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Indazolylamino Quinazolines and Pyridopyrimidines as Inhibitors of the EGFr and C-erbB-2

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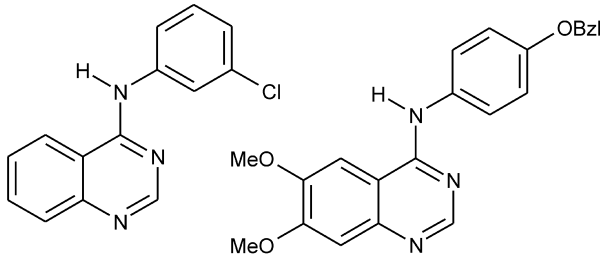
Abstract—Described herein is the design and synthesis of indazolylaminopyridopyrimidines and quinazolines as inhibitors of the class 1 tyrosine kinase receptor family. Data is presented for N^4-(1-benzyl-1*H*-indazol-5-yl)-N^6,N^6-dimethylpyrido[3,4-*d*]pyrimidine-4,6-diamine **3B**. This compound inhibited EGFr and c-erbB-2 enzymes selectively over other kinases. It inhibited the proliferation of a range of tumour cell lines in vitro and the growth of BT474 xenografts in SCID mice.

One in 12 women will develop breast cancer at some-time in their lifetime. In Europe and the US, it is one of the three most prevalent forms of cancer, with over 200,000 cases occurring in 1994.[1a] Current chemotherapy regimes involve the use of cytotoxics and antiestrogens, both with inherent selectivity disadvantages. Increased levels of c-erbB-2 and epidermal growth factor receptor (EGFr) each occur in a significant range of cancers, for example breast and non-small cell lung cancers. Their overexpression correlates with shorter time to relapse, resistance to hormone, chemotherapy and reduced survival.[1b]

c-erbB-2 and EGFr are members of the epidermal growth factor receptor (EGFr) subfamily of protein tyrosine kinases. Their overexpression in cells causes transformation, such that the cells will grow in the absence of growth factors and in anchorage-independent conditions. A kinase-inactive receptor does not have these effects. An inhibitor of this family of kinases should block signalling from the receptor, subsequent transformation and inappropriate growth. The specificity of such a compound for c-erbB-2 and EGFr dependent proliferation would reduce the likelihood of severe adverse effects and allow longer-term, effective treatment. Herceptin (trastuzumab), Genentech's humanised monoclonal antibody, is approved in patients with metastatic breast cancer overexpressing the c-erbB-2 growth factor receptor.[2] Small molecule inhibitors of EGFr, Iressa and OSI-774, have now progressed through clinical trial without serious adverse events.[2b] Inhibition of the tyrosine kinase activity of this receptor would appear to represent a tractable target for a small molecule inhibitor. The epidemiological evidence implicating both EGFr and c-erbB-2 in a diverse range of tumour types would suggest a clinical benefit in inhibiting the kinase domains of both receptors.[1]



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Optimisation of 4557W

Screening identified quinazoline 4557W as a potent inhibitor of both c-erbB-2 and EGFr [c-erbB-2 0.079 μM, EGFr 0.020 μM (isolated enzyme), 2.0 μM in HB4aC5.2 cells, 1.2 μM in BT474, both overexpressing c-erbB-2; 2.5 μM in HN5 cells overexpressing EGFr]. This combined EGFr/c-erbB-2 potency was in direct contrast to smaller, previously reported anilinoquinazolines like CAQ[3,4] and subsequent analogues which have demonstrated selectivity for EGFr over c-erbB-2.[6–8] A binding hypothesis was constructed for this compound. We utilised our knowledge of P38 binding (since reported[5]) based upon the principle of quinazoline binding through N1 to Thr798, the residue equivalent to Met109 in P38 as shown in Figure 1.

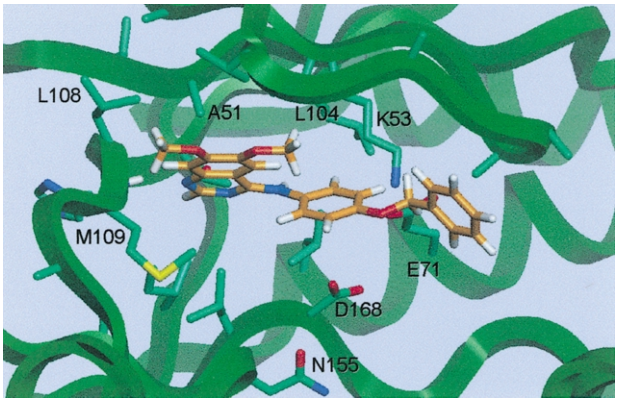


Figure 1. A model of 4557W binding to a protein kinase (P38). The complex between P38 and the quinazoline GI261634[5] was used as a starting point for modelling. The quinazoline 4-amino bond was rotated ~180° from the conformation seen in that structure in order to accommodate the increased bulk of the benzyl group in the back of the hydrophobic pocket. The complex was then energy minimised keeping the protein and the quinazoline rings fixed. The hydrogen bond between quinazoline N1 and the backbone NH of Met109 is shown as a dotted magenta line. Nearby P38 side chains are shown and labelled (P38 numbering) where they are conserved in c-erbB-2.

This binding hypothesis has the benzyloxyaniline accommodated in the back of the hydrophobic pocket with the 6,7-dimethoxy groups pointing towards the lip of the ATP binding cleft. We decided to investigate the effect of this proposed hydrophobic binding interaction by imposing a conformational restriction into the aniline fragment. A bicyclic nucleus was chosen to replace the aniline system and reduce the number of rotatable bonds in this region from 3 to 2. Subsequently a range of bicyclics **A**–**G** were combined with quinazoline and equivalent 'bottom halves' **1**, **2** and **3** (Fig. 2).

Several key structural features became apparent in this exercise. Greater intrinsic potency was observed for 6,7-dimethoxyquinazolines and pyridopyrimidines, in line with those previously reported for EGFr inhibition.[6,7] In general, 1-substitution of the bicyclic anilino system was very potent, for example in the case of indole and indazole. However in the case of benzimidazole, 2-substitution was clearly the preferred regioisomer with potency observed with all three bottom halves. Although the precise reason for this remains unclear, one can speculate upon the potential H-bonding effect of Lys53 in Figure 1 (the equivalent residue in c-erbB-2 is Lys753) compensating for a subsequent conformational alteration in the hydrophobic region of the binding pocket.

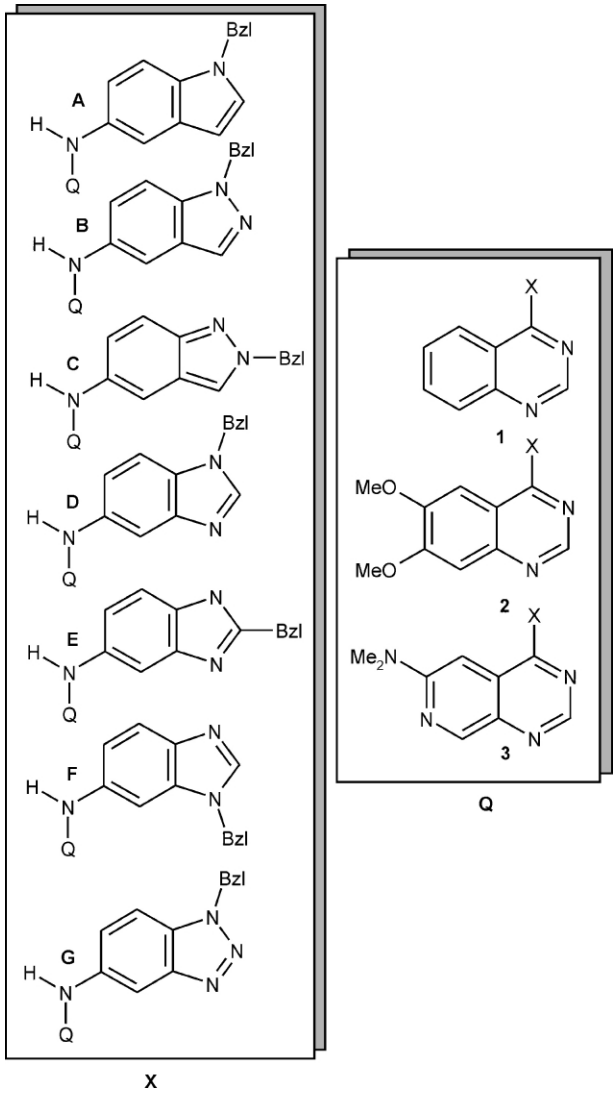


Figure 2. Top half aniline X and quinazoline equivalent Q combinations. Data for selected examples are shown in Table 1.

Of particular interest from this evaluation were the indazolyl systems exemplified by **1B**, **2B** and **3B**. The enhanced cell potency of compound **3B** (GW974, in the c-erbB-2 overexpressing HB4a.c5.2 cell line) over other analogues shown in Table 1, made it worthy of further evaluation. This compound was taken forward into pharmacokinetic and further in vitro evaluation.

GW974 Evaluation In Vitro

GW974 was evaluated against a range of isolated kinases (Table 2) and found to be selective for the EGFr subfamily. This was a common trait of this class with routine selectivity observed in the range 100–10,000-fold.[8] The compound also exhibited potency across a

range of cell types, including several (CaLu3, HN5 and BT474) derived from clinical extracts. Selectivity was also observed against a non-oncogenic MRC5 cell line. Data are shown in Table 2.

GW974 Pharmacokinetic Evaluation

In pharmacokinetic evaluation, GW974 exhibited an improved profile over its quinazoline counterparts. It exhibited improved bioavailability (24%) over its dimethoxyquinazoline analogue **2B** (7%) in initial studies in the rat. Further PK data in the mouse and dog for this compound are shown in Table 3. Comparable PK was observed in mouse and dog. Low to moderate volumes of distribution and moderate clearances were characteristic of all three species.

Table 1. Enzyme potency (c-erbB-2), cell potency and selectivity figures in the rat for selected compounds

Q	X	c-erbB-2 inhibition IC_{50} (μM)[a]	Cell potency/selectivity IC_{50} (μM)[b]
1	A	0.09	1.3/10
1	B	0.01	NT
1	D	6.6	27/39
1	E	0.56	1.3/36
1	F	4.4	21/27
2	A	0.049	0.35/12.5
2	B	0.001	0.29/>5.6
2	C	0.290	3.1/8.9
2	E	0.01	1.1/19
3	A	0.026	NT[c]
3	B	0.027	0.097/>5.6
3	E	0.049	1.86/18.5

[a]Values are means of at least 2 experiments. Assays are run at K_m concentrations of ATP and substrate.
[b]Potencies and selectivities relate to inhibition of proliferation of HB4a cells overexpressing c-erbB-2 relative to a mutant RAS HB4a control.
[c]NT, not tested.

Table 2. In vitro evaluation of GW974. Enzyme potency for several kinases and cell potencies

Kinase enzyme	IC_{50} (μM)[a]	Cell line (overexpressing[b])	IC_{50} (μM)
c-erbB-2	0.018	HB4a.c5.2 (erbB2)	0.097
EGFr	0.001	HB4a.R4.2 (RAS)	30.0
c-erbB-4	0.02	CaLu3 (erbB2)	0.39
CDK1	>8.0	HN5(egfr)	0.21
CDK2	>8.0	BT474 (erbB2)	0.07
Mek	9.7	MRC5 (non trans.)	>50.0
Src	>50.0	—	—
Raf	49.0	—	—

[a]Values are means of at least 2 experiments. Assays are run at K_m concentrations of ATP and substrate.
[b]Brackets indicate class 1 RTK overexpressed in each cell type.

Table 3. Pharmacokinetic data for compound **3B** (GW974)[a]

PK parameter	Mouse	Rat	Dog
Cl (mL/min/kg)	>27	19	34
Vdss	>0.15	0.19	0.87
iv T1/2 (min)	20	18	44
F (%)	>23	24	82

[a]Compound administered in β-cyclodextrin sulphate, DMSO water suspensions at 1 mg/kg iv, 10 mg/kg po.

GW974 Evaluation In Vivo

GW974 was evaluated alongside close analogues (**2B** and **3A**) in the fast growing N87 gastric tumour xenograft in SCID mice. At doses of 10 mg/kg po, bid a 50% inhibition of tumour growth was observed over the 20-day dosing period. Analogues **2B** and **3A** showed 20 and 0% inhibition, respectively, a result perhaps associated with their poorer oral absorption profiles. In the BT474 breast tumour xenograft model, GW974 gave complete inhibition of tumour growth 10 mg/kg po bid over the 20-day dosing period. A longer-term study with initial dosing at 30 mg/kg po bid produced tumour shrinkage. A maintenance dose of 10 mg/kg for 15 days followed. Upon cessation of dosing (at day 80), no tumour regrowth was observed (Fig. 3). The precise explanation of this effect is uncertain but may be a demonstration of the apoptosis inducing potential of c-erbB-2 inhibition, reported for anti-HER2 monoclonal antibodies.[10]

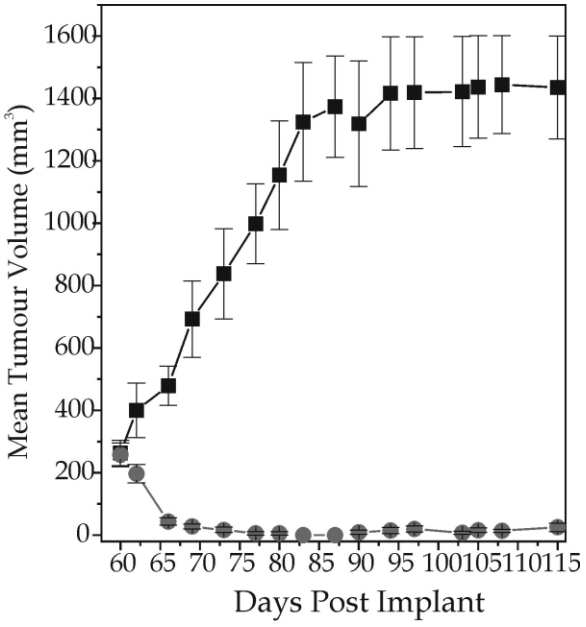


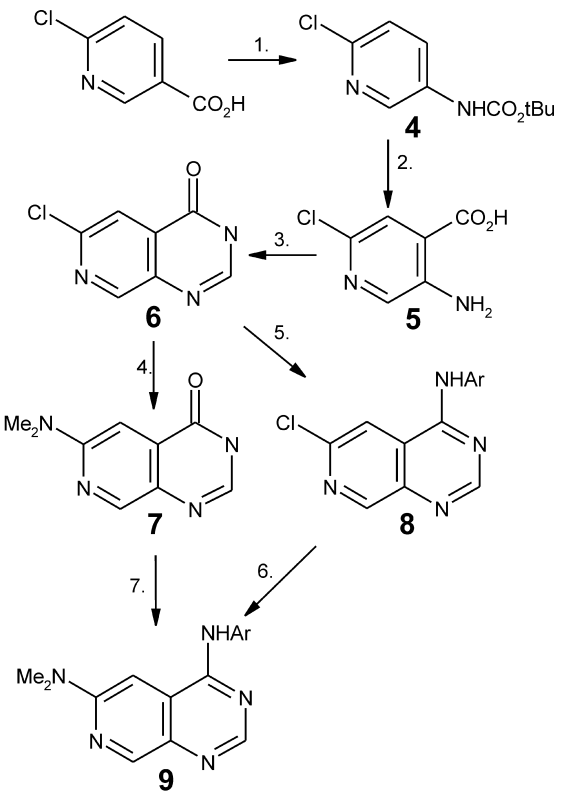
Figure 3. Dosing of **3B** (Gw974) in the BT474 c-erbB-2 overexpressing xenograft model in SCID mice. compound was dosed at 30 mg/kg for 5 days, at 10 mg/kg for 15 days. Dosing ceased at day 80. All doses were well tolerated. Only one control animal remained alive at day 100.

In addition, inhibition of the growth of EGFr overexpressing xenografts was observed with this compound. For example, 80% inhibition of growth of an HN5 EGFr cell based xenograft was demonstrated in SCID mice at 10 mg/kg, bid, oral dosing for 20 days.

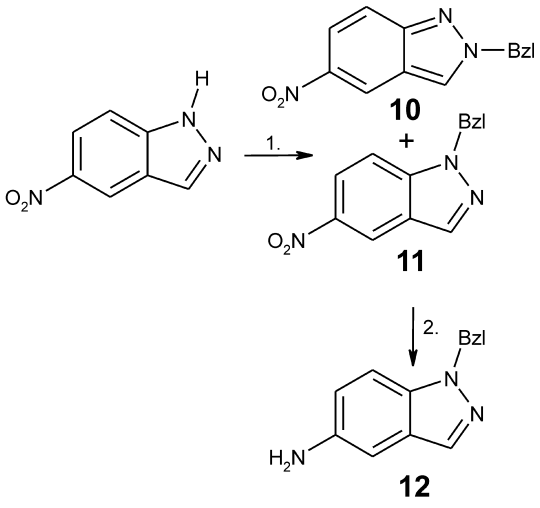
Synthetic Chemistry

Quinazoline analogues were prepared via previously described procedures.[9] Pyridopyrimidines were prepared

from 6-chloronicotinic acid (Scheme 1). Curtius rearrangement and ortholithiation of the Boc-amine **4** allowed a facile preparation of the anthranilic acid equivalent **5**. Reaction with formamidine acetate furnished the chloropyridopyrimidinone **6**. Chlorination and aniline introduction in acetonitrile to give **8** could be followed by reaction with dimethylamine to give **9** (GW974 where NHAr is **12**). Alternatively, in order to allow efficient parallel synthesis of the analogues described herein, chloropyridopyrimidinone **6** could be reacted with dimethylamine to furnish the dimethylaminopyridopyrimidinone **7**. Subsequent chlorination could then be followed by parallel introduction of a variety of anilines.



Scheme 1. (1) $(PhO)_2PON_3$, Et_3N, *tert*-BuOH, Δ, 83%; (2) (i) *n*-BuLi, TMEDA, toluene, −60 to −15; (ii) CO_2, 65%; (iii) aq NaOH, 85%; (3) formamidine acetate, AcOH, 79%; (4) aq Me_2NH, EtOH, MeCN, 90%; (5) (i) $POCl_3$, Et_3N, 90%; (ii) $ArNH_2$, MeCN, Δ, 60–90%; (6) aq. Me_2NH, EtOH, MeCN, 90%; (7) (i) $POCl_3$, Et_3N, 90%; (ii) $ArNH_2$, MeCN, Δ, 60–90%.



Scheme 2. (1) K_2CO_3, benzyl bromide followed by aq acetone recrystallisation, 30%; (2) H_2, Pd/C, 95%.

Synthesis of aniline top halves was straightforward as exemplified in Scheme 2 for the *N*-benzyl indazolylamine **12**. Alkylation of the nitro bicycle (5-nitroindazole shown) was followed by hydrogenation. In the cases of anilines required for **B**, **C**, **D** and **F** in Figure 3, these were prepared as mixtures and separated by flash chromatography. Indazole aniline **B** could also be isolated free of the 2 regioisomer by an acetone–water recrystallisation in 30% yield from the 5-nitroindazole. 2-Benzyl benzimidazole aniline for **E** in Figure 3 was prepared by known methods.[11]

Summary

Described herein is the identification and evaluation of GW974, a potent inhibitor of the tyrosine kinase activity of EGFr/c-erbB-2. This in vitro potency transcribed to in vivo potency in xenografts overexpressing both receptor kinases. At higher doses in the BT474 xenograft, tumour shrinkage was observed and found to be irreversible, that is no regrowth of tumours was observed upon cessation of dosing. GW974 represents valuable progress in the identification of combined EGFr/c-erbB-2 inhibitors as an approach to cancer chemotherapy with low toxicity.

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