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Research Letter

Synthesis and *In Vitro* Evaluation of N-Aryl Pyrido-Quinazolines Derivatives as Potent Epidermal Growth Factor Receptor Inhibitors

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A series of pyrido-quinazolines have been synthesised, characterized, and tested for their in vitro epidermal growth factor receptor (EGFR) tyrosine kinase inhibitory activity. The compounds were prepared from Alkylideno/arylideno-bis-ureas. Their final structure of the compounds was elucidated on the basis of spectral studies (IR, ^{1}H NMR, FT-IR, and EI-MS). The cellular EGFR internalization response of selected compounds was evaluated using HeLa cells. Most of the synthesized compounds displayed potent EGFR-TK inhibitory activity and structurally halogenated derivatives had a pronounced effect in inhibiting EGFR internalization.

Key words: anticancerous, epidermal growth factor receptor, HeLa, quinazolines, spectroscopy

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The quinazoline skeletons, present in a variety of biologically active compounds, are pharmacologically very attractive as reflected by activity in various assays. They are well known for a wide range of biological properties, including hypnotic, sedative, analgesic, anticonvulsant, antibacterial, antidiabetic, anti-inflammatory, and antitumor (1–11). They also demonstrate several other useful and interesting properties such as hypertensive adrenergic blocker, selective phosphodiesterase inhibitor, against prostate disorders, dihydrofolate reductase inhibitor (12–20), etc. In addition, some derivatives are calcium antagonists and share the common property of interfering with the influx of extracellular calcium via the calcium L channel. Recently, quinazoline chemistry has found new direction due to some resemblance with folic acid (21,22).

Epidermal growth factor receptor (EGFR) a transmembrane glycoprotein is composed of a glycosylated N-terminus, having three binding regions an extracellular ligand binding region, a hydrophobic transmembrane region and a C-terminal intracellular regions. When epidermal growth factors (EGF) bind to EGFR, the TK domain is activated, and this triggers a chain of different signal transduction events inside the cell that eventually leads to cell growth, proliferation, and differentiation. Elevated receptor tyrosine kinase activity is often observed in malignant tumors. Tyrosine kinases (TK) are enzymes that bind ATP and catalyze the transfer of g-phosphate to tyrosine residues on proteins, thereby regulating their activity and function. Several tyrosine kinases can be targets for cancer chemotherapy, the most important being the receptor tyrosine kinases.

In continuation of our research straightly, we are designing novel quinazolines analogues having more efficacy to inhibit EGFR for treatment of various cancers.

Materials and Method

All chemicals used in this study are of analytical grade purchased from Sigma. All the solvents were used after distillation. Thin layer chromatography (TLC) was run on the silica get coated aluminum sheets (silica gel 60 F_{254}, E Merck, Germany) and visualized under UV light. FTIR spectra were recorded on the FT-IR Perking Elmer Spectrum BX Spectrophotometer with KBr disks. NMR spectra were measured in $CDCl_3$ by Bruker 200 MHz apparatus with Me_4Si as an internal standard. EI-MS spectra were recorded on a JEOL SX102/DA (KV 10 mA) instrument. Elemental analysis was performed on elemental analyzer Gmbh variable system. Radio complexation and radio chemical purity was checked by instant strip chromatography (silica gel impregnated paper chromatography) with ITLC-SG (Gellman sciences, Ann arbar, MI, USA). The gamma scintillation counting was performed at Electronic Corporation of India Ltd. Gamma Ray Spectrometer K 2700 B. All the reaction steps were monitored by TLC [chloroform/methanol/hexane: 4:3:1]. Distilled water is used during whole of the procedure.

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Synthesis

Total synthesis of Aryl pyrido-quinazolines derivatives are described in chemical Scheme 1, started with synthesis of Alkylideno/arylideno-bis-ureas. A mixture of an aldehyde (0.01 mol) and urea (0.2 mol) in ethanol was heated under reflux 4 h. Subsequently, ethanol was distilled off and residual thick oily material was cooled to 0 °C. It solidified in about 1 h. After washing initially with 1% NaOH solution and finally with cold water, the resultant solid was filtered. The crude material was dried at 100 °C and recrystallized from diluted ethanol.

Alkylideno/arylideno-bis-urea (0.06 mol) (**1**) and p-amino-benzoic acid (0.06 mol) were mixed together and heated at 145–150 °C for 4 h. A clear liquid was obtained on heating, which on cooling to room temperature solidified. It was treated with diluted HCl (50 mL) and stirred very well. After filtering off the solid, it was washed with water several times and treated with an aqueous solution of sodium bicarbonate (10%). Vigorous effervescence as a result of evolution of carbon dioxide occurred, which subsided on adding more solution of sodium bicarbonate. When the effervescence completely ceased, the solid was filtered off. This solid was rejected, and the filtrate was acidified with diluted hydrochloric acid. On complete neutralization, a solid separated out which was filtered off and washed with water. It was dried at 100 °C and recrystallized from glacial acetic acid. The intermediate compound4-aryl-6-carboxylato-1,2,3,4-tetrahydroquinazoline (**2**) of this category are recorded in Table 1 along with their characterization data.

Synthesis of 4-Aryl-8,9-diphenyl-1,4-dihydro-3H-7-oxa-1,3-diazaanthracene-2,6-dianes (3)

A mixture of 4-aryl-6-carboxylato-1,2,3,4-tetrahydroquinazoline (0.02 mol) (**2**) and benzoin (desyl alcohol) (0.02 mol) in polyphosphoric acid (10 mL) was heated at 100 °C for 5 h. During heating, the contents were occasionally stirred. Subsequently, the reaction mixture was poured into ice-cold water (100 mL) and left as such for 1 h. A solid separated out, which was filtered off and washed initially with 10% aqueous sodium bicarbonate solution (50 mL) and finally with water (2 × 25 mL). The solid thus obtained was dried under vacuum and recrystallized from methanol.

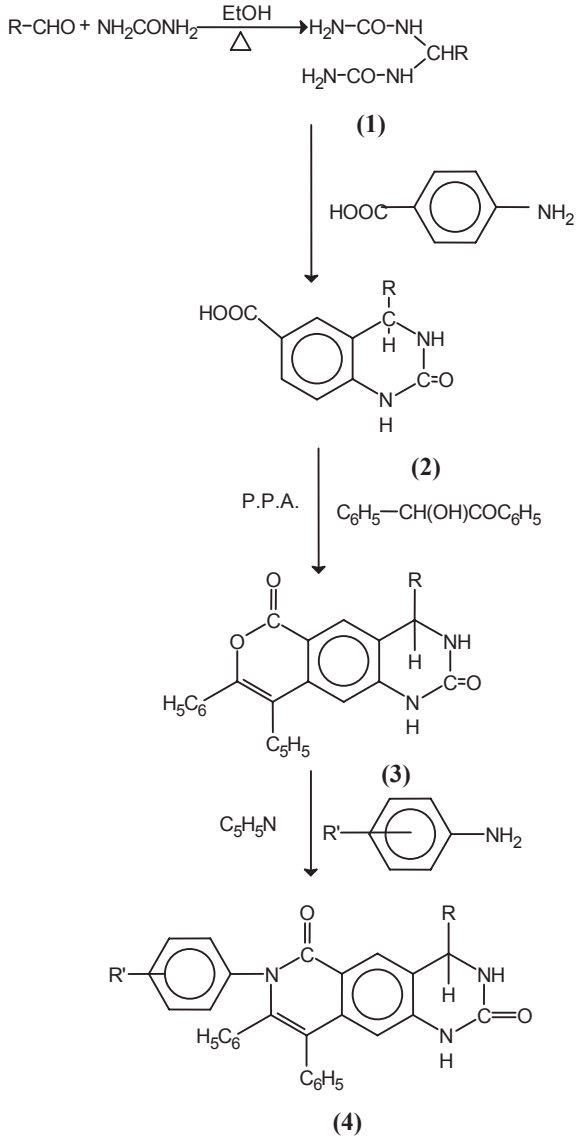
N-Aryl-8,9-diphenyl (-2-oxo-pyrido-[g]-4-aryl-2-oxo-1,3-dihydro-quinazolines 4(A–G)

The titled compounds (**4**) were synthesized by heating under reflux a mixture of 4-aryl-8,9-diphenyl-1,4-dihydro-3H-7-oxa-1,3-diazanthracene-2,6-dione **3** (0.01 mol) and aromatic primary amine (0.02) in anhydrous pyridine (30 mL) for 6 h. The solution was cooled to room temperature and acidified with dil. HCl (50 mL). A solid separated out which was filtered off and washed with water successively (4 × 25 mL). It was air-dried and recrystallized from diluted ethanol. The final compounds of this category are present in Table 1 along with their data for characterization.

Biological studies of N-Aryl pyrido-quinazolines derivatives

In vitro *serum stability assay*

The Pharmacokinetics of the synthesized compounds were determined by tracer methods through technetium



Compd. No.	R	R'	Molecular formula
4(A)	Phenyl	Hydrogen	$C_{35}H_{25}N_3O_2$
4(B)	Phenyl	Chloro	$C_{35}H_{24}N_3O_2Cl$
4(C)	Phenyl	Methoxy	$C_{36}H_{27}N_3O_3$
4(D)	Phenyl	Methyl	$C_{36}H_{27}N_3O_2$
4(E)	Hydrogen	Hydogen	$C_{29}H_{21}N_3O_2$
4(F)	Hydrogen	Chloro	$C_{29}H_{20}N_3O_2Cl$
4(G)	Hydrogen	Methoxy	$C_{30}H_{23}N_3O_3$

Scheme 1: Synthesis of N-Aryl pyrido-quinazolines derivatives.

Table 1: Characterization data of N-Aryl-pyrido [g] – quinazolines

Compound no.	R	R'	M.P. °C	Yield (%)	Molecular formula	% *N* Found(Calcd)	I.R.	^{1}H NMR
1a	Phenyl	–	178	65	$C_9H_{12}N_4O_2$	26.52 (26.92)	–	6.0 (s, 2H, –NH_2) 6.98 (t, 1H, –CH) 7.09–7.20 (m, 5H, ArH)
1b	Hydrogen	–	169	70	$C_3H_8N_4O_2$	42.15 (42.42)	–	6.01 (s, 2H, –NH_2) 6.8 (t, 2H, –CH_2)
2a	Phenyl	–	284	60	$C_{15}H_{12}N_2O_3$	10.20 (10.45)	–	6.0 (s, 1H, –NH) 6.3 (d, 1H, –NH) 7.2–7.8 (m, 8H, Ar-H) 6.4 (d, 1H, –CH)
2b	Hydrogen	–	249	67	$C_9H_8N_2O_3$	14.33 (14.58)	–	4.12 (d, 2H, –CH_2) 6.13 (s, 1H, NH) 6.01 (t, 1H, NH) 7.30–7.80 (m, 3H, Ar-H)
3a	Phenyl	–	145	53	$C_{29}H_{20}N_2O_3$	5.99 (6.31)	1740 (O-C=O δ lactones) 3032 (Aromatic C-H str. H) 1125 (Cyclic ether C-O-C) 1665 (C=O) 3465 (NH str.)	4.40 (d, 2H, -CH_2) 6.14 (s, 1H, -NH) 6.3 (d, 1H, NH) 7.10–7.9 (m, 11H, ArH)
3b	Hydrogen	–	136	59	$C_{23}H_{16}N_2O_3$	7.31 (7.61)	1742 (O-C=O δ lactones) 3036 (Aromatic C-H) 1125 (Cyclic ether C-O-C) 1670 (C=O)	6.0 (d, 1H, -CH) 6.11 (s, 1H, -NH) 6.29 (d, 1H, –NH) 7.09–7.90 (m, 17H, ArH)
4a	Phenyl	Hydrogen	128	45	$C_{35}H_{25}N_3O_2$	7.89 (8.09)	–	6.9–7.9 (m, 22H, ArH) 6.01 (s, 1H, -NH), 6.10 (d, 1H, -NH)
4b	Phenyl	Chloro	140	42	$C_{35}H_{24}N_3O_2Cl$	7.37 (7.59)	–	6.0 (s, 1H, NH), 6.09 (d, 1H, NH), 7.10–7.89 (m, 21H, ArH)
4c	Phenyl	Methoxy	170	40	$C_{36}H_{27}N_3O_3$	7.34 (7.65)	1360 (OCH_3) 3470 (NH str.) 1565 (NH bend) 1695 (C=O amide), 1640 (C=O δ lactam) 3020 (Aromatic C-H)	3.70 (s, 3H, -OCH_3) 6.0 (S, 1H, -NH) 6.0 (s, 1H, -NH) 6.19 (d, 1H, -NH) 6.99–7.81 (m, 21H, ArH)
4d	Phenyl	Methyl	130	40	$C_{36}H_{27}N_3O_2$	7.39 (7.83)	2930 (C-H of CH_3) 3465 (NH str.) 1660 (NH bend) 1700 (C=O sec amide) 1635 (C=O δ lactum) 3035 (Aromatic C-H)	2.40 (s, 3H, -CH_3) 5.98 (s, 1H, NH) 6.9 (d, 1H, -NH) 7.10–7.79 (m, 21H, Ar-H)
4e	Hydrogen	Hydrogen	220	38	$C_{29}H_{21}N_3O_2$	9.29 (9.48)	–	4.30 (d, -2H, -CH_2) 6.03 (s, 1H, NH) 6.11 (d, 1H, NH) 7.11–7.67 (m, 17H, Ar-H)
4f	Hydrogen	Chloro	194	49	$C_{29}H_{20}N_3O_2Cl$	8.50 (8.79)	–	4.38 (d, 2H, CH_2) 6.03 (s, 1H, NH) 6.09 (d, 1H, -NH) 7.11–7.70 (m, 16H, -ArH)
4g	Hydrogen	Methoxy	186	40	$C_{30}H_{23}N_3O_3$	8.41 (8.88)		

Table 1: continued

Compound no.	R	R'	M.P. °C	Yield (%)	Molecular formula	% *N* Found(Calcd)	I.R.	^{1}H NMR
							1367 (OCH_3) 3474 (NH str.) 1530 (NH bend) 1680 (C=O amide) 1658 (C=O δ lactum) 3030 (Aromatic C-H)	3.74 (s, 3H, –OCH_3) 4.32 (d, 2H, -CH_2) 6.6 (s, 1H, -NH) 6.9 (d, 1H, -NH) 7.10–7.90 (m, 16H, ArH)

labeling (23–25). The fresh human serum was prepared by allowing blood collected from healthy volunteers to clot for 1 h at 37° C in a humidified incubator maintained at 5% carbon dioxide, 95% air. Then, the sample was centrifuged at 400 rpm and the serum was filtered through 0.22 micron syringe filter into sterile plastic culture tubes. The above freshly prepared technetium radio complexes were incubated in fresh human serum at physiological conditions that is at 37 °C at a concentration of 100 nM/mL and then analyzed by ITLC-SG at different time intervals to detect any dissociation of complex. Percentage of free pertechnetate at a particular time point that was estimated using saline and acetone as mobile phase, represented percentage dissociation of the complex at that particular time point in serum.

Blood kinetic studies

The blood clearance study was performed in normal rabbit, weighing 2–2.5 kg. Five MBq of the ^{99m}Tc labeled compounds (0.3 mL) was administered intravenously through the dorsal ear vein. At different time intervals about 0.5-mL blood samples were withdrawn from the dorsal vein of other ear, and radioactivity was measured in the gamma counter. The data from the experiment were expressed as percentage of administered dose at each time interval.

Biodistribution study in mice

Albino mice strain (A) (taken in triplicate set) was used for the tissue distribution studies. Animal handling and experimentation were carried out as per the guidelines of the Institutional Animal Ethics Committee.

An equal dose of 10 μCi of labeled test compound was injected in mice through tail vein of each animal. At different time intervals, mice were sacrificed, blood was collected, and different tissue and organs were dissected and analyzed. The radioactivity was measured in a gamma counter. The actual amount of radioactivity administered to each animal was calculated by subtracting the activity left in the tail from the activity injected. Radioactivity accumulated in each organ was expressed as percentage administered dose per gram of tissue. Total volume of the blood was calculated as 7% of the body weight.

Anticaner activity

The synthesized compounds were tested against (HeLa) cell line. Routine culture maintenance and experimental studies were carried out at 37 °C in a cell incubator with humid atmosphere at 5% CO_2. Cell propagation was achieved in Dubecco's modified eagle minimal medium (DMEM) with phenol red, 10% fetal bovine serum, L-glutamine, penicillin, streptomycin, and gentamycin as described in previous literature. Before any experiment, the cells were transferred for 4 days to a defined medium, containing phenol red free DMEM, supplemented with 10% charcoal-stripped. Estrogen (17 β-estrodiol) in concentration up to 100 μg added to defined medium. Doxorubicin is taken as standard. The MTT assay with 3-(4, 5-dimethylthiazole-2-yl)-2, 5-phenyltetrazolium bromide was used to determine the number of viable cells. For assay, HeLa cells (1×10^4 cells/well) were platted in a 96-well tissue culture plate and exposed to the compounds under investigation. Cells were processed with the MTT assay for 24, 48, and 72 h of incubation. In brief, 10 μL of MTT (final concentration = 250 μg/mL) in phosphate-buffered saline (PBS) was added to every well containing 100-μL cell suspension in medium, and the cultures were allowed to incubate at 37 °C for 5 h. The reaction mixture was carefully taken out and 100 μL of DMSO was added to each well and pipetted up and down several times unless it became homogenic. After 10 minutes, the color was read at 540 nm using spectrophotometer plate reader (Bio-Rad, Tokyo, Japan). The inhibitory effect on cell proliferation was determined after 72 h of treatment with various concentrations (0.1–300 nM) of the tested compound.

EGFR kinase activity assay

To study biological activity, compounds were evaluated against EGFR kinase activity assays. Briefly, 96-well plates were precoated with a synthetic substrate poly-Glu-Tyr (Sigma, 0.25 mg/mL) overnight at 37 °C. Ten microliters of EGFR-TK extract, reaction medium and tested compound (20 μL) were added. The reaction mixtures were incubated for 30 min at room temperature while being shaken. Kinase reaction was quenched by removal of the reaction mixture and then the wells were washed with washing buffer for three times. Phosphorylated tyrosine substrate was blocked in PBS containing 3% BSA for 30 min, washed with washing buffer (PBS containing 0.1% Tween 20) for

three times, detected by adding antiphosphotyrosine antibody for 1 h. Then antibody was removed, and wells were washed with washing buffer for three times. The optical density was measured at 450 nm by an ELISA reader. Experiment with triplicate data was performed. IC50 values were calculated for test compounds using a regression analysis of the concentration/inhibition data.

Result and Discussion

All intermediates as well as final quinazoline analogues were analyzed by different spectroscopic technique such as IR, NMR, mass spectroscopy, and by elemental analysis. Comparing the TLC with the starting materials, which resulted a single spot different from the starting materials, checked the synthesized ligand. The spectral evidence confirms the presence of different functionalities (IR at 3351, 1640, 1467/cm). Similarly, NMR multiplet in the range of (6.2–8) p.p.m. of 5–15 hydrogen also confirms the presence of aromatic rings. It also confirms the proposed stoichiometry and structure for the pyrido-quinazolone. Integral of NMR confirms the number of protons in pyrido-quinazolones as well as coupling constant confirms the nature of double bonds.

Modern medicine demands progressively more sophisticated methods for the accurate diagnosis of diseased states, and there is a massive worldwide research effort into developing and improving imaging techniques.

Preliminary Complexation of novel synthesized compounds with ^{99m}Tc was found to give sufficiently stable complexes under physiological conditions. The *in vitro* serum stability of the radio complexes is necessary parameter meant to measure the effectiveness of chelating moiety to co-ordinate the radio metal. Generally, there is transchelation of radio metal to serum proteins particularly albumin. *In vitro* serum stability of the complexes clearly indicates that initially there was fall in the stability of the complex but further showing a constant stability. Initial fall in the labeling efficiency after addition of fresh serum could be attributed to the trans-chelation that could have taken place in serum due to high affinity of plasma proteins for metal ions.

The retention of drug in the blood of the animal depends upon the pharmacological and physical properties of the drugs. Nearly, all the pyrido-quinazolone shows a very rapid clearance of radioactivity from the blood. Approximately 55–65% of activity was removed within 1 h and more than 90% in 4 h. It shows rapid kinetics, which may be attributed to the hydrophilic nature of the drug radio metal complexes.

Biodistribution of the radio complexes is an important phenomenon to study because it gives an idea about its excretory metabolic pathway and *in vivo* distribution of the radio complex drug. Accumulation of low amount of radio-

Table 2: Effects of compounds **4[A]**–**4[G]** on epidermal growth factor receptor (EGFR)-TK activity and HeLa cell proliferation

Compound	IC_{50} (μM) EGFR-TK	IC_{50} (nM) HeLa
4[A]	0.238 ± 0.002	8.05 ± 1.24
4[B]	0.132 ± 0.005	4.51 ± 0.51
4[C]	0.051 ± 0.003	35.40 ± 1.34
4[D]	0.185 ± 0.006	11.02 ± 0.59
4[E]	0.152 ± 0.005	38.49 ± 1.02
4[F]	0.085 ± 0.006	49.32 ± 0.59
4[G]	0.140 ± 0.005	23.49 ± 1.02

The values are the mean ± SD of independent experiments. Concentration of compound resulting in 50% inhibition of EGFR-TK activity.

activity in the stomach precludes the presence of free pertechnetate, which indicates *in vivo* stability of preparation. The percentage distribution of drug in various organs of mice is shown as percentage of injected dose per organ or tissue at different time interval. The drug localized in the liver and kidneys, with the passage of time the activity in kidney amplified for most of the compounds, while in intestine, there were negligible increase in activity. This shows that the major route of excretion of activity is through kidneys. With passage of time, there was an increase in accumulation of activity in urinary bladder. Besides this, there was retention of radioactivity in liver for considerable period, indicating that metabolism of drugs probably takes place in liver, but the excretion of drugs and metabolites is mainly through kidney. Accumulation of drugs in liver may also be because of protein binding nature of drugs. Very slight accumulation of activity was observed in lungs, spleen, and stomach. Negligible accumulation occurs in heart and brain.

The cell proliferation was measured by MTT assay, and the results were expressed as IC_{50} values. The activity data are given in Table 2. The inhibition of the EGFR activity by 4[A] - 4[G] were evaluated in human breast cancer cell line, HeLa. These cells are also known to over express EGFR, which leads to continuous activation of the EGFR pathway involved in cell proliferation. The inhibitory effects on HeLa cell proliferation were determined after 72 h of treatment with various concentrations (10^{-5}–10^{-10} M) of the tested compound, and the results were expressed as IC_{50} values ranging from (4.5–50 nM). It was found that for these molecules cell-based numbers are lower than the enzyme numbers, which may be due to their interactive nature of diaza type of compounds and further validates the need of investigation to increase the insight for mechanistic aspect of these molecules.

Conclusion

Here, we describe the synthesis of designed compounds, which are able to irreversibly block epidermal growth factor receptor (EGFR) that may effective for cancer treatment.

The synthetic way for the preparation of the pyrido-quinazolone derivatives is easy and convenient. Additional investigation to increase their efficacy as anticancerous activity and to improve the pharmacokinetics performance of these new pyrido-quinazolone derivatives may result in potent drugs becoming available for commercial exploitation and importance of molecule as multimodal application.

Conflict of interest

This is also certified that there is no conflict of interest between the authors.

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