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Effects of tyrosine kinase inhibitors on protein kinase-independent systems

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Tyrosine kinase inhibitors have been widely used to probe the role of tyrosine phosphorylation in cellular signalling. These inhibitors exhibit an apparent specificity for tyrosine kinases over the serine/threonine kinases but little is known about their effects on other enzymes or biological systems. We demonstrate that genistein, erbstatin and α-cyanocinnamamides (tyrphostins) have inhibitory effects on fatty acid synthesis, lactate transport, mitochondrial oxidative phosphorylation and aldehyde dehydrogenase. We propose, therefore, that results obtained using tyrosine kinase inhibitors should be interpreted with caution, particularly if used at concentrations sufficient to inhibit these non-protein kinase-dependent events.

Tyrosine kinase; Inhibitor; Insulin; Fat cell; Lactate transport; Cell growth

1. INTRODUCTION

Protein phosphorylation on serine, threonine and tyrosine residues is central to the regulation of a wide range of cellular processes including metabolism, the cell cycle, gene transcription/translation, and signal transduction by growth factor receptors and several oncogene products. Over 100 protein kinases have been characterized and cloned [1,2], but determination of their role in cellular processes has often remained elusive. This has encouraged the development of low molecular weight inhibitors, some of which appear to exhibit reasonable specificity for individual protein kinases despite their apparent similarity within the kinase core structure [3,4].

Inhibitors of protein kinases have been developed that interact at the ATP or peptide substrate binding site, or at domains involved in allosteric regulation (reviewed in [4]). Erbstatin and genistein are examples of inhibitors that are competitive with substrate and ATP respectively, and which inhibit EGF receptor autophosphorylation more effectively than the cAMP-dependent protein kinase [5,6]. It might be predicted that inhibitors directed at the substrate binding site would be more specific than those acting at the ATP binding site. A series of such compounds with common hydroxy *cis*-cinnamamonitrile backbone structures, have been investigated intensively and have been termed tyrphostins by Yaish et al. [7] (see also ref. [8], e.g. inhibitors RG50864, ST271 and ST638 of Table I). These inhibitors exhibit both competitive and non-competitive inhibitory kinetics with the peptide substrate and, in most cases, are considerably more potent against the EGF receptor than the insulin receptor or serine/threonine-specific protein kinases [7–10]. Indeed it has been reported that some tyrphostins can discriminate between tyrosine kinases such as the EGF receptor and c-*erb*B2, which share considerable amino acid sequence homology [11]. Furthermore, the potency of several tyrosine kinase inhibitors (e.g. ST271 and t-Boc tyrosine aminomalonate) against the insulin receptor have been reported to be generally rather low (K_i > 0.5 mM; [7,12]).

This degree of selectivity suggests that such compounds could serve as useful indicators of the involvement of tyrosine phosphorylation in specific cellular processes. For example tyrosine kinase inhibitors have anti-proliferative effects when added to EGF-stimulated cells in culture [11,13], block long-term potentiation in hippocampal slices [14] and inhibit the lipogenic efects of insulin without apparent effects on the anti-lipolytic actions of the hormone [12].

The demonstration that tyrosine phosphorylation is involved in these highly complex biological processes is implicitly dependent on the specificity of the inhibitors for tyrosine kinases over other cellular processes. Although genistein can inhibit β-galactosidase [15] and

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Abbreviations: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; MOPS, morpholinopropane sulphonic acid; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenyl hydrazone.

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topoisomerases I and II [16], there is little information about the effects of the tyrosine kinase inhibitors (or other protein kinase inhibitors in general) on other cellular processes.

During our preliminary studies on erbstatin, genistein and the tyrphostins RG50864, ST271 and ST638 on insulin signalling pathways it became apparent that these inhibitors exhibited a series of effects on cells which could not be explained by protein kinase inhibition. Initially we found that some of the compounds inhibited basal rates of fatty acid synthesis in isolated fat cells. As the tyrphostins are structurally related to α-cyano-4-hydroxycinnamate and its derivatives, which are potent inhibitors of mitochondrial pyruvate transport (and as a consequence fatty acid synthesis; [17,18]), plasma membrane H^+-lactate co-transporters [17], and aldehyde dehydrogenase [19] we thus investigated the effects of these inhibitors on pyruvate/lactate transport, and aldehyde dehydrogenase. During the course of this work some of the inhibitors were also found to inhibit mitochondrial oxidative phosphorylation.

2. MATERIALS AND METHODS

2.1. *Materials*

The tyrphostin inhibitors RG50864 [20](also coded 47 in ref. [21]), ST271 (coded 8 in ref. [22]) and ST638 (coded 13 in ref. [22]) were synthesized as described. Erbstatin was prepared according to the procedure of Anderson et al. [23]. Genistein was purchased from Apin Chemicals. All inhibitors were judged pure by spectral and thin-layer chromatography criteria and were dissolved in Me_2SO; in all experiments appropriate controls with Me_2SO were performed.

2.2. *Assays of fatty acid synthesis in isolated fat cells*

This was performed essentially as described by Rutter and Denton [24]. Isolated rat epididymal fat cells (~ 0.1 g dry weight) were incubated at 37°C in 1 ml buffer (140 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 2.4 mM NaH_2PO_4, 10 mM HEPES, 15 mM $NaHCO_3$, 1 mM glucose, 1% (w/v) BSA, pH 7.4) containing 0.1–0.5 μCi D-[U-^{14}C]glucose and insulin as required. Incubations were continued for 30 min and radioactivity associated with triacylglycerol fatty acids and glycerol determined [25].

2.3. *Measurement of oxygen consumption by isolated liver mitochondria*

Liver mitochondria were prepared from Wistar rats (250 g) as described [26]. Rates of oxygen uptake by mitochondria (approx. 1 mg protein/ml) were measured using a Clark-type oxygen electrode at 37°C in 1 ml buffer containing 125 mM KCl, 10 mM MOPS, 5.5 mM Tris, 2.5 mM $MgCl_2$, 2.5 mM potassium phosphate and 0.5 mM EGTA pH 7.2. Uncoupling activity of compounds was assessed by measuring their stimulation of oxygen uptake in the presence of 5 mM L-glutamate and 1 mM D-malate but no ADP. Respiratory chain inhibition was assayed in the presence of 1 mM ADP and substrates (5 mM pyruvate/1 mM malate or 5 mM glutamate/1 mM malate or 5 mM Tris-succinate/1 μg/ml rotenone or 10 mM ascorbate/0.3 mM TMPD).

2.4. *Measurement of lactate transport*

Assay of the specific monocarboxylate transporter in rat erythrocytes was performed using a centrifuge stop technique [27]. In these experiments initial rates of uptake of 0.5 mM [U-^{14}C]-L-lactate were measured over 15 s at 7°C. Inhibitors were preincubated with the cell suspension (10% haematocrit) for 2 min prior to measurement of transport.

2.5 *Assay of aldehyde dehydrogenase activity*

The low K_m rat liver mitochondrial aldehyde dehydrogenase was purified by α-cyanocinnamate affinity chromatography [19]. The enzyme was assayed spectrophotometrically at 30°C, by following the production of NADH as A_{340} in buffer containing 100 mM NaCl, 20 mM MOPS, 0.5 mM EDTA, pH 7.4, and the substrates acetaldehyde (0.1 mM) and NAD^+ (0.5 mM). Inhibitors were present only during the period of the assay. Most of the inhibitors had considerable absorbance at 340 nm restricting their use to concentrations less than 100 μM.

3. RESULTS AND DISCUSSION

3.1. *Effects of tyrosine kinase inhibitors on fatty acid synthesis*

The inhibitors RG50864 and ST271 were examined for their effects on fatty acid synthesis from D-[U-^{14}C]glucose in isolated fat cells. The inhibitors had pronounced effects on both basal and insulin-stimulated fatty acid synthesis, but neither inhibitor caused any significant reduction in the magnitude of the insulin effect of on this process (approx. 3- to 4-fold in the presence or absence of 400 μM inhibitor; see Fig. 1).

The mechanism underlying the effects of the inhibitors on fatty acid synthesis is not known. The tyrphostins are structurally related to α-cyano-4-hydroxycinnamate and its derivatives which inhibit mitochondrial pyruvate transport and, as a consequence, fatty acid synthesis [17,18]. If the inhibitory effects of the tyrphostins on fatty acid synthesis were due to a similar mechanism an increase in lactate and pyruvate output into the medium might be expected. However, this does not appear to be the case since we observed no significant increase in extracellular lactate and pyruvate concentrations (results not shown). Furthermore, we observed no significant change in glyceride glycerol synthesis (results not shown) which also implies that the site of action of the inhibitors on fatty acid synthesis may be beyond the point of action of α-cyanocinnamate (i.e. post-mitochondrial pyruvate transport).

Interestingly, genistein has been reported to inhibit insulin-stimulated glucose oxidation without effect on insulin receptor autophosphorylation in isolated fat cells 28]. In that study the basal rates of glucose oxidation were too low to conclude whether or not basal rates were similarly affected. Genistein is an analogue of phloretin and quercetin, well characterised and potent inhibitors of glucose transport [29,30]. It remains to be established whether this effect on glucose oxidation might be explained by a direct inhibition of the glucose transporter, rather than through inhibition of other insulin-stimulated protein serine/threonine kinases as suggested by Abler et al. [28].

The structural similarity between the tyrphostins and α-cyanocinnamate, and the results shown in Fig. 1,

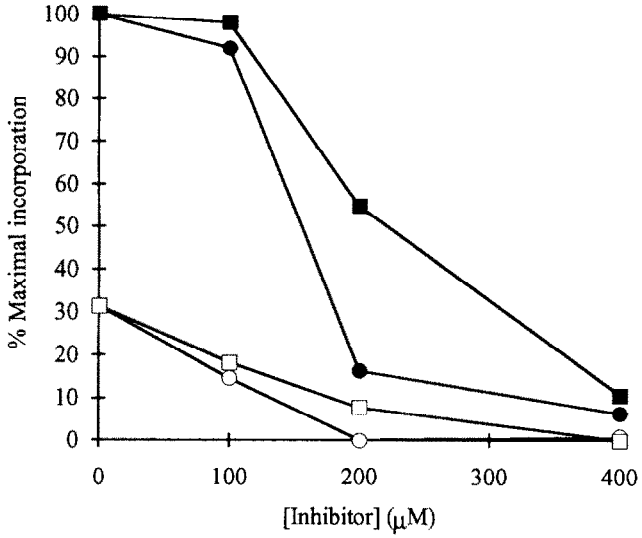


Fig. 1. Effects of tyrosine kinase inhibitors on glucose incorporation into fat in isolated fat cells. Isolated epididymal fat cells were incubated with D-[U-^{14}C]glucose and the indicated concentrations of inhibitor RG50864 (circles) or ST271 (squares) in the absence (open symbols) or presence (filled symbols) of insulin. The data points represent means of two determinations. Quantitatively similar results were obtained in two further experiments where the effects of insulin were 4- to 7-fold. Experimental details are given in section 2.

prompted us to perform a more detailed examination of systems which are affected by α-cyanocinnamates. These included pyruvate transport and respiratory chain activity of isolated mitochondria, plasma membrane H^+-lactate co-transport, and aldehyde dehydrogenase.

3.2. *Effects of tyrosine kinase inhibitors on mitochondrial pyruvate transport and respiratory chain activity*

Mitochondrial pyruvate transport was measured indirectly by determining rates of pyruvate oxidation in isolated mitochondria. While inhibitor RG50864 had no significant effect, ST271, ST638 and genistein were inhibitors of pyruvate oxidation (200 μM inhibitor gave approx. 67%, 83% and 60% inhibition of oxygen uptake, respectively). In order to investigate the nature of this inhibition we carried out further experiments with isolated mitochondria. As shown in Table I, four of the inhibitors were found to be uncouplers of oxidative phosphorylation. Indeed, the compounds achieved approx. 90% of the maximal extent of uncoupling observed with FCCP. Similar properties have been reported previously for SF6847 (as ST271 but with a nitrile group in place of the amide) although the EC_{50} was significantly lower (10 nM) [31]. This is, perhaps, not surprising in view of the weak acid nature of the phenolic hydroxyl and the electron withdrawing nature of the nitrile group. At higher concentrations, however, the compounds were found to be respiratory chain inhibitors; in the presence of 1 mM ADP and glutamate/malate as oxidative substrates, we found that ST271, ST638 and genistein (all at 200 μM) exhibited 60%, 85% and 58% inhibition of oxygen uptake, respectively. Similarly, when succinate was used as a respiratory substrate, inhibitors ST271, ST638 and genistein (at 200 μM) caused approx. 62%, 50% and 37% inhibition of oxygen uptake, respectively. No inhibition of ascorbate/TMPD oxidation was observed with any of the inhibitors. Thus the inhibitors have complex effects on mitochondrial respiratory chain activity. Depending on the conditions used they can both uncouple oxidative phosphorylation as well as act as respiratory chain inhibitors at complex III although a further site of action at complex I cannot be ruled out. Interestingly, we also found that staurosporine, a potent but non-specific inhibitor of protein kinases at nanomolar concentrations [32], also inhibited the respiratory chain; 1 μM staurosporine gave approx. 80% inhibition of glutamate/malate oxidation.

3.3. *Effects of tyrosine kinase inhibitors on H^+-lactate co-transport and aldehyde dehydrogenase*

The effects of the inhibitors on H^+-lactate co-transport into isolated erythrocytes was examined at a physiological lactate concentration. This process involves a transporter which has similar properties to the pyruvate transporter of the inner mitochondrial membrane and, indeed, transporters with similar properties are present in almost all mammalian cells [33]. The most potent inhibitor was ST271 with an EC_{50} value in the micromolar range (Table I). By contrast, ST638 was the most potent inhibitor of aldehyde dehydrogenase with ST271 having little detectable effect up to 100 μM (Table I).

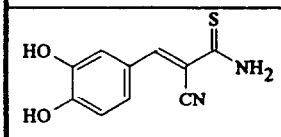
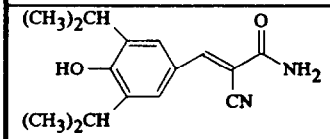
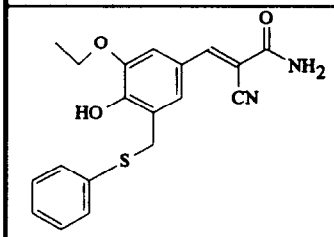
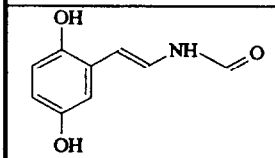
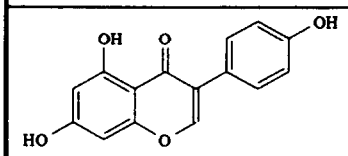
Erbstatin had no apparent effects on the respiratory chain or aldehyde dehydrogenase, but it did have a weak inhibitory effect on lactate transport (Table I). However, after periods of storage as a solution at 4°C,

presumably as a result of decomposition to an uncharacterized derivative, erbstatin became a relatively potent inhibitor (EC_{50} approx. 18 μM) of glutamate/malate oxidation by isolated mitchondia. In contrast genistein, a widely used tyrosine kinase inhibitor which is reported to be competitive with respect to ATP [5], had multiple inhibitory effects on respiratory chain activity, lactate transport and aldehyde dehydrogenase (Table I).

4.4. *General conclusions*

It is clear from these studies that the tyrosine kinase inhibitors used have multiple effects on protein kinase-independent events (lactate transport, respiratory chain activity and aldehyde dehydrogenase). Inhibition of fatty acid synthesis could, perhaps, be explained by the inhibition of protein-serine/threonine kinases involved in the regulation of acetyl-CoA carboxylase and/or pyruvate dehydrogenase, the two key regulatory steps in this pathway, although we cannot rule out an effect of the inhibitors on cellular ATP levels.

These apparently non-specific effects of the tyrosine kinase inhibitors is of some concern. Tyrosine kinase inhibitors have been used in several situations to infer the involvement of tyrosine kinase-dependent signalling. For example, genistein, at a concentration of 100 μM, has been reported to block long term potentiation in the hippocampus [14] and CD40/Bp50 B-cell receptor signalling [34]; this is a concentration which is significantly above the EC_{50} value for inhibition of lactate transport (8.5 μM; Table I). Similarly, Hill et al. [35] have reported that 100 μM genistein inhibits PDGF-stimulated DNA synthesis in mouse fibroblasts. Furthermore, 100 μM ST638 and 80 μM RG50864 have been shown to inhibit the growth of A431 and HER14 cells, respectively [9,13,20]. The behaviour of these tyrosine kinase inhibitors could be explained through their ability to inhibit lactate transport, rather than via inhibition of a tyrosine kinase, particularly in the case of genistein and ST271. For example, many cultured cell lines possess few mitochondria and rely heavily on glycolysis and thus an inhibition of H^+-linked efflux of the lactate produced could result in intracellular acidification, inhibition of glycolysis and a consequent drop in cellular ATP levels. Indeed, another inhibitor of lactate

	Inhibitor name	Mitochonrial uncoupling[a,d]	Lactate transport[b]	Aldehyde dehydrogenase[b,f]
	RG50864	50.0 ± 0.9	126 ± 7[f]	19.8 ± 1.1
	ST271	27.6 ± 1.8	7.2 ± 1.0[f]	> 100[c]
	ST638	58.1 ± 7.6	46.6 ± 9.2[e] (0.50 ± 0.06)	6.0 ± 0.8
	Erbstatin	N.E.	132 ± 34[e] (0.60 ± 0.10)	N.E.
	Genistein	68.9 ± 2.8	8.5 ± 2.0[f]	30 ± 3.0

transport, quercetin (also a tyrosine kinase inhibitor) [36], causes cytoplasmic acidification and inhibition of glycolysis in Ehrlich ascites cells [37].

Inhibition of aldehyde dehydrogenases could also have important effects in certain experimental systems. Most notably, these enzymes are involved in the synthesis of the neurotransmitter γ-aminobutyric acid [38], and in the metabolism of aldehydes derived from histamine and dopamine [39]. Whether the concentrations of the inhibitors could reach levels high enough to uncouple or inhibit the respiratory chain in intact cells is not known, although genistein has been reported to have no significant effect on ATP levels in isolated fat cells [28]. However, importantly, little is known about the rates of uptake of these compounds into cells, whether they become accumulated within the cell, how rapidly they are metabolised and, finally, whether the intermediary or end products of their metabolism have effects on other cellular processes not described in the present study. Thus an interaction of these inhibitors with the respiratory chain cannot be ruled out when they are added at extracellular concentrations approaching 100 μM.

Whilst some of the effects of the compounds might be predicted based on their structural similarity to known inhibitors of lactate/pyruvate transport and aldehyde dehydrogenase, many of the effects we have observed were unexpected. This was particularly the case with genistein which clearly has effects on ATP-independent processes. Thus it remains quite possible that these compounds could inhibit cellular processes other than those described in the present study, which might also be important in signal transduction events and cellular metabolism.

In conclusion, results derived from the use of tyrosine kinase inhibitors should be interpreted with great caution until more is known about their effects on other non-protein kinase-dependent events. The involvement of a tyrosine kinase in a cellular process/signalling pathway cannot be concluded from the use of tyrosine kinase inhibitors alone until such knowledge is more complete.

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