cells post-treatment. A549 cells subjected to compound treatment for 24h at 2 µM concentration were collected, fixed and stained for FACS analysis. Untreated cells were employed as negative control.

Effect on EGFR/STAT3 pathway To investigate the pathway responsible for cell death and arrest, A549 cells were treated with hybrids **6b**, **6h**, and **6i** at 2 µM concentration for 24 h, followed by total protein lysate extraction. Protein lysate was subjected to western blot analysis. Western blot analysis revealed elevated levels of EGFR and P-EGFR upon treatment with hybrids in comparison to erlotinib-treated cells (Fig. 3). STAT3 is a well-known transcription factor responsible for cell survival and proliferation, and is commonly over expressed in most cancer types. Interestingly, significant down regulation of STAT3 and P-STAT3 upon treatment was observed. Further, AKT, a key component of cellular survival pathways was observed to be down-regulated. Both, **6h** and **6i** have shown the significant downregulation compared to the untreated cells. Among the compounds studied, **6b**, **6h** and **6i** demonstrated a significant effect on the modulation of the EGFR/STAT3 axis and AKT pathway (Fig. 3). They also show a high degree of correlation with the erlotinib-treated cells. Also, a non-cancerous HEK cell line was employed to study the deleterious effects on normal cells and much to our interest, there was no significant alteration in the expression of EGFR/STAT3 pathway (Supplementary Fig 2A).

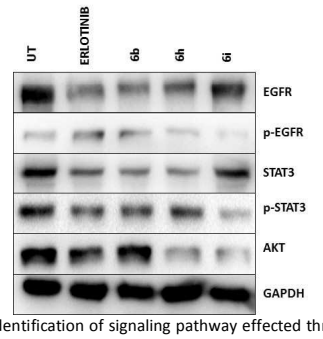


Fig. 3 Identification of signaling pathway effected through Western blot analysis. A549 cells were treated with **6b**, **6h** and **6i** at 2 µM final concentration and collected 24 h post-treatment. Total protein was isolated and analyzed by Western blotting using corresponding antibody. Compounds **6b** and **6h** revealed significantly reduced levels of p-EGFR, p-STAT3 and AKT. Erlotinib was utilized as positive control, whereas untreated cells were considered as negative control.

Effect on the co-localization of EGFR and STAT3 To further clarify the deregulation of the EGRF/STAT axis in the treated A549 cells, the cellular protein levels and the localization of p-EGFR and STAT3 proteins were identified by immunofluorescence method. Cells after 24h treatment were fixed, stained with EGFR/STAT3 tagged with Cy3-conjugated secondary antibody fluorophore were analyzed with aid of laser scanning confocal microscopy. Hybrids **6b**, **6h** and **6i** treated cells showed a significant downregulated of EGFR as well as STAT3, in comparison to the positive control (erlotinib) treated cells (Fig. 4). This clearly shows the reduced co-localization of EGFR and STAT3 upon treatment with chalcone-linked pyrazolo[1,5-*a*]pyrimidine hybrids.

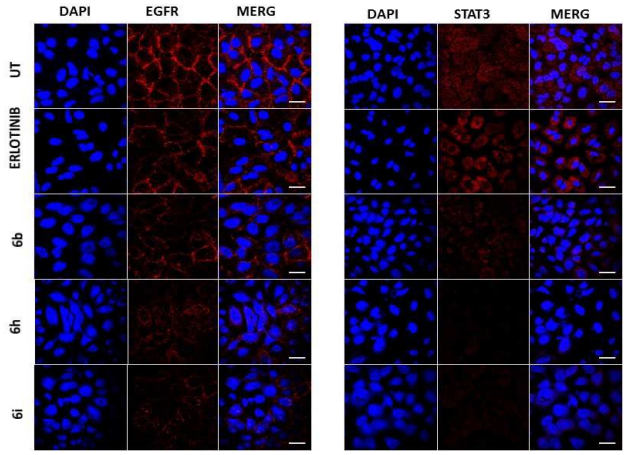


Fig. 4 Regulation of EGFR and STAT3 in cells 24 h post-treatment. A549 cells were fixed, stained and observed for 24 h post treatment with compounds **6b**, **6h** and **6i** at 2 µM final concentration under confocal microscope (FLOW VIEW FV 1000 series). Cy3 dye was used to stain both EGFR and STAT3, whereas DAPI as nuclear stain. Compounds **6h** and **6i** showed profound inhibitory effect on levels of EGFR and STAT3.

Effect on pro-apoptotic and anti-apoptotic proteins To understand the role of chalcone-linked pyrazolo[1,5-*a*]pyrimidine hybrids in regulating apoptosis, A549 cells were treated and extracted protein lysate was subjected to analysis at both transcriptional and translation level of key apoptotic proteins like p53, p21, BAX, caspase-3 and Bcl-2.The test compounds significantly increased the levels of p53, p21 and BAX and decreased the expression of Bcl2 (Fig. 5a, 5b). The increase in the levels of many of these proapoptotic proteins also showed a concomitant decrease in the levels of antiapoptotic protein Bcl-2 upon compound treatment. This regulation of apoptosis at both transcriptional and translational level shows the prominent effect of chalcone-linked pyrazolo[1,5-*a*]pyrimidine hybrids in inducing apoptosis. To understand the effect on normal cells, non-cancerous HEK cell line was studied with the same compounds and there was no significant alteration in the expression pattern in comparison to the untreated control cells (Supplementary Fig 2B).

View Article Online
DOI: 10.1039/C7MD00193B

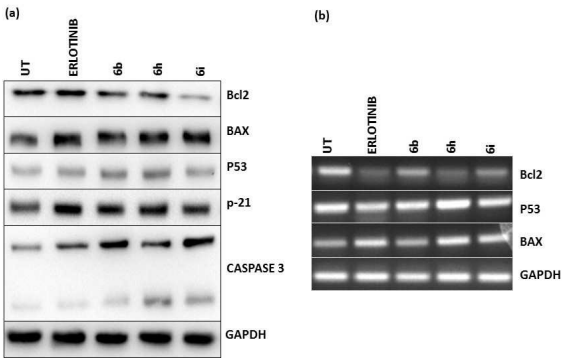


Fig. 5 Analysis of pro-apoptotic and anti-apoptotic proteins post 24h treatment. **(a)** Total lysate was isolated from A549 cells after 24h treatment with compounds **6b**, **6h** and **6i** at 2 µM final concentration and analyzed by Western blotting using corresponding antibody. Compounds 6b and 6i show clear up-regulation of apoptotic proteins including Bax, p53, p21and caspase-3, whereas down-regulation of anti-apoptotic protein Bcl2 was observed. Erlotinib was employed as positive control and untreated as negative control. **(b)** RNA was isolated from cells using Trizol reagent and cDNA was synthesized. End point RT-PCR results showing p53 and Bax upregulation in cells treated with compounds **6b** and **6h** and **6i**. Significant reduction in levels of anti-apoptotic protein, Bcl2.

Molecular Docking studies

Molecular docking studies were performed to get the insight of interactions between chalcone-linked pyrazolo[1,5-*a*]pyrimidine hybrids and EGFR. Molecular docking studies were carried out for all hybrids (**6a-q**, and **7a-k**). Docking pose of **6j** displayed that these hybrids bind well at the ATP binding site of EGFR (Fig. 6B). The C2 phenyl ring was buried in specificity pocket where it was surrounded by Val702, Ala719, Ile720, Lys721, Met742, Leu764, Ile765 and Thr766 amino acids. The superimposed pose of **6j** with cocrystal ligand erlotinib showed that C2 phenyl ring of **6j** was overlapping on the acetylphenyl group of erlotinib (Fig. 6C). The C7 phenyl ring is oriented towards the hinge region and surrounded by Leu694, Val702, Ala719, Gln767, Leu768, Met769, Pro770, Gly772, and Leu820 amino acids. 3-Methoxy and 4-methoxy groups were forming hydrogen bonds with –NH of Met769. This hydrogen bond is crucial for EGFR inhibitors and is present in all the drug molecules and ATP as well. The C5 phenylprop-2-en-1-one was oriented towards the 'A loop' and forms a close contact with Cys773, Arg817 and Asn818 amino acids and the carbonyl carbon was forming a hydrogen bond with Cys773. In MTT assay it was observed that **6a-q** series has superior activity than the **7a-k** series. In the superimposed pose for the hybrids of **6a-q** series, hydrogen bonding was observed between the carbonyl carbon and Cys773 amino acid, whereas, for hybrids in **7a-k** series such hydrogen bonding was not present (Fig. 6D). This is due to the difference in the geometrical orientation of the enone group in **6a-q** and **7a-k** and may be a reason for the superior activity of compounds **6a-q** over **7a-k**. The activity order for C7 phenyl substitutions was 3,4-dimethoxy > 4-methoxy > 3,4,5-trimethoxy. From the docking studies it was observed that 3,4-dimethoxy substituted hybrids were forming two hydrogen bonds with Met769. On the other hand, 4-methoxy substituted hybrids were forming only one hydrogen bond (Supporting information Fig 3a). The superimposed pose of 3,4-dimethoxy and 4-methoxy explain the higher activity of 3,4-dimethoxy substituted hybrids but it does not explain the least activity of 3,4,5-trimethoxy substituted hybrids. However, most of the drugs acting on EGFR having dialkoxy substitution supports the dialkoxy substitution is optimum for the activity (structures of drugs having dialkoxy substitution are given in supplementary information Fig 4). In the series, least active hybrid is **6p** with 3,4,5-trimethoxy substitution on both C7 phenyl ring and C5 phenylprop-2-en-1-one. The docking pose shows that carbonyl group of **6p** was not forming the hydrogen bond with the Cys773 which supports the least activity of **6p** (Supporting information Fig 3b). Docking studies showed that these hybrids bind well in the ATP binding site of EGFR with the required hydrophobic interactions in the specificity pocket and hydrogen bonding with Met769 amino acid in the hinge region and explained the SAR of the series.

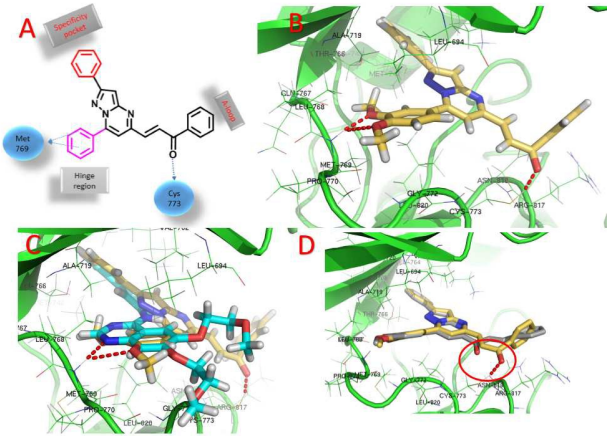


Fig. 6 Docking poses on EGFR A) 2D structure of chalcone linked-pyrazolo[1,5-*a*]pyrimidine with observed interactions with EGFR B) Binding pose of **6j** (yellow color, EGFR in green color and hydrogen bonds in red dotted lines) C) Superimposed pose of **6j** and erlotinib D) Superimposed pose of **6j** and **7a** showing the difference in hydrogen bonding between carbonyl carbon and Cys773.

Conclusion

A series of chalcone-linked pyrazolo[1,5-*a*]pyrimidines (**6a-q** and **7a-k**) was synthesized and evaluated for its cytotoxic activity. Most of the hybrids exhibited prominant anti-proliferative potential with IC_{50} ranging from 2.6 µM to 34.9 µM. In the series, **6b**, **6h** and **6i** showed promising cytotoxicity and were advanced for further mechanistic studies. Cell cycle analysis displayed a significant accumulation of cells at G2/M phase of cell cycle. Western blot analysis and immunostaining assay suggested the downregulation of EGFR, p-EGFR, STAT3, and p-STAT3 with a stronger downregulation of EGFR/ STAT3 axis. Western blot analysis and RT-PCR also showed the up-regulation of apoptotic proteins like p53, p21 and BAX whereas downregulation of antiapoptotic proteins such as Bcl-2 and caspase-3 at both the transcriptional and translation level. Our study establishes the role of chalcone-linked pyrazolo[1,5-*a*]pyrimidine hybrids in lung carcinoma cell line,

View Article Online
DOI: 10.1039/C7MD00193B

A459 in inducing cellular arrest and activation of apoptosis. Molecular docking studies provided insight into the binding mode of these molecules at the APT binding site of EGFR and shed light on the superior activity of compounds **6a-q** in comparison to those of **7a-k** series. These studies suggest the potential of synthesized hybrid molecules for development into promising anticancer agents.

Acknowledgements

The author C.B acknowledges the Department of Pharmaceuticals (Ministry of Chemicals and Fertilizers, Govt. of India) for the financial support & the CSIR-IICT, Hyderabad for providing facilities. K.R.G thanks UGC for his fellowship. This work was financially supported by the CSIR 12th FYP CSC0111 (SMiLE). The authors thank Y. Suresh for the Flow Cytometry and Confocal Microscopy studies.

Conflict of Interest

The authors declare no competing interests.

References

1. L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent and A. Jemal, *CA Cancer J. Clin.*, 2015, **65**, 87-108.
2. F. Bray, A. Jemal, N. Grey, J. Ferlay and D. Forman, *Lancet. Oncol.*, 2012, **13**, 790-801.
3. J. Zugazagoitia, C. Guedes, S. Ponce, I. Ferrer, S. Molina-Pinelo and L. Paz-Ares, *Clin. Ther.*, 2016, **38**, 1551-1566.
4. F. Biemar and M. Foti, *Cancer Biol. Med.*, 2013, **10**, 183.
5. A. Backes, B. Zech, B. Felber, B. Klebl and G. Müller, *Exp. Op. Drug Disc.*, 2008, **3**, 1427-1449.
6. A. Gschwind, O. M. Fischer and A. Ullrich, *Nat. Rev. Cancer*, 2004, **4**, 361-370.
7. A. Backes, B. Zech, B. Felber, B. Klebl and G. Müller, *Exp. Op. Drug Disc.*, 2008, **3**, 1409-1425.
8. C. Yewale, D. Baradia, I. Vhora, S. Patil and A. Misra, *Biomaterials*, 2013, **34**, 8690-8707.
9. P. Warnault, A. Yasri, M. Coisy-Quivy, G. Cheve, C. Bories, B. Fauvel and R. Benhida, *Curr. Med. Chem.*, 2013, **20**, 2043-2067.
10. G. Speake, B. Holloway and G. Costello, *Curr. Opin. Pharmacol.*, 2005, **5**, 343-349.
11. J. Dowell, J. D. Minna and P. Kirkpatrick, *Nat. Rev. Drug Discov.*, 2005, **4**, 13-14.
12. T. W. Wong, F. Y. Lee, C. Yu, F. R. Luo, S. Oppenheimer, H. Zhang, R. A. Smykla, H. Mastalerz, B. E. Fink and J. T. Hunt, *Clin. Cancer Res.*, 2006, **12**, 6186-6193.
13. Y. Yarden and M. X. Sliwkowski, *Nat. Rev. Mol. Cell Biol.*, 2001, **2**, 127-137.
14. T. Holbro and N. E. Hynes, *Annu. Rev. Pharmacol. Toxicol.*, 2004, **44**, 195-217.
15. M. A. Olayioye, R. M. Neve, H. A. Lane and N. E. Hynes, *EMBO J.*, 2000, **19**, 3159-3167.
16. S. Temam, H. Kawaguchi, A. K. El-Naggar, J. Jelinek, H. Tang, D. D. Liu, W. Lang, J.-P. Issa, J. J. Lee and L. Mao, *J. Clin. Oncol.*, 2007, **25**, 2164-2170.
17. E. Tanaka, Y. Hashimoto, T. Ito, T. Okumura, T. Kan, G. Watanabe, M. Imamura, J. Inazawa and Y. Shimada, *Clin. Cancer Res.*, 2005, **11**, 1827-1834.
18. M. Kurai, T. Shiozawa, H.-C. Shih, T. Miyamoto, Y.-Z. Feng, H. Kashima, A. Suzuki and I. Konishi, *Hum. Pathol.*, 2005, **36**, 1281-1288.
19. A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu and M. J. Thun, *CA Cancer J. Clin.*, 2007, **57**, 43-66.
20. S. V. Sharma, D. W. Bell, J. Settleman and D. A. Haber, *Nat. Rev. Cancer*, 2007, **7**, 169-181.
21. M. F. Rimawi, P. B. Shetty, H. L. Weiss, R. Schiff, C. K. Osborne, G. C. Chamness and R. M. Elledge, *Cancer*, 2010, **116**, 1234-1242.
22. D. S. Salomon, R. Brandt, F. Ciardiello and N. Normanno, *Crit. Rev. Oncol. Hematol.*, 1995, **19**, 183-232.
23. G. Di Carlo, N. Mascolo, A. A. Izzo and F. Capasso, *Life sci.*, 1999, **65**, 337-353.
24. D. K. Mahapatra, V. Asati and S. K. Bharti, *Eur. J. Med. Chem.*, 2015, **92**, 839-865.
25. D. K. Mahapatra, S. K. Bharti and V. Asati, *Eur. J. Med. Chem.*, 2015, **98**, 69-114.
26. D. K. Mahapatra, S. K. Bharti and V. Asati, *Eur. J. Med. Chem.*, 2015, **101**, 496-524.
27. S. Ducki, *IDrugs*, 2007, **10**, 42.
28. T. Ji, C. Lin, L. S. Krill, R. Eskander, Y. Guo, X. Zi and B. H. Hoang, *Mol. Cancer*, 2013, **12**, 55.
29. E.-B. Yang, K. Zhang, L. Y. Cheng and P. Mack, *Biochem. Biophys. Res. Commun.*, 1998, **245**, 435-438.
30. D. A. Heathcote, H. Patel, S. H. Kroll, P. Hazel, M. Periyasamy, M. Alikian, S. K. Kanneganti, A. S. Jogalekar, B. Scheiper and M. Barbazanges, *J. Med. Chem.*, 2010, **53**, 8508-8522.
31. M. Gao, L. Duan, J. Luo, L. Zhang, X. Lu, Y. Zhang, Z. Zhang, Z. Tu, Y. Xu and X. Ren, *J. Med. Chem.*, 2013, **56**, 3281-3295.
32. S. Selleri, F. Bruni, C. Costagli, A. Costanzo, G. Guerrini, G. Ciciani, P. Gratteri, F. Besnard, B. Costa and M. Montali, *J. Med. Chem.*, 2005, **48**, 6756-6760.
33. P. Popik, E. Kostakis, M. Krawczyk, G. Nowak, B. Szewczyk, P. Krieter, Z. Chen, S. J. Russek, T. T. Gibbs and D. H. Farb, *J. Pharmacol. Exp. Ther.*, 2006, **319**, 1244-1252.
34. S. Bondock, W. Fadaly and M. A. Metwally, *Eur. J. Med. Chem.*, 2010, **45**, 3692-3701.
35. H. Behbehani, H. M. Ibrahim, S. Makhseed and H. Mahmoud, *Eur. J. Med. Chem.*, 2011, **46**, 1813-1820.
36. S. Cherukupalli, R. Karpoormath, B. Chandrasekaran, G. A. Hampannavar, N. Thapliyal and V. N. Palakollu, *Eur. J. Med. Chem.*, 2016.
37. A. Kamal, J. R. Tamboli, V. L. Nayak, S. Adil, M. Vishnuvardhan and S. Ramakrishna, *Bioorg. Med. Chem. Lett.*, 2013, **23**, 3208-3215.
38. P. S Brahmkshatriya, P. Dobes, J. Fanfrlik, J. Rezac, K. Paruch, A. Bronowska, M. Lepsík and P. Hobza, *Curr. Comput. Aided Drug Des.*, 2013, **9**, 118-129.

View Article Online
DOI: 10.1039/C7MD00193B

39. S. J. McClue, D. Blake, R. Clarke, A. Cowan, L. Cummings, P. M. Fischer, M. MacKenzie, J. Melville, K. Stewart and S. Wang, *Int. J. Cancer*, 2002, **102**, 463-468.
40. A. Kamal, J. R. Tamboli, M. J. Ramaiah, S. Adil, G. Koteswara Rao, A. Viswanath, A. Mallareddy, S. Pushpavalli and M. Pal-Bhadra, *ChemMedChem*, 2012, **7**, 1453-1464.
41. C. Viegas-Junior, A. Danuello, V. da Silva Bolzani, E. J. Barreiro and C. A. M. Fraga, *Curr. Med. Chem.*, 2007, **14**, 1829-1852.
42. Y. Bansal and O. Silakari, *Eur. J. Med. Chem.*, 2014, **76**, 31-42.
43. L. F. Tietze, H. P. Bell and S. Chandrasekhar, *Angew. Chem. Int. Ed.*, 2003, **42**, 3996-4028.
44. F. W. Muregi and A. Ishih, *Drug develop. res.*, 2010, **71**, 20-32.
45. K. Nepali, S. Sharma, M. Sharma, P. Bedi and K. Dhar, *Eur. J. Med. Chem.*, 2014, **77**, 422-487.

View Article Online
DOI: 10.1039/C7MD00193B

Synthesis and biological evaluation of chalcone-linked pyrazolo[1,5-*a*]pyrimidines as anticancer agents

Chandrakant Bagul, Garikapati Koteswara Rao, Venkata Krishna Kanth Makani, Jaki R. Tamboli, Manika Pal-Bhadra and Ahmed Kamal