Available online at www.sciencedirect.com
ScienceDirect
European Journal of Medicinal Chemistry 43 (2008) 1478–1488
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

Syntheses of 4-(indole-3-yl)quinazolines – A new class of epidermal growth factor receptor tyrosine kinase inhibitors

Anja Lüth*, Werner Löwe

Freie Universität Berlin, Institut für Pharmazie, Königin-Luise-Strasse 2+4, 14195 Berlin, Germany

Received 21 November 2006; received in revised form 28 August 2007; accepted 10 September 2007
Available online 29 September 2007

Abstract

The epidermal growth factor (EGF) family of membrane receptors has been identified as a key element in the complex signaling network that is utilized by various classes of cell–surface receptors. The synthesis and pharmacological results of 4-(indole-3-yl)quinazolines are described. The synthesized compounds are new high potent EGFR-tyrosine kinase inhibitors with excellent cytotoxic properties at different cell lines. Furthermore the 4-(indole-3-yl)quinazolines show some tendencies to inhibit the HER-2 TK, too. Moreover this substance class has remarkable strong fluorescence properties.

Keywords: Tyrosine kinase inhibitor; EGFR; HER-2; ATP antagonist

1. Introduction

The epidermal growth factor (EGF) family of membrane receptors has been well characterized in cancer research. Conclusive evidence from studies in the last two decades has demonstrated that dysregulation of the epidermal growth factor receptor (EGFR, also known as HER-1, ErbB-1) is associated with the principal characteristics of cancer, including autonomous cell growth, invasion and angiogenesis. The EGFR is a transmembrane glycoprotein with an extracellular ligand-binding domain and an intracellular domain with tyrosine kinase activity for signal transduction. Ligand binding activates the receptor and its signaling pathways leading to the activation or modulation of cellular processes [1,2]. The receptor is expressed on healthy cells (40–100 EGFR/cell) as well as on malignant tissues (more than 1 000 000 EGFR/cell) [3]. EGF and transforming growth factor-α (TGF-α) are the most important stimulatory ligands for EGFR. Overexpression of EGFR has been demonstrated in a wide variety of malignant cells, and this increase in receptor levels has been associated with a poor clinical prognosis.

Thus, therapeutic strategies to inhibit EGFR and EGFR-related pathways have been pursued, including the development of ATP-competitive small molecule inhibitors of the intracellular tyrosine kinase domain of the receptor or inhibitors of downstream effectors of EGFR signaling pathways, like Gefitinib **1** (IC_{50} 20 nM) and Erlotinib **2** (IC_{50} 1 nM) as shown in Fig. 1 [4,5]. The 4-anilinoquinazoline derivatives are both selective and effective inhibitors of the EGFR tyrosine kinase.

Most of the EGFR-tyrosine kinase inhibitors have the same 4-anilinoquinazoline skeleton, only the substituents and the side chains are variable. Therefore, the replacement of the aniline structure by an indole nucleus could rigid the resulting structure by only one single bonding between the indole and the quinazoline nucleus. On the other hand, hydrogen bonding between the indole–NH and the peptide backbone of the EGF receptor could afford specific conformations, improving the inhibitory activities of the resulting derivatives.

In an earlier work by the Rhone-Poulenc Rorer group three 4-(indole-3-yl)quinazolines have already been described. Here it was discovered that 4-(indole-3-yl)-substitution at the

* Corresponding author. Tel.: +49 30 838 54109; fax: +49 30 838 53854.
E-mail address: anja.lueth@fu-berlin.de (A. Lüth).

0223-5234/$ - see front matter © 2007 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2007.09.018

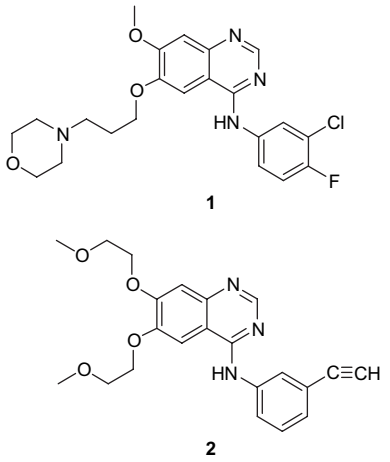


Fig. 1. The 4-anilinoquinazolines Gefitinib **1** and Erlotinib **2**.

quinazoline moiety is not compatible with good activity [6]. For example, the Rhone-Poulenc Rorer ''compound 90'' was evaluated for its activity toward EGFR and HER-2 tyrosine kinases and was described in a review article by Bridges as follows: ''Compound 90 represents a more fundamental change in the pharmacophore and claims moderate ErbB-2 activity with little or no EGFR activity'' (Fig. 2) [7]. But these mentioned compounds possess no halogen substitution pattern. Therefore the benzene ring of the indole moiety of 4-(indole-3-yl)quinazolines should be substituted by halogen, preferably by fluorine and chlorine, which are essential for the activity of the comparable 4-anilinoquinazolines like Gefitinib.

2. Results and discussion

2.1. Chemistry

Since an earlier work by the Parke-Davis group with quinazoline-based inhibitors of EGFR established that 6,7-dialkoxy substitution is compatible with good activity, we decided to retain this feature in our initial compounds [8]. Therefore compound **4** was synthesized according to the procedure of Zeneca Ltd depicted in Scheme 1 [9]. Starting with 6,7-dimethoxyanthranilic acid the amino group was condensed with formamidine acetate in 2-methoxyethanol, followed by treatment with thionyl chloride and *N*,*N*-dimethylformamide to give 4-chloro-6,7-dimethoxyquinazoline (**4**). On the other side, it was necessary to prepare the differently substituted indoles **16**–**18** as outlined in Scheme 2 [10,11].

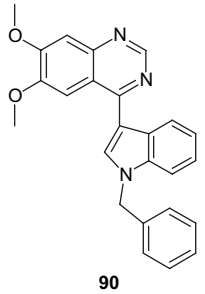
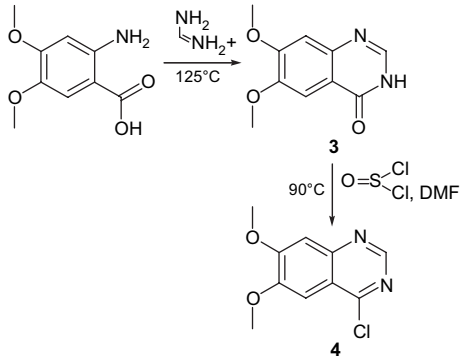


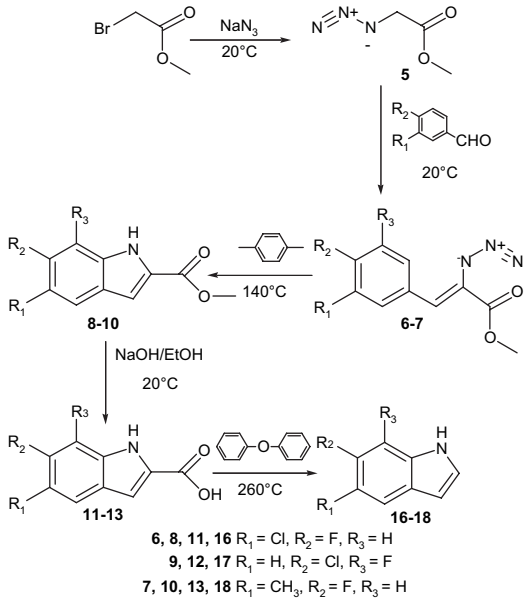
Fig. 2. Rhone-Poulenc Rorer ''compound **90**''.



Scheme 1. Syntheses of 4-chloro-6,7-dimethoxyquinazoline **4**.

The syntheses of the indoles are based on a procedure reported by Hemetsberger-Knittel and co-workers [12]. Treatment of methylazidoacetate **5** with substituted benzaldehydes gave α,β-unsaturated cinnamic acid esters **6**–**7**. Refluxing compounds **6**–**7** in xylene resulted in cyclization of the cinnamic esters to give the indole-2-carboxylic acid esters **8**–**10**. The intramolecular cyclization of the cinnamic acid ester **6** did not occur regiospecific in position 6 of the benzene ring but also in position 2. At this point of the synthesis, a mixture of two indole carboxylic esters was isolated. After that, the hydrolysis of the esters afforded the corresponding indole carboxylic acids **11**–**13** which were decarboxylated to give the desired indoles **16**–**18**. The resulting indole isomers were easily separated by sc-chromatography.

The syntheses of 4-(indole-3-yl)quinazolines (**19**–**22**, **28**, **36**) are based on a chemoselective and regioselective hetarylation reaction. Searching for a cross-coupling approach to connect the quinazoline and indole moieties, an interesting method was discovered, which afforded a hetarylation reaction with the help of Grignard compounds. In the key step

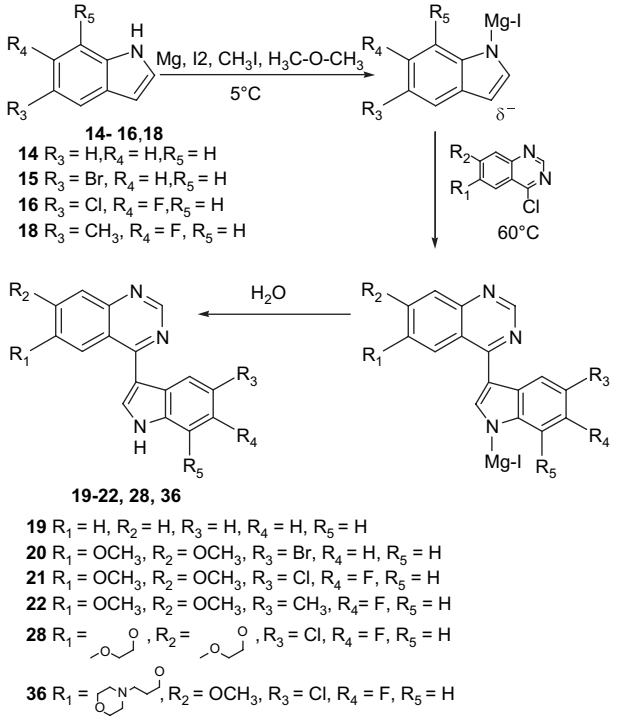


Scheme 2. Syntheses of indoles **16**–**18**.

metalated indolyl-magnesium compounds were reacted with 4-chloroquinazolines to give 4-(indole-3-yl)quinazolines **19**–**22**, **28**, **36** as shown in Scheme 3. To prove that method, firstly we prepared the unsubstituted compound **19**. In this case the indolyl-magnesium compound was reacted with 4-chloroquinazoline to give the desired 4-(indole-3-yl)quinazoline **19**. After a successful isolation of compound **19** it was proved, whether indoles with halogen substitution retain its halogen-pattern permanently under hetarylation conditions. Therefore compound **20** was synthesized from the above used 4-chloro-6,7-dimethoxyquinazoline **4** and commercially available 5-bromoindole.

Because of the successful synthesis of the halo substituted compound **20** the hetarylation reaction with help of Grignard compounds is suitable to yield all the desired substituted 4-(indole-3-yl)quinazolines. The first part of the syntheses of this new class deals with the preparation of 4-(indole-3-yl)quinazolines **19**, **20**–**22** and **27** with constant quinazoline and variable indole substitution. Because of the excellent pharmacologically profile of **21** indole **16** was used for the following hetarylations.

The second section of the work deals with the syntheses of 4-(indole-3-yl)quinazolines, showing different quinazoline and constant indole substitution patterns. In this part at first compound **28** was synthesized, whose quinazoline structure is comparable with that of Erlotinib (**2**). The quinazoline component **27** was completely synthesized before the cross-coupling reaction. Therefore, ethyl-3,4-dihydroxybenzoate was alkylated with bromoethylmethylether to give compound **23**. Subsequently the nitro group was introduced in **23** by an electrophilic substitution at the aromatic benzene ring resulting in substance **24**. After reduction of the nitro group to give compound **25** the following condensation of the anthranilic acid moiety with formamidine acetate yielded in the cyclized quinazolinone **26**, which was then chlorinated in position 4 in the presence of thionyl chloride and *N*,*N*-dimethylformamide to give compound **27**. In the final step the desired compound **28** was separated after the above-mentioned cross-coupling reaction with the indole moiety **16** (see Scheme 4).



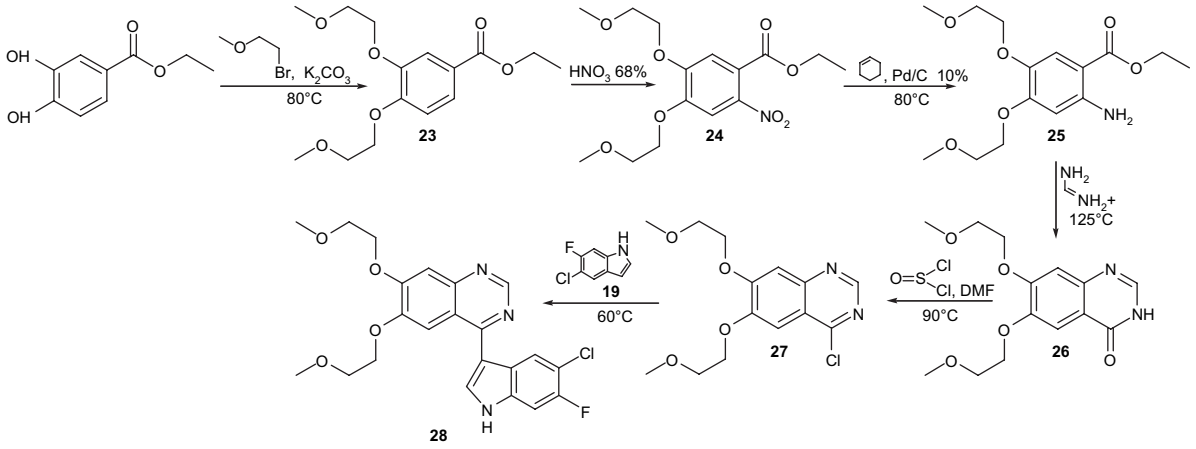
Scheme 3. Hetarylation reaction between the indole and quinazoline part.

In case of the Gefitinib-like compound **36** we had to prepare a special 4-chloroquinazoline with a suitable 6-position for the substitution of a specific side chain, comparable with that of Gefitinib (**1**, Scheme 5). Here the hetarylation was not the final step during the synthesis. The quinazoline side chain at position 6 of **36** was introduced only after cross-coupling reactions. Firstly, the condensation reaction of the 3,4-dimethoxyanthranilic acid with formamidine acetate gave the quinazolinone **29**. Treatment with methane sulphuric acid in the presence of methionine dealkylates only one methoxy group in the position 6 of **29**. The resulting free phenolic function of compound **30** was protected using acetic anhydride in the presence of pyridine to give compound **31**. The following halogenation in position 4 was performed with thionyl chloride and *N*,*N*-dimethylformamide to yield the 4-chloro-compound **32**. Subsequently, 5-chloro-6-fluoroindole (**16**) was metalated using the Grignard compound methylmagnesium iodide to activate the 3-position of the indole heterocycle for the electrophilic hetarylation reaction with the 4-chloroquinazoline **32**. After the successful cross-coupling reaction resulting in substance **33** the prepared 4-(3-chloropropyl)morpholine side chain was introduced in the 6-position of the quinazoline moiety of **34**. In this case, firstly the indole–NH in compound **33** was protected with BOC to introduce the side chain regiospecific in the desired position 6 of compound **34**. It was difficult to unprotect the BOC group of **35** because of the inclusion of solvents or inorganic salts. Therefore that reaction was done with formic acid and Triton B which were suitable to neutralize the acidic solution. In this case the voluminous cation could not be included in the podand-like structure of compound **36**.

The synthesized 4-(indole-3-yl)quinazolines show remarkable strong fluorescence in the ultraviolet light. For example the fluorescence of compound **36** is comparable with that of quinine-sulphate (Fig. 3).

2.2. In vitro EGFR inhibition activity

Compounds **20**–**22**, **28** and **36** were evaluated for their ability to inhibit EGFR tyrosine kinase by MDS Pharma Services, Taiwan [13,14]. The results are reported in Table 1. The assay was based on the inhibition of phosphorylation of polyglutamic acid/tyrosine [poly(glu/tyr, 4:1)] by EGFR tyrosine kinase and was performed in an IC_{50} semi-quantitative analysis (five concentrations in duplicate: 10 tubes). The IC_{50} values of the EGFR-TK inhibition (131–533 nM) show that compounds **20**–**22**, **28** and **36** are relatively potent inhibitors of EGFR-TK activity. Because of the bromine induced polarity we suppose, but we cannot prove it, that the cytotoxicity of **20** is somewhat weaker compared with the more lipophilic



Scheme 4. Syntheses of the Erlotinib-like compound **28**.

chloro-fluoroindole-substitution pattern of compounds **21** and **36**.

2.3. In vitro HER-2 inhibition activity

Compounds **21** and **36** were additionally tested for their HER-2-tyrosine kinase inhibition activity by a two-point measure (primary screen) by MDS Pharma Services, Taiwan [15,16]. Compound **21** inhibited the HER-2 tyrosine kinase at a concentration of 100 nM with a rate of 19% and substance **36** at the same concentration with a result of 3%. These results indicate that compounds **21** and **36** could be discussed as dual EGFR-TK and HER-2-TK inhibitors.

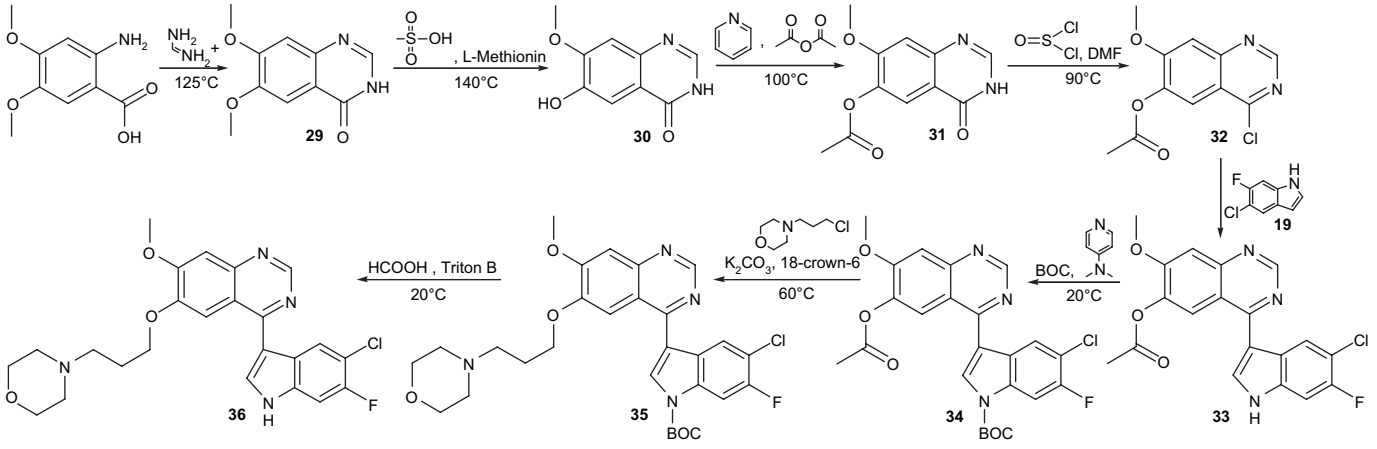
2.4. In vitro cytotoxicity assay

Compounds **21** and **36** were submitted for in vitro biological testing against 60 cancer cell lines provided by the National Cancer Institute-Developmental Therapeutics Program (NCI-DTP). The NCI-DTP screening procedure is described in detail elsewhere [17–19]. The results, summarized in Table 2, reveal that compounds **21** and **36** exhibited excellent growth inhibition ($GI_{50} < 10^{-6}$ M) against different cancer cell lines. Compound **36** showed a very strong growth inhibition ($GI_{50} \sim 10^{-8}$ M) against lung cancer cell lines EKVX and NCI-H322M. The same phenomenon ($GI_{50} \sim 10^{-8}$ M) was observed for cell lines of CNS cancer (SNB-75) and renal cancer (TK-10). The other mentioned cell lines in Table 2 were inhibited by compound **36** at concentrations in the range of 10^{-6}–10^{-7} M (GI_{50}). In case of compound **21** the GI_{50} values are observed between 10^{-6} and 10^{-7} M. Surprisingly the GI_{50} of CNS cancer cell line SNB-75 is weaker ($\sim 10^{-7}$ M) than the comparable result with compound **36** ($\sim 10^{-8}$ M). These results give no remark of a connection between the cytotoxicity and the EGFR-TK inhibition. The mechanism of action which is responsible for the cytotoxic properties remains yet unclear.

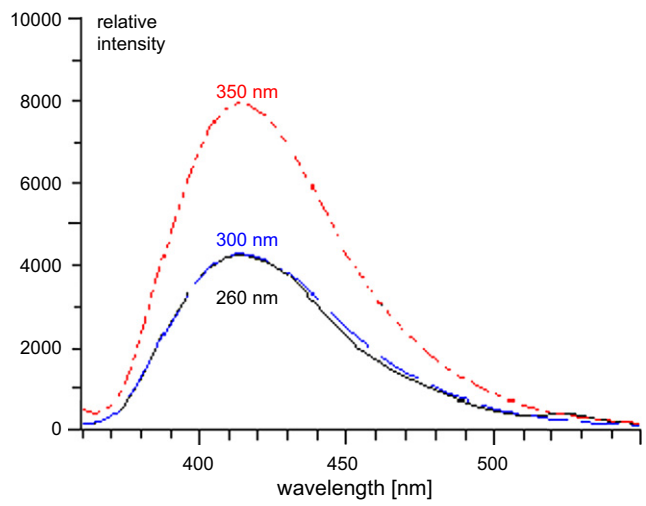
3. Conclusions

Our new synthetic pathways are suitable to guarantee the desired 4-(indole-3-yl)quinazolines with a halogen substitution pattern in the molecules.

The synthesized compounds **20**–**22**, **28** and **36** were tested for their in vitro EGFR-tyrosine kinase inhibition. Furthermore compounds **21** and **36** were evaluated for HER-2-tyrosine

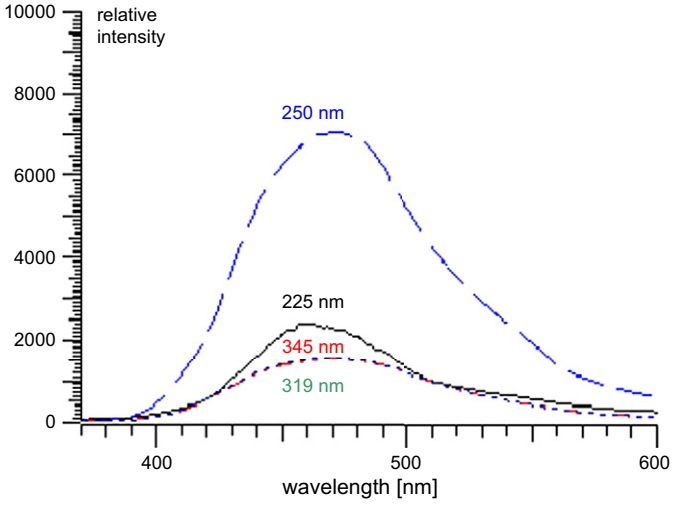


Scheme 5. Syntheses of the Gefitinib-like compound **36**.



36

excitation	emission	intensity
260	413.8	4258
300	413.6	4298
350	413.6	7948



quinine-sulphate

excitation	emission	intensity
225	461.4	2416
250	470.6	7089
319	468.6	1542
345	468.6	1556

Fig. 3. Fluorescence analysis of compound **36** at a concentration of 5 μm and quinine-sulphate at a concentration of 1 μm.

kinase inhibition and cytotoxicity testing. Partly the results show a relatively potent inhibition of the EGFR tyrosine kinase and for **28** and **36** also a tendency for inhibition of the HER-2 tyrosine kinase. Remarkable are the outstanding cytotoxic qualities at different cancer cell lines with GI_{50} values between 10^{-6} and 10^{-8} M.

The results of the EGFR-TK inhibition and the cytotoxity give this new substance class a very interesting option in treatment of EGFR-dependent tumors.

It is conspicuous that the compounds show strong fluorescences.

The EGF receptor appears more flexible, as assumed until now. Therefore more structure variations in position 4 of the quinazolines are possible to yield quite new structures of modern and innovative EGFR-TK inhibitors.

4. Experimental section

*4.1. 6,7-Dimethoxy-3,4-dihydroquinazoline-4-on (**3**)*

2-Amino-4,5-dimethoxybenzoic acid (6.30 g, 38.14 mmol) and formamidine acetate (8.50 g, 81.65 mmol) in 2-methoxyethanol (100 mL) were heated under reflux for 8 h. After evaporation of 2-methoxyethanol the resulting residue was stirred with ammonia (10%). The residue was isolated, washed with water and dried to give 5.90 g (89.5%) of a redbrown solid. mp: 300 °C; ^{1}H NMR (DMSO-d_6): δ 12.64 (s, 1H, NH), 7.99 (s, 1H, H-2), 7.44 (s, 1H, H-5), 7.13 (s, 1H, H-8) 3.89 (s, 6H, OCH_3); MS: *m/z* 206.4 (M^+•). Anal.: ($C_{10}H_{10}N_2O_3$) C, H, N.

*4.2. 4-Chloro-6,7-dimethoxyquinazoline (**4**)*

A stirred mixture of **3** (2.00 g, 9.67 mmol), thionyl chloride (30 mL) and *N*,*N*-dimethylformamide (0.6 mL) was heated under reflux for 4 h. The organic layer was evaporated. The residue was washed with diethyl ether and dried to give 1.80 g (82.8%) of a beige solid. ^{1}H NMR (DMSO-d_6): δ 8.89 (s, 1H, H-2), 7.47 (s, 1H, H-5), 7.41 (s, 1H, H-8), 4.01 (s, 6H, OCH_3); MS: *m/z* 223.9 (M^+•).

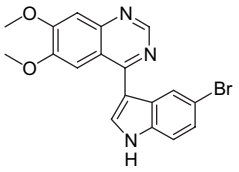
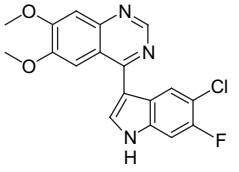
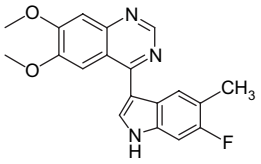
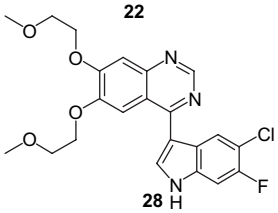
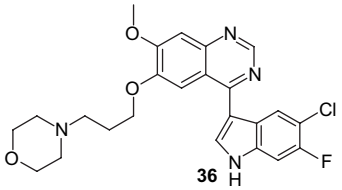
*4.3. Methylazidoacetate (**5**)*

Sodium azide (44.00 g, 676.81 mmol) and methylbromoacetate (96.90 g, 687.44 mmol) were suspended in *N*,*N*-dimethylformamide and stirred at ambient temperature for 16 h. The reaction mixture was given into ice water and was extracted with diethylether. The organic layer was washed with water and dried. After evaporation of *N*,*N*-dimethylformamide the crude product was distillated under vacuo to give 64.20 g (82.4%) of a colourless oil. ^{1}H NMR (DMSO-d_6): δ 3.90 (s, 2H, CH_2), 3.81 (s, 3H, OCH_3); MS: *m/z* 115.1 (M^+•).

*4.4. Methyl-α-azido-3-chloro-4-fluorocinnamate (**6**)*

Under cooling sodium (1.35 g) was solved in methanol (30 mL). A mixture of 3-chloro-4-fluorobenzaldehyde (4.65 g, 29.33 mmol) and **5** (6.75 g, 58.65 mmol) in methanol (10 mL) was dropwise given to the sodium methanolate. The mixture was stirred at ambient temperature for 3 h. After neutralization with hydrochloric acid (2 N) the reaction mixture

Table 1
IC_{50} of EGFR-tyrosine kinase inhibition of compounds **20**–**22**, **28** and **36** and HER-2-tyrosine kinase inhibition in % at a concentration of 100 nm of compounds **21** and **36**

Compound	IC_{50} of the EGFR-TK inhibition (nM)
 **20**	131
 **21**	209 (HER-2-TK inhibition at a concentration of 100 nm: 3%)
 **22**	533
 **28**	472
 **36**	333 (HER-2-TK inhibition at a concentration of 100 nm: 19%)

Reference compounds: EGFR-TK inhibition: Tyrphostin 47 – IC_{50} 1.31 μM; HER-2-TK inhibition: Tyrphostin 47 – IC_{50} 5.70 μM.

was extracted with ethyl acetate. The organic layer was washed with water and dried. After evaporation of ethyl acetate at a temperature less than 40 °C the resulting residue was chromatographed on silica gel with ethyl acetate/*n*-hexane (1:3) to give 4.55 g (60.7%) of white crystals. mp: 58 °C; ^{1}H NMR (DMSO-d_6): δ 8.13 (dd, $^4J = 2.16$ Hz, $J_{H-F} = 7.42$ Hz, 1H, H-2), 7.90 (m, 1H, H-6), 7.47 (t, $^3J = 9.01$ Hz, 1H, H-5), 6.93 (s, 1H, CH aliph), 3.86 (s, 3H, OCH_3); MS: *m/z* 255.1 (M^+•). Anal.: ($C_{10}H_7ClFN_3O_2$) C, H, N.

4.5. Methyl-α-azido-4-fluoro-3-methylcinnamate (7)

This compound was prepared from 4-fluoro-3-methylbenzaldehyde and **5** as previously described. The crude yield was 4.74 g (67.0%) of light yellow crystals. mp: 61 °C; ^{1}H NMR (DMSO-d_6): δ 7.85 (dd, $^4J = 2.32$ Hz, $J_{H-F} = 7.69$ Hz, 1H, H-2), 7.79 (m, 1H, H-6), 7.22 (t, $^3J = 9.14$ Hz, 1H, H-5), 6.93 (s, 1H, CH aliph), 3.88 (s, 3H, OCH_3); MS: *m/z* 235.3 (M^+•). Anal.: ($C_{11}H_{10}FN_3O_2$) C, H, N.

Table 2
In vitro cytotoxicity of compounds **21** and **36** in selected cancer cell lines

Cell line	GI_{50} (M)	
	21	**36**
Non-small lung cancer		
EKVX	4.10×10^{-7}	8.97×10^{-8}
HOP-92	1.75×10^{-6}	5.04×10^{-7}
NCI-H322M	2.55×10^{-7}	3.80×10^{-8}
Ovarian cancer		
IGROV1	4.24×10^{-7}	1.08×10^{-7}
SK-OV-3	7.11×10^{-7}	5.92×10^{-7}
Renal cancer		
A498	4.92×10^{-7}	2.90×10^{-6}
ACHN	2.22×10^{-7}	2.15×10^{-7}
TK-10	3.25×10^{-7}	9.76×10^{-8}
Colon cancer		
HT29	8.48×10^{-7}	3.70×10^{-6}
Prostate cancer		
PC-3	7.66×10^{-7}	4.90×10^{-7}
CNS cancer		
SNB-75	2.04×10^{-7}	4.93×10^{-8}

4.6. Methyl-5-chloro-6-fluoroindole-2-carboxylate (8) and methyl-7-chloro-6-fluoroindole-2-carboxylate (9)

A mixture of **6** (2.38 g, 10.11 mmol) and xylene (180 mL) was heated under reflux for 45 min. The solvent was removed. There were obtained 1.00 g (47.8%) of an almost 1:1 mixture of **8** and **9** as white crystals which was used in the next step without further purification. ^{1}H NMR: (DMSO-d_6) δ 12.37 (s, 1H, NH, **9**), 12.22 (s, 1H, NH, **8**), 7.91 (d, $J_{H-F} = 7.47$ Hz, 1H, H-4, **8**), 7.67 (m, 1H, H-4, **9**), 7.35 (d, $J_{H-F} = 9.87$ Hz, 1H, H-7, **8**), 7.29 (s, 1H, H-3, **9**), 7.17 (t, $^3J = 9.20$ Hz, 1H, H-5, **9**), 7.15 (s, 1H, H-3, **8**), 3.89 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3); MS: *m/z* 227.0 (M^+•).

4.7. Methyl-6-fluoro-5-methylindole-2-carboxylate (10)

This compound was prepared from **7** as previously described. The crude yield was 1.00 g (47.8%) of white crystals. ^{1}H NMR (DMSO-d_6): δ 11.82 (s, 1H, NH), 7.46 (d, $J_{H-F} =$ 8.37 Hz, 1H, H-4), 7.03 (d, $J_{H-F} = 10.73$ Hz, 1H, H-7), 7.01 (s, 1H, H-3), 3.79 (s, 3H, OCH_3), 2.20 (s, 1H, CH_3); MS: *m/z* 207.3 (M^+•).

4.8. 5-Chloro-6-fluoroindole-2-carboxylic acid (11) and 7-chloro-6-fluoroindole-2-carboxylic acid (12)

A suspension of an almost 1:1 mixture of **8** and **9** (2.04 g, 8.96 mmol) in ethanol (90 mL) and sodium hydroxide solution (2 N, 45 mL) was stirred at ambient temperature for 2 h. The alcohol was evaporated and the residue was treated with

hydrochloric acid (2 N). The crystals were isolated, washed with water and dried. There were obtained 1.8 g (94.1%) of an almost 1:1 mixture of **11** and **12** as white solid. ^{1}H NMR: (DMSO-d_6): δ 13.10 (s, 2H, COOH, **11**, **12**) 12.18 (s, 1H, NH, **12**), 12.02 (s, 1H, NH, **11**), 7.88 (d, J_{H-F} = 7.48 Hz, 1H, H-4, **11**), 7.65 (m, 1H, H-4, **12**), 7.33 (d, J_{H-F} = 9.94 Hz, 1H, H-7, **11**), 7.21 (s, 1H, H-3, **12**), 7.13 (t, ^3J = 9.07 Hz, 1H, H-5, **12**), 7.08 (s, 1H, H-3, **11**); MS: *m/z* 212.9 (M^+•).

4.9. 6-Fluoro-5-methylindole-2-carboxylic acid (*13*)

This compound was prepared from **10** as previously described. The crude yield was 1.72 g (90.4%) as white solid. ^{1}H NMR (DMSO-d_6): δ (ppm) 12.91 (s, 1H, COOH) 11.75 (s, 1H, NH), 7.50 (d, J_{H-F} = 8.09 Hz, 1H, H-4), 7.08 (d, J_{H-F} = 10.97 Hz, 1H, H-7), 7.01 (s, 1H, H-3), 2.28 (s, 3H, CH_3); MS: *m/z* 193.5 (M^+•).

4.10. 5-Chloro-6-fluoroindole (*16*) and 7-chloro-6-fluoroindole (*17*)

A suspension of 1.92 g (8.99 mmol) of an almost 1:1 mixture of **11** and **12** in diphenyl ether (45 mL) was stirred at 260 °C for 4 h. The reaction mixture was chromatographed over silica gel with *n*-hexane and *n*-hexane/toluene (3:1). There were obtained 0.70 g (45.9%) of **16** as white and 0.40 g (26.3%) of **17** as light brown crystals. **16**: mp: 83 °C; ^{1}H NMR (DMSO-d_6): δ 11.32 (s, 1H, NH), 7.70 (d, J_{H-F} = 7.40 Hz, 1H, H-4), 7.41 (t, J = 5.70 Hz, 1H, H-2), 7.38 (d, J_{H-F} = 10.22 Hz, 1H, H-7), 6.43 (t, J = 5.02 Hz, 1H, H-3); MS: *m/z* 169.2 (M^+•). Anal.: (C_8H_5ClFN) C, H, N.; **17**: mp: 42 °C; ^{1}H NMR (DMSO-d_6): δ 11.61 (s, 1H, NH), 7.53 (m, 1H, H-4), 7.42 (t, J = 5.46 Hz, 1H, H-2) 7.04 (t, ^3J = 8.79 Hz, 1H, H-5), 6.54 (t, J = 4.94 Hz, 1H, H-3); MS: *m/z* 169.1 (M^+•). Anal.: (C_8H_5ClFN) C, H, N.

4.11. 6-Fluoro-5-methylindole (*18*)

This compound was prepared from **13** as previously described. The crude yield was 0.9 g (60.7%) as light yellow crystals. ^{1}H NMR (DMSO-d_6): δ 11.05 (s, 1H, NH), 7.42 (d, J_{H-F} = 7.68 Hz, 1H, H-4), 7.33 (t, J = 5.48 Hz, 1H, H-2), 7.16 (d, J_{H-F} = 10.88 Hz, 1H, H-7), 6.40 (t, J = 4.13 Hz, 1H, H-3), 2.44 (s, 1H, CH_3); MS: *m/z* 169.1 (M^+•). Anal.: (C_9H_8FN) C, H, N.

4.12. 4-(Indole-3-yl)quinazoline (*19*)

Under cooling magnesium (0.25 g) and iodine were stirred with a mixture from methyliodide (1.2 mL) and diethyl ether (5 mL) for 15 min. Afterwards a solution of indole (0.25 g, 2.13 mmol) in diethyl ether (15 mL) was given to the reaction mixture and was stirred for 15 min. Subsequently 4-chloroquinazoline (0.30 g, 1.82 mmol) was added in portions. The mixture was heated under reflux for 1 h and then was added to ice water. After extraction with ethyl acetate the organic layer was dried and the ethyl acetate was evaporated. The crude product was chromatographed over silica gel with ethyl acetate/ethanol (9:1). There were obtained 0.2 g (45.1%) of a yellow solid. mp: 91 °C; ^{1}H NMR (DMSO-d_6): δ 12.16 (s, 1H, NH), 9.34 (s, 1H, H-2), 8.58 (d, ^3J = 8.44 Hz, 1H, H-5), 8.34 (d, ^3J = 6.90 Hz, 1H, H-4′), 8.32 (d, J = 2.88 Hz, 1H, H-2′), 8.09 (t, ^3J = 6.34 Hz, 1H, H-6), 8.05 (d, ^3J = 8.53 Hz, 1H, H-8), 7.80 (t, ^3J = 8.36 Hz, 1H, H-7), 7.61 (d, ^3J = 8.36 Hz, 1H, H-7′), 7.32 (t, ^3J = 8.06 Hz, 1H, H-5′), 7.25 (t, ^3J = 7.44 Hz, 1H, H-6′); MS: *m/z* 245.0 (M^+•). Anal.: ($C_{16}H_{11}N_3$) C, H, N.

4.13. 4-(5-Bromoindole-3-yl)-6,7-dimethoxyquinazoline (*20*)

Under cooling magnesium (0.25 g) and iodine were stirred with a mixture from methyliodide (1.2 mL) and diethyl ether (5 mL) for 15 min. Afterwards a solution of 5-bromoindole (0.50 g, 2.50 mmol) in diethyl ether (15 mL) was given to the reaction mixture and was stirred for 15 min. Subsequently **4** (0.70 g, 3.11 mmol) was added in portions. The mixture was heated under reflux for 1 h and then was added to ice water. After extraction with ethyl acetate the organic layer was dried and the ethyl acetate was evaporated. The crude product was chromatographed over silica gel with ethyl acetate/ethanol (9:1) and was recrystallized from ethyl acetate/*n*-hexane. There were obtained 0.15 g (14.2%) of yellow crystals. mp: 256 °C; ^{1}H NMR (DMSO-d_6): δ 12.08 (s, 1H, NH), 9.09 (s, 1H, H-2), 8.40 (d, 1H, ^4J = 2.47 Hz, H-4′), 8.39 (d, 1H, J = 1.31 Hz, H-2′), 7.68 (s, 1H, H-5), 7.51 (d, 1H, ^3J = 8.58 Hz, H-7′), 7.38 (s, 1H, H-8), 7.36 (dd, 1H, ^3J = 9.19 Hz, ^4J = 1.81 Hz, H-6′); MS: *m/z* 383.1 (M^+•). Anal.: ($C_{18}H_{14}BrN_3O_2$) C, H, N.

4.14. 4-(5-Chloro-6-fluoroindole-3-yl)-6,7-dimethoxyquinazoline (*21*)

This compound was prepared from **16** and **4** as previously described. There were obtained 0.12 g (12.7%) of yellow crystals. mp: 245 °C; ^{1}H NMR (DMSO-d_6): δ 12.09 (s, 1H, NH), 9.09 (s, 1H, H-2), 8.44 (d, 1H, J = 2.70 Hz, H-2′), 8.37 (d, 1H, J_{H-F} = 7.63 Hz, H-4′), 7.68 (s, 1H, H-5), 7.53 (d, 1H, J_{H-F} = 9.82 Hz, H-7′), 7.39 (s, 1H, H-8), 4.04 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3); MS: *m/z* 357.2 (M^+•). Anal.: ($C_{18}H_{13}ClFN_3O_2$) C, H, N.

4.15. 4-(6-Fluoro-5-methylindole-3-yl)-6,7-dimethoxyquinazoline (*22*)

This compound was prepared from **18** and **4** as previously described. There were obtained 0.24 g (22.8%) of yellow crystals. mp: 251 °C; ^{1}H NMR (DMSO-d_6): δ 11.86 (s, 1H, NH), 9.12 (s, 1H, H-2), 8.31 (d, 1H, J = 2.62 Hz, H-2′), 8.10 (d, 1H, J_{H-F} = 7.89 Hz, H-4′), 7.73 (s, 1H, H-5), 7.43 (s, 1H, H-8), 7.32 (d, 1H, J_{H-F} = 10.45 Hz, H-7′), 4.06 (s, 3H, OCH_3), 3.98 (s, 3H, OCH_3), 2.40 (s, 3H, CH_3); MS: *m/z* 337.0 (M^+•). Anal.: ($C_{19}H_{16}FN_3O_2$) C, H, N.

4.16. Ethyl-3,4-di-(2-methoxyethoxy)benzoate (**23**)

Ethyl-3,4-dihydroxybenzoate (7.29 g, 40.02 mmol), bromoethylmethylether (11.3 mL) and potassium carbonate anhydrous (16.60 g) in acetonitrile (100 mL) were heated under reflux for 20 h. After filtration the solvent was evaporated and the crude product was chromatographed over silica gel with *n*-hexane/ethyl acetate (3:2). There were obtained 10.00 g (83.6%) as colourless wax-like mass. mp: 53 °C; ^{1}H NMR (DMSO-d_6): δ 7.57 (dd, $^4J = 1.96$ Hz, $^3J = 8.46$ Hz, 1H, H-6), 7.47 (d, $^4J = 1.95$ Hz, 1H, H-2), 7.09 (d, $^3J = 8.50$ Hz, 1H, H-5), 4.25 (q, 2H, CH_2), 4.17 (t, 4H, H-2′, H-2″), 4.13 (t, 4H, H-3′, H-3″), 3.66 (m, 6H, H-5′, H-5″), 1.28 (t, 3H, CH_3); MS: *m/z* 298.5 (M^+•). Anal.: ($C_{15}H_{22}O_6$) C, H, N.

4.17. Ethyl-4,5-di-(2-methoxyethoxy)-2-nitrobenzoate (**24**)

Compound **23** (6.15 g, 20.62 mmol) was added to cooled nitric acid (65%, 75 mL) in portions and stirred under cooling for 2 h. The reaction mixture was put into water and extracted with dichloromethane. After evaporation of the solvent the crude product was chromatographed over silica gel with *n*-hexane/ethyl acetate (3:2). There were obtained 6.90 g (97.4%) of a brown oil. ^{1}H NMR (DMSO-d_6): δ 7.67 (s, 1H, H-3), 7.34 (s, 1H, H-6), 4.27 (m, 10H, CH_2, H-2′, H-2″, H-3′, H-3″), 3.68 (m, 6H, H-5′, H-5″), 1.26 (t, 3H, CH_3); MS: *m/z* 343.0 (M^+•). Anal.: ($C_{15}H_{21}NO_8$) C, H, N.

4.18. Ethyl-2-amino-4,5-di-(2-methoxyethoxy) benzoate (**25**)

Compound **24** (7.30 g, 21.26 mmol), palladium/carbon (10%, 2.8 g) and cylohexene (50 mL) in ethanol (500 mL) were heated under reflux for 9 h. After filtration and evaporation of the solvent the crude product was chromatographed over silica gel with *n*-hexane/ethyl acetate (3:2). There were obtained 4.40 g (66.0%) of a brown oil. ^{1}H NMR (DMSO-d_6): δ 7.20 (s, 1H, H-3), 6.43 (s, 2H, NH_2), 6.35 (s, 1H, H-6), 4.20 (q, 2H, CH_2), 4.05 (t, 2H, H-2′), 3.93 (t, 2H, H-2″), 3.67 (t, 2H, H-3′), 3.58 (t, 2H, H-3″), 3.40 (m, 3H, H-5′), 3.35 (m, 3H, H-5″), 1.27 (t, 3H, CH_3); MS: *m/z* 313.0 (M^+•). Anal.: ($C_{15}H_{23}NO_6$) C, H, N.

4.19. 6,7-Di-(2-methoxyethoxy)-3,4-dihydroquinazoline-4-on (**26**)

Compound **25** (6.30 g, 21.41 mmol) and formamidine acetate (8.5 g, 81.65 mmol) in 2-methoxyethanol (100 mL) were heated under reflux for 8 h. After evaporation of the solvent the crude product was chromatographed over silica gel with dichloromethane/methanol (9:1). There were obtained 4.00 g (63.5%) of a white solid. mp: 182 °C; ^{1}H NMR (DMSO-d_6): δ 12.07 (s, 1H, NH), 7.98 (s, 1H, H-2), 7.46 (s, 1H, H-5), 7.16 (s, 1H, H-8), 4.25 (t, 4H, H-2′, H-2″), 4.19 (t, 4H, H-3′, H-3″), 3.71 (m, 6H, H-5′, H-5″); MS: *m/z* 294.2 (M^+•). Anal.: ($C_{14}H_{18}N_2O_5$) C, H, N.

4.20. 4-Chloro-6,7-di-(2-methoxyethoxy)quinazoline (**27**)

A stirred mixture of **26** (2.00 g, 6.80 mmol), thionyl chloride (30 mL) and *N*,*N*-dimethylformamide (0.6 mL) was heated at reflux for 4 h. The liquid layer was evaporated. The residue was washed with diethyl ether and dried to give 1.10 g (51.8%) of a beige solid. ^{1}H NMR (DMSO-d_6): δ 8.88 (s, 1H, H-2), 7.50 (s, 1H, H-5), 7.46 (s, 1H, H-8), 4.36 (m, 4H, H-2′, H-2″), 3.76 (m, 4H, H-3′, H-3″), 3.35 (m, 6H, H-5′, H-5″); MS: *m/z* 312.2 (M^+•).

4.21. 4-(5-Chloro-6-fluoro-indole-3-yl)-6,7-di-(2-methoxyethoxy)quinazoline (**28**)

Under cooling magnesium (0.25 g) and iodine were stirred with a mixture from methyliodide (1.2 mL) and diethyl ether (5 mL) for 15 min. Afterwards a solution of **16** (0.50 g, 2.99 mmol) in diethyl ether (15 mL) was given to the reaction mixture and was stirred for 15 min. Subsequently **27** (0.70 g, 3.11 mmol) was added in portions. The mixture was heated under reflux for 1 h and then was added to ice water. After extraction with ethyl acetate the organic layer was dried and the ethyl acetate was evaporated. The crude product was chromatographed over silica gel with ethyl acetate/ethanol (9:1) and was recrystallisated from ethyl acetate/*n*-hexane. There were obtained 0.10 g (9.8%) of yellow crystals. mp: 197 °C; ^{1}H NMR (DMSO-d_6): δ 12.11 (s, 1H, NH), 9.09 (s, 1H, H-2), 8.41 (d, 1H, $J = 3.01$ Hz, H-2′), 8.38 (d, 1H, $J_{H-F} = 7.64$ Hz, H-4′), 7.73 (s, 1H, H-5), 7.55 (d, 1H, $J_{H-F} = 9.93$ Hz, H-7′), 7.41 (s, 1H, H-8), 4.36 (t, 4H, H-2″, H-2‴), 4.32 (t, 4H, H-3″, H-3‴), 3.76 (m, 6H, H-5″, H-5‴); MS: *m/z* 445.1 (M^+•). Anal.: ($C_{22}H_{21}ClFN_3O_4$) C, H, N.

4.22. 6,7-Dimethoxy-3,4-dihydroquinazoline-4-on (**29**)

2-Amino-4,5-dimethoxybenzoic acid (6.30 g, 38.14 mmol) and formamidine acetate (8.50 g, 81.65 mmol) in 100 mL 2-methoxyethanol were heated at reflux for 8 h. After evaporation of 2-methoxyethanol the resulting residue was stirred with ammonia (10%). The residue was isolated, washed with water and dried to give 5.90 g (89.5%) of a redbrown solid. mp: 300 °C; ^{1}H NMR (DMSO-d_6): δ 12.64 (s, 1H, NH), 7.99 (s, 1H, H-2), 7.44 (s, 1H, H-5), 7.13 (s, 1H, H-8) 3.89 (s, 6H, OCH_3); MS: *m/z* 206.4 (M^+•). Anal.: ($C_{10}H_{10}N_2O_3$) C, H, N.

4.23. 6-Hydroxy-7-methoxy-3,4-dihydroquinazoline-4-on (**30**)

Compound **29** (1.50 g, 7.27 mmol) was added portionwise to stirred methanesulphonic acid (10 mL). L-Methionine (1.25 g) was added and the resultant mixture was stirred and heated under reflux for 6 h. The mixture was cooled to ambient temperature and poured onto a mixture of ice and water.

The mixture was neutralized by the addition of an aqueous sodium hydroxide solution (40%). The precipitate was isolated, washed with water and dried. There were obtained 0.40 g (28.6%) of a white solid. mp: 293 °C; ^{1}H NMR (DMSO-d_6): δ 11.92 (s, 1H, NH), 9.80 (s, 1H, OH), 7.90 (s, 1H, H-2), 7.38 (s, 1H, H-5), 7.09 (s, 1H, H-8) 3.90 (s, 3H, OCH_3); MS: *m/z* 192.2 (M^+•). Anal.: ($C_9H_8N_2O_3$) C, H, N.

*4.24. 6-Acetoxy-7-methoxy-3,4-dihydroquinazoline-4-on (**31**)*

A mixture of **30** (6.75 g, 35.13 mmol), acetic anhydride (55 mL) and pyridine (6.7 mL) was stirred and heated to 100 °C for 2 h. The mixture was poured onto a mixture of ice and water. The precipitate was isolated, washed with water and dried. The crude product was chromatographed over silica gel with dichloromethane/methanol (9:1). There were obtained 3.40 g (41.3%) of a white solid. mp: 293 °C; ^{1}H NMR (DMSO-d_6): δ 12.19 (s, 1H, NH), 8.08 (s, 1H, H-2), 7.75 (s, 1H, H-5), 7.28 (s, 1H, H-8) 3.91 (s, 3H, OCH_3), 2.30 (s, 3H, COOR); MS: *m/z* 234.1 (M^+•). Anal.: ($C_{11}H_{10}N_2O_4$) C, H, N.

*4.25. 4-Chloro-6-acetoxy-7-methoxyquinazoline (**32**)*

A stirred mixture of **31** (2.00 g, 8.54 mmol), thionyl chloride (30 mL) and *N*,*N*-dimethylformamide (0.6 mL) was heated at reflux for 4 h. The liquid layer was evaporated. The residue was washed with diethyl ether and dried to give 1.70 g (78.8%) of a beige solid. ^{1}H NMR (DMSO-d_6): δ 9.02 (s, 1H, H-2), 8.03 (s, 1H, H-5), 7.65 (s, 1H, H-8), 4.03 (s, 3H, OCH_3), 2.36 (s, 3H, CH_3COOR); MS: *m/z* 251.1 (M^+•).

*4.26. 4-(5-Chloro-6-fluor-indol-3-yl)-6-acetoxy-7-methoxyquinazoline (**33**)*

Under cooling magnesium (0.25 g) and iodine were stirred with a mixture from methyliodide (1.2 mL) and diethyl ether (5 mL) for 15 min. Afterwards a solution of **16** (0.50 g, 2.99 mmol) in diethyl ether (15 mL) was given to the reaction mixture and was stirred for 15 min. Subsequently **32** (0.70 g, 2.77 mmol) was added in portions. The mixture was heated under reflux for 1 h and then was added to ice water. After extraction with ethyl acetate the organic layer was dried and the ethyl acetate was evaporated. The crude product was chromatographed over silica gel with ethyl acetate/ethanol (9:1) and was recrystallisated from ethyl acetate/*n*-hexane. There were obtained 0.23 g (21.7%) of yellow crystals. mp: 190 °C; ^{1}H NMR (DMSO-d_6): δ 12.28 (s, 1H, NH), 9.24 (s, 1H, H-2), 8.44 (d, 1H, $J_{H-F} = 7.62$ Hz, H-4′), 8.34 (d, 1H, $J = 2.88$ Hz, H-2′), 8.23 (s, 1H, H-5), 7.56 (d, 1H, $J_{H-F} = 10.00$ Hz, H-7′), 7.55 (s, 1H, H-8), 4.02 (s, 3H, OCH_3), 2.34 (s, 3H, CH_3COOR); MS: *m/z* 343.2 (M^+•). Anal.: ($C_{19}H_{13}ClFN_3O_3$) C, H, N.

*4.27. tert-Butyl-3-(6-acetoxy-7-methoxyquinazoline-4-yl)-5-chloro-6-fluoroindole-1-carboxy-late (**34**)*

A mixture of **33** (0.21 g, 0.83 mmol), di-*tert*-butylpyrocarbonate and 4-dimethylamino-pyridine in acetonitrile (70 mL) was stirred at ambient temperature for 24 h. The solvent was evaporated. were obtained 0.20 g (49.4%) of yellow crystals. ^{1}H NMR (DMSO-d_6): δ 9.30 (s, 1H, H-2), 8.32 (s, 1H, H-2′), 8.21 (d, 1H, $J_{H-F} = 7.52$ Hz, H-4′), 8.12 (s, 1H, H-5), 8.05 (d, 1H, $J_{H-F} = 10.27$ Hz, H-7′), 7.62 (s, 1H, H-8), 4.03 (s, 3H, OCH_3), 2.33 (s, 3H, CH_3COOR), 1.68 (s, 9H); MS: *m/z* 484.8 (M^+•).

*4.28. tert-Butyl-5-chloro-6-fluoro-3-[7-methoxy-6-(3-morpholin-4-ylpropoxy)-quinazoline-4-yl]-indole-1-carboxylate (**35**)*

A mixture of **34** (0.20 g, 0.41 mmol), potassium carbonate anhydrous, 18-crown-6 and 4-(3-chloropropyl)morpholin (5 mL) in acetonitrile (50 mL) was heated at 60 °C for 4 h. After filtration the solvent was evaporated and the crude product was chromatographed over silica gel with ethyl acetate/ethanol (9:1). There were obtained 0.22 g (95.1%) of light yellow crystals. mp: 156 °C; ^{1}H NMR (DMSO-d_6): δ 9.17 (s, 1H, H-2), 8.46 (s, 1H, H-2′), 8.26 (d, 1H, $J_{H-F} = 7.52$ Hz, H-4′), 8.10 (d, 1H, $J_{H-F} = 10.26$ Hz, H-7′), 7.61 (s, 1H, H-5), 7.45 (s, 1H, H-8), 4.16 (t, 2H, H-1′), 4.03 (s, 3H, OCH_3), 3.54 (t, 4H, H-6), 2.42 (t, 2H, H-3′), 2.33 (t, 4H, H-3, H-5), 1.96 (m, 2H, H-2′), 1.67 (s, 9H, $C(CH_3)_3$); MS: *m/z* 570.9 (M^+•). Anal.: ($C_{29}H_{32}ClFN_4O_5$) C, H, N.

*4.29. 4-(5-Chloro-6-fluoroindole-3-yl)-7-methoxy-6-(3-morpholin-4-ylpropoxy)quinazoline (**36**)*

Compound **35** (0.22 g, 0.39 mmol) in formic acid (30 mL) was stirred at ambient temperature for 24 h. The reaction mixture was poured onto a mixture of Triton B, ice and water with a pH > 8. After extraction with diethyl ether and ethyl acetate the organic layer was dried over molecular sieve for 2 d. After evaporation the crude product was chromatographed over silica gel with diethyl ether/ethyl acetate/methanol (3:1:1) and was recrystallisated from ethyl acetate/*n*-hexane to give 0.04 g (20.5%) of yellow needles. mp: 186 °C; ^{1}H NMR (DMSO-d_6): δ 12.10 (s, 1H, NH), 9.09 (s, 1H, H-2), 8.37 (d, $J = 2.74$ Hz, 1H, H-2′), 8.33 (d, 1H, $J_{H-F} = 7.59$ Hz, H-4′), 7.65 (s, 1H, H-5), 7.54 (d, 1H, $J_{H-F} = 10.00$ Hz, H-7′), 7.39 (s, 1H, H-8), 4.19 (t, 2H, H-1′), 4.01 (s, 3H, OCH_3), 3.54 (t, 4H, H2, H-6), 2.43 (t, 2H, H-3′), 2.34 (t, 4H, H-3, H-5), 1.96 (m, 2H, H-2′); MS: *m/z* 470.7 (M^+•). Anal.: ($C_{24}H_{24}ClFN_4O_3$) C, H, N.

4.30. Analyses indicated by the symbols of the elements or functions were within ±0.4% of the theoretical values

4.30.1. In vitro EGFR-tyrosine kinase activity

The EGFR-tyrosine kinase inhibitory activity assay was carried out by MDS Pharma Services, Taiwan (R.O.C.).

Enzyme source	Human A31 cells
Substrate	10 μg/mL polyglutamic acid/tyrosine [poly(glu/tyr, 4:1)]
Vehicle	1% dimethylsulfoxide
Pre-incubation time/temp.	None
Incubation time/temp.	60 min/25 °C
Incubation buffer	50 mM Hepes, 20 mM $MgCl_2$, 0.2 mM Na_3VO_4, pH 7.4
Quantitation method	ELISA quantitation of poly(glu:p-tyr)
Significance criteria	≥50% of maximal stimulation or inhibition

The epidermal growth factor was purchased from Sigma (prod. number: E2645). The enzyme assay was performed in wells coated with poly(glu/tyr) substrate. Incubation buffer and test compound were added into the wells. Finally, enzyme was added and then ATP to start the reaction. For the calculation of the inhibitory activities, each assay contained two wells for determination of the maximum phosphorylation rate (p-tyr_{max}: in the absence of inhibitor) and two wells for determination of the minimum phosphorylation rate (p-tyr_{min}: in the absence of enzyme), as well. Quantitation of phosphortyrosine (p-tyr) was determinated via ELISA [13,14,20–23].

4.31. Evaluation for cytotoxic activity against a panel of 60 human cancer cell lines

Evaluation of anticancer activity was performed at the National Cancer Institute (Bethesda, MD, USA). The compounds were tested in an in vitro 60-cell line anticancer assay over a 5 log dose range. The cell lines are derived from nine human cancer cell types: leukaemia, NSCLC, colon, CNS, melanoma, ovarian, renal, prostate and breast. The tumor cell lines are grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mL L-glutamine. For a typical screening experiment, cells are inoculated into 96 well microtiter plates in 100 μM at plating densities ranging from 5000 to 40 000 cells/well depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h prior to addition of experimental drugs.

After 24 h, two plates of each cell line are fixed in situ with TCA to represent a measurement of the cell population for each cell line at the time of drug addition (T_z). Experimental drugs are solubilized in DMSO at 400-fold the desired final maximum test concentration. At the time of drug addition, an aliquot of concentrate is thawed and diluted to twice the desired final maximum test concentration with complete medium containing 50 μg/mL gentamicin. Additionally four, 10-fold or 1/2 log serial dilutions are made to provide a total of five drug concentrations plus control. Aliquots of 100 μL of these different drug dilutions are added to appropriate microtiter wells already containing 100 μL of medium, resulting in the required final drug concentration. Following drug addition, the plates are incubated for an additional 48 h at the above-mentioned conditions. For adherent cells, the assay is terminated by the addition of cold TCA. Cells are fixed in situ by the gentle addition of 50 μL of cold 50% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The supernatant is discarded, and the plates are washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (100 μL) at 0.4% (w/v) in 1% acetic acid is added to each well, and the plates are incubated for 10 min at room temperature. After staining, unbound dye is removed by washing five times with 1% acetic acid and the plates are air dried. Bound stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automatic plate reader at a wavelength of 515 nm.

For suspension cells, the methodology is the same except that the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 μL of 80% TCA (final concentration 16% TCA). Using the seven absorbance measurements [time zero (T_z), control growth (C), and test growth in the presence of drug at five concentration levels (T_i)], the percentage growth is calculated at each of the drug concentration levels.

Percentage growth is calculated as $[(T_i - T_z)/C - T_z)] \times 100$ for concentrations for which $T_i > T_z$ and $[(T_i - T_z)/T_z)] \times 100$ for concentrations for which $T_i < T_z$. Three dose response parameters are calculated for each experimental agent. Growth inhibition (TGI) is calculated from $T_i = T_z$. The LC_{50} concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment is calculated from $[(T_i - T_z)/T_z)] \times 100 = -50$.

Values are calculated for each of these three parameters if the level of activity is reached; however, if the effect is not reached or exceeded, the value for that parameter is expressed as greater or less than the maximum or minimum concentration tested [19,24].

Acknowledgment

The authors thank the National Cancer Institute in Bethesda (USA) for testing the anticancer activities at 60 human cancer cell lines.

References

[1] J. Schlessinger, Cell 103 (2000) 211–225.
[2] N. Prenzel, Endocr. Relat. Cancer 8 (2001) 11–31.
[3] M.W. Pedersen, H.S. Poulsen, Sci. Med. (2002) 206–217.
[4] R. Tibes, J. Trent, R. Kurzrock, Annu. Rev. Pharmacol. Toxicol. 45 (2005) 357–385.
[5] R. Perez-Soler, J. Clin. Oncol. 22 (2004) 3238–3247.
[6] M.R. Myers, A.P. Spada, M.P. Maguire, P.E. Persons, WO 96/39145, US-Patent, 1996.
[7] A.J. Bridges, Chem. Rev. 101 (2001) 2541–2571.
[8] A.J. Bridges, J. Med. Chem. 39 (1996) 267–276.
[9] A.J. Barker, EP 0566226, GB-Patent, 1993.
[10] A.W. Faull, J. Kettle, US 6569 888, GB-Patent, 2003.
[11] M. Boes, US 5494 928, CH-Patent, 1996.
[12] K. Kondo, Chem. Pharm. Bull. 47 (1999) 1227–1231.
[13] K.R. Cheng, J.G. Koland, J. Biol. Chem. 271 (1996) 311–318.
[14] K. Farley, Anal. Biochem. 203 (1992) 151–157.
[15] C.I. Bargmann, M.C. Hung, R.A. Weinberg, Nature 319 (1986) 226–230.
[16] E. Liu, Oncogene 7 (1992) 1027–1032.

[17] <http://dtp.nci.nih.gov>.
[18] M.R. Boyd, K.D. Pall, Drug Dev. Res. 34 (1995) 91–109.
[19] M.C. Alley, D.A. Scuderio, A. Monks, M.L. Hursey, M.J. Czerwinski, D.C. Fine, B.J. Abott, J.G. Mayo, R.H. Shoemaker, M.R. Boyd, Cancer Res. 48 (1988) 589–601.
[20] J.F. Geissler, J. Biol. Chem. 265 (1990) 22255–22261.
[21] P.B. Wedegaertner, G.N. Gill, J. Biol. Chem. 264 (1989) 11346–11353.
[22] P. Yaish, Science 242 (1988) 933–935.
[23] A. Lin, MDS Pharma Services, Taiwan, R.O.C., correspondence, 2005.
[24] M.R. Grever, S.A. Schepartz, B.A. Chabner, Semin. Oncol. 19 (1992) 622–638.