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Specific inhibition of epidermal growth factor receptor tyrosine kinase by 4-anilinoquinazolines

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Summary

Since the mitogenic action of EGF is mediated by ligand-induced autophosphorylation of the EGF receptor (EGFR), and EGFR is commonly overexpressed in solid human tumours, inhibitors of receptor tyrosine kinase activity (RTK) could prove to be effective antitumour agents. Screening of a compound library using an EGF-RTK enzyme prepared from human tumour derived A431 cells identified a series of potent (IC_{50} <1μM) enzyme inhibitors. These inhibitors are quinazolines bearing a variety of substituted anilines at the 4-position. The most potent 4-anilinoquinazolines ($IC_{50} \cong$ 20nM) have small non-polar meta substituents on the aniline ring, and are competitive with ATP and non-competitive with substrate. The growth inhibitory activity of these agents was assessed in vitro using KB cells (human oral squamous tumour) grown in the absence or presence of EGF. A selected compound, 4-(3-chloroanilino)quinazoline (CAQ), inhibited EGF-stimulated growth in a concentration dependent manner and complete blockade was observed at concentrations (1-10 μM) which had no effect on basal growth. Selectivity of growth inhibition by CAQ was further exemplified in IGF1-stimulated KB cells where no effect was detected at concentrations which completely blocked EGF-stimulated growth. Similarly, CAQ blocked TGFα-stimulated growth in MCF-7 human breast cancer cells without affecting insulin-stimulated growth. These studies define a novel class of EGF-RTK inhibitors which are also potent and selective inhibitors of EGF-stimulated human tumour cell growth *in vitro*.

Introduction

Since the first report almost ten years ago that the presence of the epidermal growth factor receptor (EGFR) in some human breast tumours indicates a poor prognosis [1], it has become clear that aberrant expression of EGFR and other members of the EGF (*erb*B) family of receptors occurs in many common solid tumours of epithelial origin including those of the breast [2,3]. These receptors are members of a large family of eukaryotic protein kinases which encompass an extracellular ligand binding domain, a short transmembrane domain, and an intracellular domain which has

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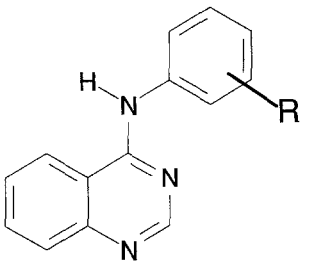
Table 1. Biological activity of anilino-quinazoline derivatives



Compound	Substituent	Inhibition of EGF-RTK IC50 (μM)	Inhibition of EGF stimulated KB cells IC50 (μM)	Inhibition of basal KB cell growth IC50 (μM)
1	H	0.55	12.5	38
2	o-Cl	1	>12.5	25
3	m-Cl	0.04	1.2	15
4	p-Cl	0.5	3.6	6
5	m-F	0.12	4.7	25
6	m-Br	0.02	0.8	12.5
7	m-I	0.07	1.1	12.5
8	m-OMe	0.7	10–100	>10
9	m-Me	0.18	2.4	16.5
10	m-NMe_2	0.52	11	12.5
11	m-OH	0.66	7.3	28
12	m-NO_2	0.07	2.7	12.5
13	m-CF_3	0.16	3.9	12.5
14	m-CN	0.2	7.6	19
15	m-SMe	0.5	7.8	12.5

tyrosine kinase (TK) activity [4]. EGF binding causes receptor dimerisation, autophosphorylation, and activation of intracellular signalling cascades which lead ultimately to cell division [4,5]. The EGFR thus represents a potential target for novel antitumour therapies directed at inhibition of its functional activity [6,7].

One such approach is directed at inhibition of the EGFR tyrosine kinase (EGF-RTK) activity to block the initiation of the mitogenic signalling cascade. A key problem in view of the ubiquity of both tyrosine and serine/threonine protein kinases in both normal and tumour cells is the selectivity of putative enzyme inhibitors. Many of the currently available inhibitors have poor selectivity and/or low potency [8]. In view of the high homology of the active kinase domains amongst different protein tyrosine kinases [9], poor selectivity of inhibitors might be predicted; however, studies by Yaish et al [10] first demonstrated that it is possible to find small molecules which effectively inhibit EGF-RTK activity without affecting insulin receptor TK activity. The studies reported here describe the properties of a novel series of inhibitors which inhibit EGF-RTK activity and selectively inhibit EGF-stimulated tumour cell growth without affecting basal or insulin-like growth factor 1 (IGF-1)/insulin stimulated growth.

Materials and methods

The preparation of EGF-RTK from A431 cells and the measurement of enzyme inhibition are fully described elsewhere [11]. All cell lines

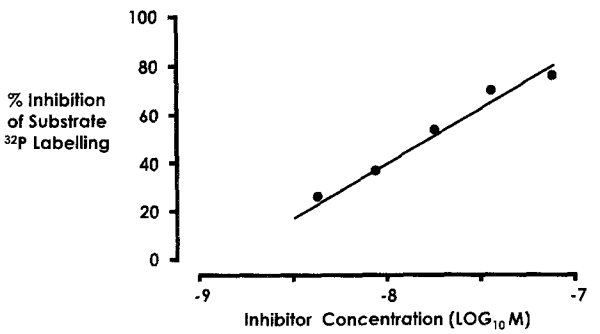


Figure 1. Inhibition of epidermal growth factor receptor tyrosine kinase activity. A solubilised preparation of EGF-RTK from A431 human tumour-derived cells was incubated with EGF in the presence of the indicated concentrations of inhibitor (CAQ = compound 3 in Table 1) prior to addition of ATP and peptide substrate. Kinase activity was measured by transfer of ^{32}P from γ^{32}P-ATP to peptide substrate as described elsewhere [11].

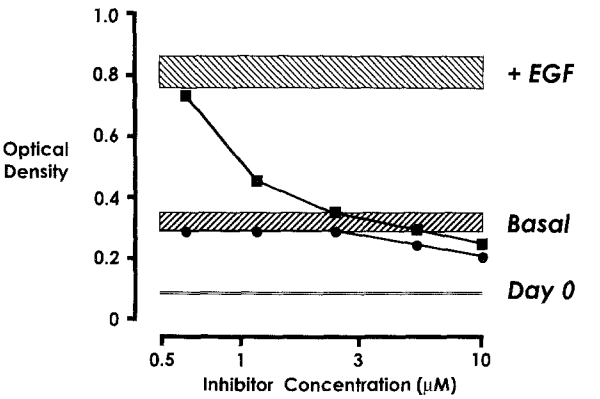


Figure 2. Effect of CAQ on basal and EGF-stimulated KB cell growth. KB cells were seeded into 96-well plates and allowed to attach in DMEM in the presence of 5% charcoal-treated fetal calf serum (CFCS). Cells were allowed to grow for 3 days in CFCS together with the indicated concentrations of CAQ in the absence (●) or presence (■) of 10 ng/ml of EGF. Cell number was estimated at the beginning and end of treatment by incubation for 1 h at 37°C with 1 mg/ml of MTT added directly to each well. The MTT product was dissolved in acid alcohol and optical density determined using an automated plate reader [12]. Values are means of quadruplicate observations and errors in this and succeeding Figures were less than 10% of the mean. Cell number proportional to optical density at the start of the experiment is shown by the lower open bar, growth in CFCS by the lower hatched bar and in the presence of EGF by the upper hatched bar.

were grown routinely in Dulbecco's MEM supplemented with 5% foetal calf serum and passaged once (MCF-7) or twice (KB and NRK49F) weekly. For experimental use, cells were aliquoted into 48 or 96-well dishes and incubated for three (KB), four (NRK), or five days (MCF-7) in 5% charcoal-treated FCS (to deplete endogenous serum growth factors) in the absence or presence of added growth factors and/or enzyme inhibitors as indicated in the Figure legends. A standard MTT assay was used as the measure of cell growth [12].

Results

The relative inhibitory potency against EGF-RTK of a series of fourteen substituted 4-anilinoquinazolines is compared with that of the parent unsubstituted compound (compound 1) in Table 1. The IC_{50} values, measured using a fixed 20μM concentration of ATP, were estimated from inhibition curves like that illustrated in Figure 1 for compound 3 (IC_{50} = 0.04μM). The nature and position of the substituent on the aniline ring substantially affects potency; in the series illustrated the most potent inhibitors were the meta-substituted halogens, for example compounds 3 and 5-7.

The human oral squamous tumour derived KB cell line provides a rapid and reproducible means to distinguish between specific and non-specific growth inhibitory effects of EGF-RTK inhibitors by comparing cell growth under EGF-stimulated and basal (serum only) conditions. A typical experiment with compound 3, 4-(3-chloroanilino)-quinazoline (CAQ), is illustrated in Figure 2. In the range 0.5-2.5μM, CAQ inhibited the EGF-stimulated component of cell growth in a concentration dependent and complete manner without affecting basal growth. At higher concentrations (≥10μM) both basal and EGF-stimulated growth was inhibited in parallel and the number of cells was reduced below the number seeded only in cultures treated with ≥50μM CAQ. Relative growth inhibitory potencies compiled in Table 1 were estimated from the concentration of each

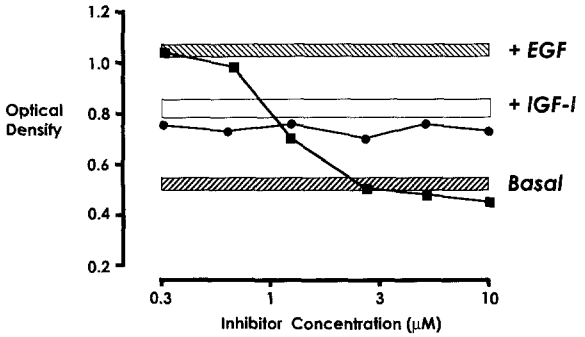


Figure 3. Effect of CAQ on IGF-1 stimulated KB cell growth. Experimental conditions and effects of CAQ on EGF-stimulated growth were as described for Figure 2. Additionally KB cells were treated with 50 ng/ml of IGF-1 in the absence (center open bar) or presence of CAQ (●).

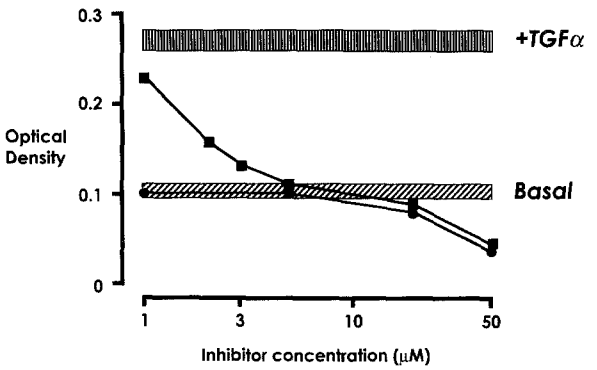


Figure 4. Effect of CAQ on TGFα-stimulated MCF-7 cells. Cells were grown in phenol red free CFCS for 5 days in the absence (lower bar) or presence of 100 ng/ml of TGFα (upper bar), and cell growth was measured using MTT as described for Figure 2. CAQ was added at the indicated concentrations in the absence (●) or presence of TGFα (■).

compound required to reduce cell growth by 50% (IC_{50} values) in the absence or presence of EGF.

The selective action of CAQ on cell growth was further evident in KB cells stimulated with IGF-1 instead of EGF. In the range 1-10μM, only a weak growth inhibition (maximum 15%), unrelated to inhibitor concentration, was recorded (Figure 3). Selectivity was further investigated by examining the action of CAQ in growth factor-stimulated MCF-7 human breast cancer cells and in NRK49F normal rat kidney derived cells.

In MCF-7 cells stimulated to grow with transforming growth factor α (TGFα), CAQ inhibited growth in a concentration dependent manner (IC_{50} ≅ 2μM), and at 4μM, where TGFα action was blocked completely, basal growth was unaffected (Figure 4). Basal growth was inhibited at 10-20μM CAQ. For insulin-stimulated MCF-7 cells, growth inhibition was first evident at 5μM CAQ (Figure 5). In NRK cells stimulated to grow with EGF or platelet derived growth factor (PDGF), 1.25μM CAQ completely blocked EGF-stimulated cell growth whilst reducing PDGF-stimulated growth by only 20% (Figure 6).

Since MCF-7 cells are growth stimulated by 17β-oestradiol as well as by TGFα, the effect of CAQ on oestradiol response was measured. CAQ was an effective growth inhibitor (Figure 7) but, in contrast to effects in TGFα treated cells, blockade of oestradiol-stimulated growth was incomplete at 5-10μM.

Discussion

The novel selective inhibitors of EGF-RTK described here were discovered by a combination of screening of the Zeneca Company Compound Collection and molecular modelling based on a proposed catalytic mechanism for the enzyme [11]. Investigation of the catalytic mechanism of CAQ indicated that it is a competitive inhibitor with respect to ATP and noncompetitive when the peptide substrate concentration is varied [11], consistent with formation of a ternary complex between the enzyme, ATP, and peptide [13]. The inhibitory potency of CAQ (IC_{50} = 0.04μM) is 10-100 fold greater than that of other recently reported EGF-RTK inhibitors [14-16]. Other chemical modifications of the quinazoline nucleus can substantially increase the *in vitro* enzyme inhibitory potency of the 4-anilinoquinazolines; for example 6,7-dimethoxy-4-(3-bromoanilino)-quinazoline (PD153035) has a reported inhibition constant of 5 pM [17].

Because the large family of receptor and non-

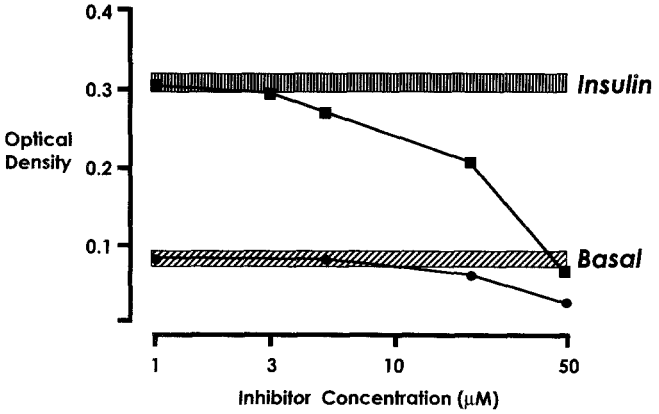


Figure 5. Effect of CAQ on insulin-stimulated MCF-7 cell growth. Cells were grown as described for Figure 4 except that insulin replaced TGFα. CAQ was added at the indicated concentrations in the absence (●) or presence of 1μg/ml insulin (■).

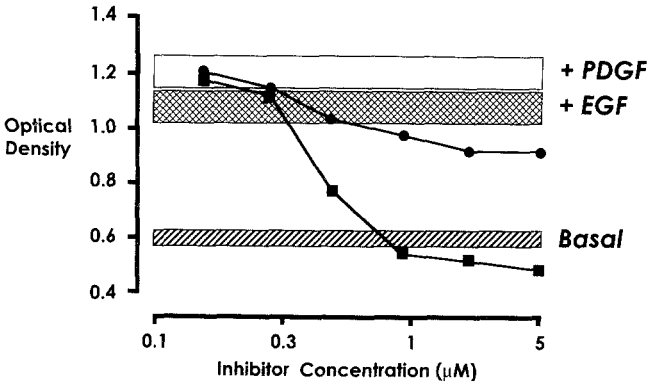


Figure 6. Effect of CAQ on EGF- and PDGF-stimulated NRK cell growth. Cells were grown for 4 days in CFCS (lower bar) or with the addition of 1 ng/ml EGF (upper hatched bar) or PDGF (upper open bar). CAQ was added at the indicated concentrations to EGF (■) or PDGF treated cells (●).

receptor tyrosine kinases share a high degree of sequence homology within their kinase domains [9], selectivity of action against the targeted enzyme is a key requirement. Selectivity has been demonstrated by comparative enzyme inhibition studies against panels of kinases [16,17]. However, since potency against an isolated enzyme may not translate into similar selectivity in whole cells [18], we chose to test both the efficacy and selectivity of our EGF-RTK inhibitors in tumour cell growth assays. The first step was the selection of the KB human tumour-derived cell line, which grows reproducibly in growth factor-depleted serum and demonstrates a further capacity to respond to the mitogenic action of EGF (Figure 2) or IGF-1 (Figure 3). These studies exemplified EGF-RTK inhibitors which selectively block EGF-stimulated growth; for example, CAQ and compounds 6 and 7 (Table 1) showed a 10-fold ratio between IC_{50} values for basal versus EGF-stimulated growth. Other close structural homologues, for example compounds 2, 4, and 8, appeared almost equally effective in basal and EGF-stimulated cells. It is also clear from Table 1 that there was no simple correlation between enzyme and cell growth inhibitory potency for the 4-anilinoquinazolines.

For CAQ, putative selectivity for EGF-induced mitogenesis was further demonstrated in IGF-1 stimulated KB cells (Figure 3), where no significant inhibition was seen until CAQ concentration exceeded 10μM. In MCF-7 cells stimulated with TGFα (Figure 4), the potency ($IC_{50} \cong 2\mu M$) and selectivity (basal growth inhibition 10-20μM) of CAQ was similar to that in EGF-stimulated KB cells. Similarly, in insulin-stimulated MCF-7 cells, 4μM CAQ, which completely blocked TGFα action, had no effect (Figure 5), and 20μM CAQ, which significantly reduced basal growth (Figure 4), produced about a 50% inhibition of the insulin effect. In NRK cells, CAQ was equally as effective against EGF-stimulated growth (IC_{50} = 1.25 μM) as in KB and MCF-7 cells, but in this case similar concentrations also partially blocked PDGF action (Figure 6).

MCF-7 cells have been used extensively as a model of hormone responsive breast cancer since their growth is stimulated both by oestrogens and growth factors. Previous studies have shown that antioestrogens partially but incompletely block the mitogenic effect of EGF [19,20] or insulin [21]. As a corollary of those studies we investigated whether CAQ influenced oestradiol-stimulated MCF-7 cell growth. The EGF-RTK inhibitor, at concentrations up to 10μM, partially but incompletely (≅80%) blocked oestradiol-induced growth (Figure 7), consistent with a mutual interaction or 'cross-talk' between the steroid and growth factor-

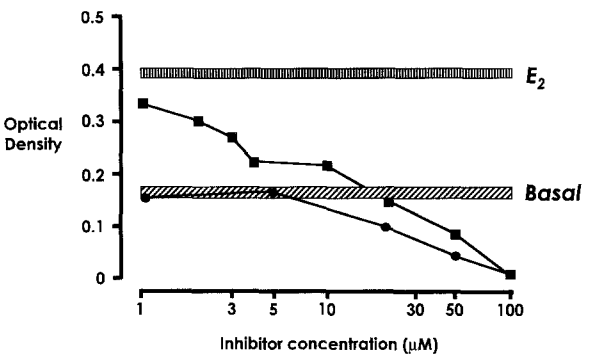


Figure 7. Effect of CAQ on oestradiol (E_2)-treated MCF-7 cells. Cells were grown as described for Figure 4 except that oestradiol replaced TGFα. CAQ was added at the indicated concentrations in the absence (●) or presence of 10 nM 17β-oestradiol (■).

dependent mitogenic pathways [22,23]. Higher concentrations of CAQ (>10μM), which affect basal and insulin-stimulated growth, completely blocked oestrogen action, consistent with a previous report that a less selective TK inhibitor, RG-13022, completely inhibited oestradiol-induced MCF-7 cell growth [24].

We conclude that the discovery of potent EGF-RTK inhibition and selective growth inhibition of EGF-stimulated tumour cells by the 4-anilinoquinazolines represents an important first stage in the search for novel antiproliferative, antitumour agents directed at signal transduction pathways. The potential for *in vivo* antitumour action of TK inhibitors has already been demonstrated with animal models [16,25], but much further work will be needed before the clinical utility of TK inhibition can be evaluated.

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