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Tyrphostins IV—Highly Potent Inhibitors of EGF Receptor Kinase. Structure–Activity Relationship Study of 4-Anilidoquinazolines

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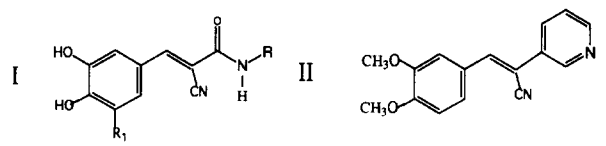
Abstract—Potent 4-anilido-substituted quinazolines which potently inhibit epidermal growth factor receptor (EGFR) kinase were prepared. Structure–activity relationship studies reveal high sensitivity to substitution at the aniline ring. Copyright © 1996 Elsevier Science Ltd

Introduction

The enhanced activities of protein tyrosine kinases (PTKs) are a hallmark of many diseases including cancer. These enhanced activities result either from overexpression of the normal kinase or are due to activating mutations.[1] The involvement of PTKs in disease states identifies them as targets for antiproliferative drugs. Indeed, numerous PTK blockers have already been described[2] and their mechanism of action studied.[3] In this communication we describe a group of PTK blockers that is highly selective for the epidermal growth factor receptor (EGFR) kinase.



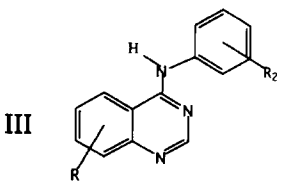
In recent years we developed a family of PTK inhibitors, tyrphostins, designed to mimic the tyrosine substrate.[2,4,5] The pharmacophores of most tyrphostins are hydrophilic catechol rings and more lipophilic-substituted cyano-vinyl radicals. Kinetic studies have shown that some tyrphostin compounds are pure competitive inhibitors vis-a-vis the tyrosine substrate and noncompetitive vis-a-vis ATP site, while many tyrphostins show competitive inhibition against both the substrate and ATP.[3]

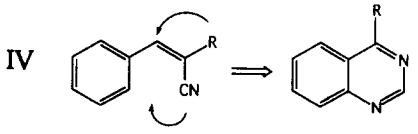
In a related group of tyrphostins, the hydrophilic catechol ring was replaced by lipophilic dichloro or dimethoxy phenyl rings to yield EGFR kinase inhibitors, effective in the low micromolar range.[6] Detailed kinetic analysis on class II compounds has not been performed.

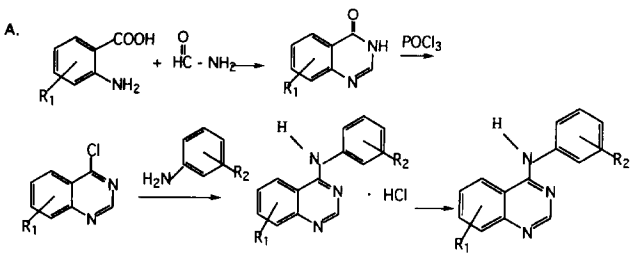
Recently, quinazoline derivatives III were reported as potent EGFR kinase inhibitors.[7–12] Quinazolines can be viewed as rigid bicyclic analogues of tyrphostins, in which the cyano vinyl is incorporated into the heterocyclic ring and the α-substituent in I moved to the β position (IV). The chemical properties of quinazolines are not expected to resemble those of tyrphostins. For example, quinazolines are not expected to be Michael acceptors.

In this study we report on the activities of an extensive series of quinazolines, and compare them with the tyrphostin EGFR inhibitors.

Quinazolines were prepared by the Neimentowski synthesis as shown in Scheme 1, followed by chlorination and substitution with various anilines. The substituents at the 2 position were prepared from 2,4-dichloroquinazoline which reacted with equimolar aniline to give mainly 2-chloro-4-anilidoquinazoline. Reaction with nucleophiles then gave the 2-substituted analogues. Only a few derivatives with substituents on the aniline ring have been described.[7–12] We, therefore, prepared a large series of quinazolines III, substituted







Scheme 1.

at both the quinazoline (R_1) and aniline ring (R_2). The biochemical and biological data for these compounds is described in Table 1.

Structure–Activity Relationship

For several quinazolines both the hydrochloride salt and the free base were prepared, and were found to have the same potency (Table 1). Substitution of the quinazoline ring indicated that the inhibition is enhanced by lipophilic groups at positions 6 and 7. Thus, with 3-chloroanilide at the 4-quinazoline position the potency order for autophosphorylation inhibition is 6-CH_3 > 6,7-di-OCH_3 > 6-Cl > H (600, 900, 2700, 4160 nM for compounds **13a**, **6b**, **12a** and **2a**). A Compound identical to **2a** was reported[7] to inhibit peptide substrate phosphorylation with IC_{50} = 40 nM. In the cellular assay for autophosphorylation ('cellular assay') inhibition, the order of inhibition was 6,7-di-OCH_3 > 6-CH_3 > H (2, 37 and 205 nM for **6b**, **13a** and **2a**). Substitution at the 8 position of the quinazoline ring, 8-CH_3 in **1a** and 6,7,8-tri-OCH_3 in **11b** abolish inhibition.

A similar pattern was observed for the 3-Br anilide derivatives **7b** and **14a**. In this case the 6,7-dimethoxy was superior to the 6-methyl in both assays (16.0 and 1.6 nM for **7b** and 1700 and 330 nM for **14a** for autophosphorylation in the cell free assay and the cellular assay, respectively).

A series of 4-anilide derivatives with substituents of different size and polarity was prepared. Comparison of the 6-methyl quinazolines shows that substitution at the 2 or 4 position of the anilide ring greatly reduces or completely abolishes EGFR autophosphorylation in the cell free assay and the cellular assay. However, compounds **27a** (4-OCH_3), **16a** (4-Br) and **22b** (3,4-methylene dioxy) exhibit moderate cellular inhibition (10,000, 14,000 and 21,000 nM respectively).

Thus, SAR points to the 6,7 positions in the quinazoline ring and the 3 position in the anilido ring as the most important positions. Polar substitution of the 6-CH_3 quinazoline derivatives at the 3-anilido position (3-amide, and 3-COOH, compounds **19a** and **20a**) show good inhibition of autophosphorylation activity (250 and 1000 nM, respectively), but no cellular activity, perhaps due to low cellular penetration. Good inhibition is shown also at both assays with 3-CF_3 (6-methyl), **15b** and 3-OCH_3 (6,7-dimethoxy), **9a**.

The most potent inhibitors were the 3-halogen derivatives. The dimethoxy substitution compounds **6b**, **7b** and **8a** with Cl, Br and I inhibit EGFR autophosphorylation with IC_{50} values 900, 16.0 and 950 nM and are extremely potent in the cellular assays (IC_{50} = 2.0, 1.6 and 1.7 nM). The 3-F analogue **5a** was less potent (IC_{50} = 90 and 60 nM).

IC_{50} values found in this study differ markedly from those reported by Rewcastle et al.[11] For example, these authors report an IC_{50} = 0.029 nM for compound **7b**, for which we find an IC_{50} = 16 nM for the inhibition of EGFR autophosphorylation of the isolated receptor. Rewcastle determined their IC_{50} values as the potency of the compound to block the phosphorylation of 200 μM of an exogenous substrate derived from phospholipase C in a 10 min assay using 10 μM ATP.[11] The assay described in this paper refers to the inhibition of EGFR autophosphorylation for 15 minutes using 50 μM ATP. Since quinazolines are ATP competitive[8] and we have used a longer incubation time, this discrepancy is at least partially accounted for by different assay conditions. Another factor could be the state of the EGFR. In this article, we use EGFR in its native membrane environment, whereas the biochemical preparation described by Rewcastle is different. Furthermore, when the kinetics of EGFR inhibition by quinazolines was studied for solubilized EGFR, a K_i = 16 nM was reported for compound **2a** when the compound was tested as a blocker of EGFR-catalysed phosphorylation of an exogenous substrate.[8] Rewcastle reported an IC_{50} value of 23 nM for compound **6b** using an exogenous substrate,[10] while we found on IC_{50} value of 900 nM for the inhibition of EGFR autophosphorylation at 25 °C using an incubation time of 30 min. Clearly, IC_{50} values, unlike true dissociation constants, depend on the conditions. IC_{50} values are expected to be higher the longer the incubation time and the higher the temperature. The values of K_i reported by us as 3 and 10 nM[2,13] are much lower and are based on the inhibition of EGFR autophosphorylation at 0 °C for very short incubation times as described in ref 13. Still all the results fall within the range similar to that reported by the Zeneca group.[8] Interestingly, the IC_{50} values for EGFR autophosphorylation are markedly different for **6b** and **7b** in vitro but similar and much lower in the cellular assay where the potency of the compounds is measured as EGFR autophosphorylation inhibitors in the intact cell. Discrepancies need to be studied further since they most probably reflect a complex behavior of the receptor and different permeation properties of the quinazolines.

The 6-methyl 3′-halogen derivatives, while still among the best inhibitors, gave reduced values and preference to the 3-chloro analogue **13a** over the 3-bromo derivative **14a** (600 and 1700 nM in the in vitro autophosphorylation assay and 37 and 330 nM in the cellular assay).

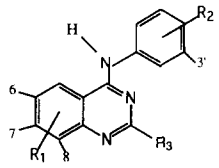
Substitution at the 2 position of the quinazoline ring with large aliphatic or aromatic electron donating

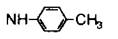
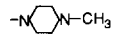
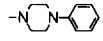
substituents (**32–35**) abolished inhibition. Even exchange of hydrogen to chlorine reduced efficacy considerably (2800 nM for **36b** and 900 nM for **6b**).

Cellular Activity and Selectivity of Quinazolines

Many of the quinazolines that have moderate to good EGFR autophosphorylation inhibitory activity in cell lysates were ineffective in inhibiting the receptor in intact cells as measured in the cellular assays (Table 1). This is probably due to inefficient penetration of the cell membrane, especially for compounds with polar groups. Some quinazolines are better inhibitors of the EGFR kinase in intact cells than on the isolated receptor in vitro. This group includes mainly the quinazolines with a 3′-halogen substituent at the anilido ring, among them the most potent inhibitors, compounds **6a**, **7a** and **8a**. These are the most potent EGFR blockers and as independently discovered by others also reveal

Table 1. Data for



No	R_1	R_2	R_3	Inhibition of EGFR autophosphorylation IC_{50} (μM)	
				A431 membranes	Cellular assay
1a[a]	8-CH_3	3-Cl	H	>100	>50
2a[b]	H	3-Cl	H	4.16	0.205
3b	H	2-Cl	H	15.3	>50
4b[b]	6,7-$(OCH_3)_2$	H	H	5.1	>50
5a[b]	6,7-$(OCH_3)_2$	3-F	H	0.090	0.060
6b[b]	6,7-$(OCH_3)_2$	3-Cl	H	0.9	0.002
7b[b]	6,7-$(OCH_3)_2$	3-Br	H	0.016	0.0016
8a[b]	6,7-$(OCH_3)_2$	3-I	H	0.95	0.0017
9a	6,7-$(OCH_3)_2$	3-OCH_3	H	6.3	1.0
10a	6,7-$(OCH_3)_2$	3,5-di-Cl	H	4.0	0.024
11b	6,7,8-$(OCH_3)_3$	3-Cl	H	>100	>50
12a	6-Cl	3-Cl	H	2.7	—
13a	6-CH_3	3-Cl	H	0.6	0.037
14a	6-CH_3	3-Br	H	1.7	0.330
15b	6-CH_3	3-CF_3	H	2.1	0.860
16a	6-CH_3	4-Br	H	—	14.0
17a	6-CH_3	4-I	H	>50	>50
18a	6-CH_3	4-CN	H	22	>50
19a	6-CH_3	3-$CONH_2$	H	0.25	>50
20a	6-CH_3	3-COOH	H	1.0	>50
21a	6-CH_3	3,4-di-CH_3	H	6.9	>45
22b	6-CH_3	3,4-OCH_2O	H	>100	21
23a	6-CH_3	2-CH_3	H	21	>50
24a	6-CH_3	4-OH	H	15	>50
25b	6-CH_3	2-Cl	H	>100	>50
26a	6-CH_3	4-COOH	H	>50	>50
27a	6-CH_3	4-OCH_3	H	9.0	10.0
28b	6-CH_3	4-CH_3	H	10.0	>50
29b	6-CH_3	H	H	11.3	>50
30b	6-CH_3	2-OH	H	13.0	>50
31b	6,7$(OCH_3)_2$	4-CH_3	Cl	16.0	4.9
32b	6,7$(OCH_3)_2$	4-CH_3		>50	3.4
33b	6,7$(OCH_3)_2$	3-Cl		>50	3.4
34b	6,7$(OCH_3)_2$	3-Cl		>50	>50
35b	6,7$(OCH_3)_2$	3-Cl		>50	>50
36b	6,7$(OCH_3)_2$	3-Cl	Cl	2.8	20

[a] Compounds designated as **a** are the hydrochloride salt, while **b** are the free base.
[b] These compounds were recently described: **2a**,[8] **4b**,[7,10] **5a**,[10] **6b**,[10–13] **7b**[9–11] **8a**.[10]

some discrepancies between their potency in different assays. It seems that the mode of action of these compounds is more complex than originally predicted. Further studies are needed to examine the origin of the differences between the various assays.

We also examined the activity of several quinazolines in cell lines expressing another tyrosine kinase, PDGF. All quinazolines exhibit a very high selectivity towards the EGF receptor compared with the PDGF receptor (not shown). For the most potent compounds **6a**, **7a** and **8a** up to 1000 fold selectivity was observed.

Conclusions

In this article we described an extensive series of quinazolines, first described by the Zeneca group.[7,8] Several quinazolines are the most potent and selective EGFR tyrosine kinase inhibitors reported to date. Only the staurosporine family inhibitors are as potent in cells, however, they are also PKC inhibitors, whereas quinazoline **2** was reported[7] as inactive against PKC kinase.

Kinetic studies have shown compounds **2** and **7** are competitive against ATP and noncompetitive towards the peptide substrate.[8,9] The potency of compound **2** was rationalized[8] by assuming a close match among compound **2** and bisubstrate inhibitors. Thus, the quinazoline nitrogens correspond to the oxygens of the γ-phosphate of ATP, the aromatic anilido ring to tyrosine ring and the anilido nitrogen to the tyrosine hydroxyl oxygen.

This model, however, does not account for the kinetic noncompetitive behavior towards the tyrosine-containing peptide substrate and the importance of the 3-halogen substituents.

In a more suitable model, one may assume that the lipophilic quinazoline moeity binds at the ATP site or close to it, perhaps the same site used by lipophilic tyrphostins (II), whereas the hydrophilic benzylidene malononitriles (I) bind at a site closer to that occupied by tyrosine containing substrate.[3] This assertion is supported by recent kinetic studies performed on representatives of this class of inhibitors.

Further fine tuning of this model may be achieved by combining structural elements of the tyrphostin and the quinazoline pharmacophores to yield true bisubstrate inhibitors, from which useful novel drugs against cancer may emerge.

Experimental

Synthetic methods

Compounds **1–29** were prepared according to Scheme 1. Illustrative synthetic procedures are given for compound **6**.

6,7-Dimethoxyquinazoline-4-one. 7 g of 4,5-dimethoxyanthranilic acid and 8 mL of formamide were heated for 2 h at 170 °C. Cold water was added and the mixture left at room temperature for 1 h. The precipitate was filtered, washed with water and dried to give a gray–white solid, 1.5 g, mp 308 °C, 20% yield. NMR (DMSO-d_6): δ 8.0 (1H, s), 7.43 (1H, s), 7.12 (1H, s), 3.89 (3H, s), 3.85 (3H, s).

4-Chloro-6,7-dimethoxyquinazoline. 0.8 g of 6,7-dimethoxyquinazoline-4-one, 1 mL of $POCl_3$ and 1 mL of dimethylaniline in 20 mL of toluene were refluxed for 4 h. Water was added to the cooled solution and NH_3 solution until slightly basic. Extraction with ethyl acetate, evaporation and recrystalization from benzene–cyclohexane gave 0.5 g of a white solid, mp 188 °C, 57% yield. NMR ($CDCl_3$): δ 8.88 (1H, s), 7.41 (1H, s), 7.36 (1H, s), 4.09 (3H, s), 4.08 (3H, s).

6,7-Dimethoxy-4-(3′-chloroanilino)quinazoline. 0.4 g, 1.8 mmol, of 4-chloro-6,7-dimethoxyquinazoline and 0.24 g, 2.0 mmol, of *m*-chloroaniline in 10 mL of ethanol were refluxed for 0.5 h, cooled, filtered and dried to give 0.52 g of a white solid, mp 270 °C, 80% yield, as the hydrochloride salt, **6a**. NMR (DMSO-d_6): δ 8.90 (1H, s), 8.28 (1H, s), 7.92 (1H, s), 7.70–7.34 (4H, m), 4.02 (3H, s), 4.01 (3H, s).

Free base 6b. 1 g of **6a** was treated with 40 mL of satd Na_2CO_3 soln and extracted with 3×50 mL of CH_2Cl_2. Evaporation of the organic phase and chromatography (silica gel, 2% CH_3OH in CH_2Cl_2) gave 0.72 g of a white solid, 80% yield, mp 177 °C. NMR ($CDCl_3$): δ 8.68 (1H, s), 7.83 (1H, s), 7.5–7.1 (5H, m), 4.0 (6H, s). MS: *m/z* 317, 315 (M^+, 20, 55), 316, 314 (M − 1, 36, 100), 280 (M − Cl, 10), 140 (21).

Biochemical methods

The inhibition of EGFR autophosphorylation in A431 membranes was performed essentially as previously described.[14] Briefly: EGFR autophosphorylation was performed in the presence of 50 μM ATP, 5 mM $MnCl_2$ in PBS for 15 min, at room temperature (22 °C). Cellular assays were perfomed also as previously described for DHER14 cells.[14] Cells were preincubated with the compounds for 45 min at room temperature prior to stimulation by EGF and processing as described.[14]

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