Design of a Selective Insulin Receptor Tyrosine Kinase Inhibitor and Its Effect on Glucose Uptake and Metabolism in Intact Cells

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ABSTRACT: An inhibitor of the insulin receptor tyrosine kinase (IRTK), (hydroxy-2-naphthalenylmethyl)phosphonic acid, was designed and synthesized and was shown to be an inhibitor of the biological effects of insulin in vitro. With a wheat germ purified human placental insulin receptor preparation, this compound inhibited the insulin-stimulated autophosphorylation of the 95-kDa β-subunit of the insulin receptor (IC_{50} = 200 μM). The ability of the kinase to phosphorylate an exogenous peptide substrate, angiotensin II, was also inhibited. Half-maximal inhibition of basal and insulin-stimulated human placental IRTK activity was found at concentrations of 150 and 100 μM, respectively, with 2 mM angiotensin II as the peptide substrate. The inhibitor was found to be specific for tyrosine kinases over serine kinases and noncompetitive with ATP. The inhibitor was converted into various (acyloxy)methyl prodrugs in order to achieve permeability through cell membranes. These prodrugs inhibited insulin-stimulated autophosphorylation of the insulin receptor 95-kDa β-subunit in intact CHO cells transfected with human insulin receptor. Inhibition of insulin-stimulated glucose oxidation in isolated rat adipocytes and 2-deoxyglucose uptake into CHO cells was observed with these prodrugs. Our data provide additional evidence for the involvement of the insulin receptor tyrosine kinase in the regulation of glucose uptake and metabolism. These results and additional data reported herein suggest that this class of prodrugs and inhibitors will be useful for modulating the activity of a variety of tyrosine kinases.

The initial step in insulin action is the interaction of the hormone with its cell surface receptor [for review, see Czech (1977)], which activates a tyrosine-specific protein kinase activity (Kasuga et al., 1982) associated with the 95-kDa β-subunit of the receptor. Tyrosine kinase activity is also associated with other membrane-bound proteins such as the epidermal growth factor receptor (Cohen et al., 1980), the platelet-derived growth factor receptor (Ek et al., 1982), and the Rous sarcoma viral gene product $pp60^{v\text{-}src}$ (Collet et al., 1978). These tyrosine kinase activities are thought to be involved in the regulation of cellular growth and metabolism.

The importance of IRTK[1] to the metabolic events mediated by insulin have been recently discussed (Espinal, 1988). Site-directed mutation of tyrosine residues 1150 and 1151 to phenylalanines in the β-subunit of the insulin receptor and transfection into Chinese hamster ovary (CHO) cells resulted in a decreased ability to autophosphorylate and decreased sensitivity to insulin (Ellis et al., 1986). Mutations in the putative ATP binding site of the β-subunit resulted in decreased insulin stimulation of glucose uptake, thymidine incorporation into DNA, and glycogen synthesis in transfected CHO cells (Ebina et al., 1987; Chou et al., 1987). Monoclonal antibodies against the β-subunit were able to block insulin-stimulated metabolic processes when microinjected into several cell lines (Morgan & Roth, 1987; Morgan et al., 1988). However, monoclonal antibodies against the α-subunit, which do not bind to the insulin binding domain, have been shown to inhibit insulin binding, yet they, paradoxically, stimulate glucose transport in adipocytes without activating the IRTK (Forsayeth et al., 1987a,b). Also, monoclonal antibody MA-10 inhibits insulin binding yet does not stimulate receptor autophosphorylation, deoxyglucose uptake, or thymidine incorporation into DNA (Russell et al., 1987). Polyclonal antibodies have been shown to stimulate glucose uptake in rat adipocytes without stimulating receptor phosphorylation or receptor kinase activity (Simpson & Hedo, 1984; Zick et al., 1984). A different polyclonal antibody (B-10) has been shown to elicit all of the biological effects attributed to insulin in wild-type CHO cells (Gherzi et al., 1987). However, this antibody did not mimic insulin in stimulating postreceptor cellular events in kinase-deficient CHO cells.

Thus, a suitable inhibitor of the IRTK could yield valuable additional information regarding which actions of insulin are mediated by the tyrosine kinase activity. Such an inhibitor might also be helpful for identifying intracellular substrates of the IRTK. Some important features for an inhibitor to have in order to be useful in this regard include (a) cell membrane permeability, (b) reasonable potency, (c) selective inhibition of tyrosine kinases over serine/threonine kinases, and (d) noncompetitive inhibition with respect to ATP (to avoid competition with relatively high levels of intracellular ATP). Previously reported inhibitors include amino acid derivatives (Tamura et al., 1984; Begum et al., 1985), peptides (Ueno et al., 1987; Walker et al., 1987), tyrosine-containing synthetic

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[1] Abbreviations: hIR, human insulin receptor; (hIR)CHO, chinese hamster ovary cells transfected with human insulin receptor; IRTK, insulin receptor tyrosine kinase; EDAc, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride; CHO, chinese hamster ovary; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethananesulfonic acid; IC_{50}, concentration of inhibitor yielding half-maximal activity; K_m, Michaelis–Menten constant; V_m, maximum velocity; EGFTK, epidermal growth factor receptor tyrosine kinase.

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polymers (Braun et al., 1984; Sahat et al., 1988; Vicario et al., 1988a), ATP-competitive inhibitors (Davis & Czech, 1985; Kruse et al., 1988a,b), and divalent metal ions (Pang & Shafer, 1985; Vicario et al., 1988b). In addition, catecholamines (Haring et al., 1986a; Obermaier et al., 1987) and phorbol esters (Bollag et al., 1986; Haring et al., 1986b; Obermaier et al., 1987; Takayama et al., 1984) have been shown to reduce IRTK activity. None of these inhibitors have the desired potency, selectivity, and cell membrane permeability.

In this paper we describe the design and synthesis of an inhibitor which possesses all of the important features listed above, including cell permeability when presented as a prodrug. The results reported herein, with this inhibitor and its prodrugs, provide further evidence for the involvement of IRTK in mediating glucose uptake and metabolism.

Materials and Methods

Materials

[γ-^{32}P]ATP (3000 Ci/mmol), [^{14}C(U)]glucose (10–15 mCi/mmol), ^{125}I-insulin (receptor grade, 2200 Ci/mmol), and [1-^{3}H]-2-deoxy-D-glucose (49.2 mCi/mmol) were purchased from Du Pont/NEN (Boston, MA). What germ coupled agarose was obtained from Vector Laboratories (Burlingham, IN) and angiotensin II (AII) from Boehringer Mannheim (Indianapolis, IN). Crystalline pork insulin (26 units/mg) was purchased from Elanco (Indianapolis, IN). Collagenase was obtained from Worthington Biochemicals (Freehold, NJ). Cyclic AMP dependent protein kinase (bovine heart, 1–2 picomolar units/mg of protein) was obtained from Sigma Chemical Corp. (St. Louis, MO). Sulfa-MBS was purchased from Pierce (Rockford, IL). EDAC was obtained from Bio-Rad (Richmond, CA). Peptide 1142–1159 was purchased from Cambridge Research Biochemicals (Cambridge, England). Peptide 1315–1330 was synthesized by Dr. D. F. Veber of Merck Sharp & Dohme Research Laboratories (West Point, PA).

Animals

Male Sprague-Dawley (CD) rats (150–200 g) were obtained from Charles River (Boston, MA). New Zealand rabbits were purchased from Hazelton Research Products (Denver, PA). Animals were allowed free access to food and water up to time of sacrifice.

Methods

Preparation of Rat Adipocytes. Intact rat adipocytes were prepared according to the procedure described by Rodbell (1964) and were derived from the distal portion of the epididymal fat pads.

CHO Cell Line. CHO cells (5×10^5) transfected with human placental insulin receptor ($\sim$18 000 receptors/cell) were plated in 35 × 10 mm tissue culture dishes in MEM α medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). Prior to use, the cells were washed with Krebs Ringer bicarbonate/Hepes, pH 7.4, containing 30 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5 mM glucose, and 1 mg/mL bovine serum albumin.

Synthesis of (Hydroxy-2-naphthalenylmethyl)phosphonic Acid and Its Prodrugs. Experimental details are included in the supplementary material.

Insulin Receptor Tyrosine Kinase Assay. Wheat germ purified human placental insulin receptor was prepared as previously described (Strout et al., 1988). IRTK-catalyzed phosphorylation of AII was determined as previously described (Vicario et al., 1988c). Briefly, wheat germ purified insulin receptor ($\sim$8 fmol of insulin binding activity) was incubated for 15 min at 23 °C in a final volume of 0.050 mL of a reaction mixture containing (at final concentrations) 20 mM Hepes (pH 7.5), 100 nM insulin, 5% glycerol, 30 mM NaCl, 2 mM $MnCl_2$, 12 mM $MgCl_2$, and 100 μM sodium orthovanadate. [γ-^{32}P]ATP (50 μM, 5 cpm/fmol) was added and the incubation continued for 15 min at 23 °C. AII (2 mM) was added and the reaction mixture incubated for 15 min at 23 °C. Phosphorylated AII was isolated by use of phosphocellulose (P81) paper (Casnelli et al., 1982). Kinase reactions were performed under conditions where the reaction rate was linear with respect to both time and enzyme concentration.

In studies where the phosphorylation of the 95-kDa β-subunit of the insulin receptor was examined, the phosphorylated β-subunit was precipitated with an antibody directed against it as previously described (Herrera & Rosen, 1986).

Antibody to Insulin Receptor β-Subunit COOH Terminus Peptide Sequence 1315–1330. The sequence STEEHIPYTHMNGGKK is identical with residues 1315–1330 of the human insulin receptor with the exception that threonine replaces tyrosine at residue 1316. The crude peptide was purified by HPLC on a C_{18} column with an acetonitrile gradient (10–80%). The molecular weight was confirmed by FAB mass spectral analysis. The peptide was conjugated to thyroglobulin with EDAC at a 50:1 peptide to protein ratio. White New Zealand rabbits were inoculated with 2.0 mg of the conjugate, boosted at 2 weeks, and bled at 6 weeks. This antibody is designated Ab 1315–1330.

Antibody Which Recognizes the Phosphorylated Insulin Receptor β-Subunit. The sequence TRDIYETDYYRKGGKGLLC is identical with residues 1142–1159 of the human insulin receptor, with the addition of cysteine at the carboxy terminus (Ullrich et al., 1985). The crude peptide was purified by HPLC on a C_{18} column with an acetonitrile gradient (10–80%). Peptide composition and molecular weight were confirmed by amino acid and FAB mass spectral analysis, respectively. The peptide was conjugated to thyroglobulin through the cysteine residue with the bifunctional coupling reagent sulfo-MBS. The ratio of peptide to protein was 30:1. New Zealand rabbits were inoculated with 2.0 mg of conjugate, boosted at 2 weeks, and bled at 6 weeks. This antibody is designated as Ab 1142–1159.

Other Methods. cAMP-dependent protein kinase activity was measured as described by Erlichmann et al. (1971) with protamine sulfate as a substrate. Protein kinase C activity was measured as described by Hannun et al. (1985). The bioassay used for measuring the conversion of [^{14}C]glucose to $^{14}CO_2$ was essentially that described by Gliemann (1965). Measurement of [^{3}H]-2-deoxyglucose uptake was performed as previously described (Cascieri et al., 1986). [^{3}H]Thymidine uptake was measured as described by Gherzi et al. (1987). Protein was measured by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

The synthesis of the parent inhibitor, (hydroxy-2-naphthalenylmethyl)phosphonic acid (**4**), and four prodrug derivatives, **8**–**11**, is outlined in Figure 1. The addition of di-*tert*-butyl phosphite (**2**) to aldehyde **1** was catalyzed by cesium fluoride (Texier-Boullet & Foucaud, 1982), giving diester **3** as a racemic mixture. The *tert*-butyl protecting groups were removed with formic acid, giving the parent inhibitor **4**. The phosphonic acid **4** was converted into the corresponding disilver salt **5** and then alkylated with 2 equiv of either iodomethyl pivalate (**6**) or iodomethyl acetate (**7**)

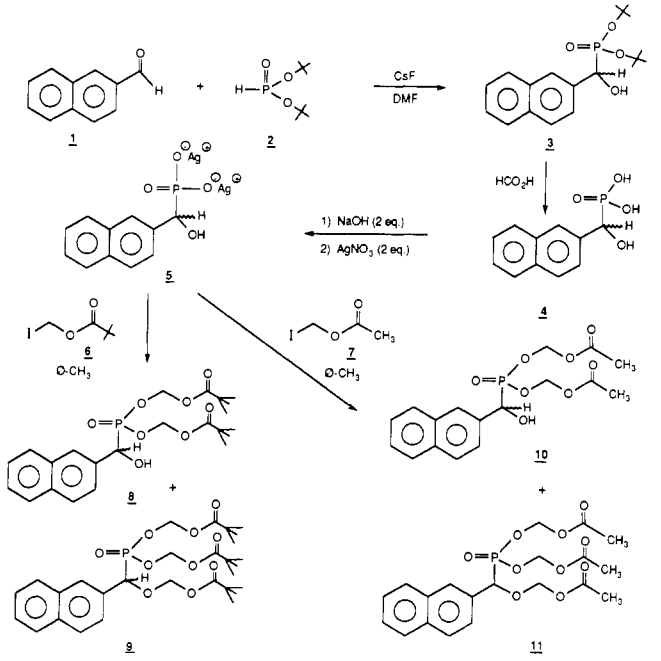

FIGURE 1: Synthesis of the parent IRTK inhibitor **4** and four prodrugs, **8**–**11**.

(Srivastva & Farquhar, 1984), yielding the corresponding di- and trialkylated products **8** and **9** or **10** and **11**, respectively. The formation of the trialkylated products was not anticipated but was a fortunate outcome of the synthesis since prodrug **11** was later found to be the most effective of the four prodrugs in cell culture. Increasing the amount of iodomethyl acetate used in the alkylation to 3.3 equiv resulted in only trialkylated product **11**.

The water solubility of the four prodrugs differed significantly, as might be expected from their structures. Prodrugs **8** and **9** had lower solubility in water (~200 and 60 μM, respectively) than did the corresponding prodrugs with fewer carbon atoms, **10** and **11** (both greater than 1.8 mM). The stability of the prodrugs in aqueous buffer and the rate of release of the parent inhibitor **4** by esterases were not measured but would be expected to differ significantly on the basis of a study of similar prodrugs of phosphates by Srivastva and Farquhar (1984).

With a wheat germ purified human placental insulin receptor preparation, inhibitor **4** decreased the insulin-dependent IRTK-catalyzed phosphorylation of 2.0 mM AII in a concentration-dependent manner (Figure 2A), with an IC_{50} of 100 μM. Somewhat higher concentrations of inhibitor **4** were required to inhibit basal (no insulin) IRTK activity for AII phosphorylation (IC_{50} = 150 μM). Only a slight inhibition of insulin-stimulated IRTK activity was observed with similar concentrations of prodrug **11** (21% inhibition at 1000 μM). Increasing (1–200 μM) concentrations of inhibitor **4** resulted in a concentration-dependent inhibition of human placental 95-kDa insulin receptor subunit autophosphorylation (Figure 2B). In this experiment, ~50% inhibition of 95-kDa insulin receptor β-subunit phosphorylation was obtained at a concentration of 200 μM inhibitor **4**.

IRTK activity was assayed in the presence of increasing concentrations (0–250 μM) of inhibitor **4** at a fixed (2.5 mM) concentration of AII substrate and varying (5, 50, and 500 μM) concentrations of metal–ATP (Figure 3A). When the data were plotted according to the method of Dixon (1953), an apparent K_i for inhibitor **4** between 68 and 215 μM was obtained (Figure 3B). These data indicated that the K_i for

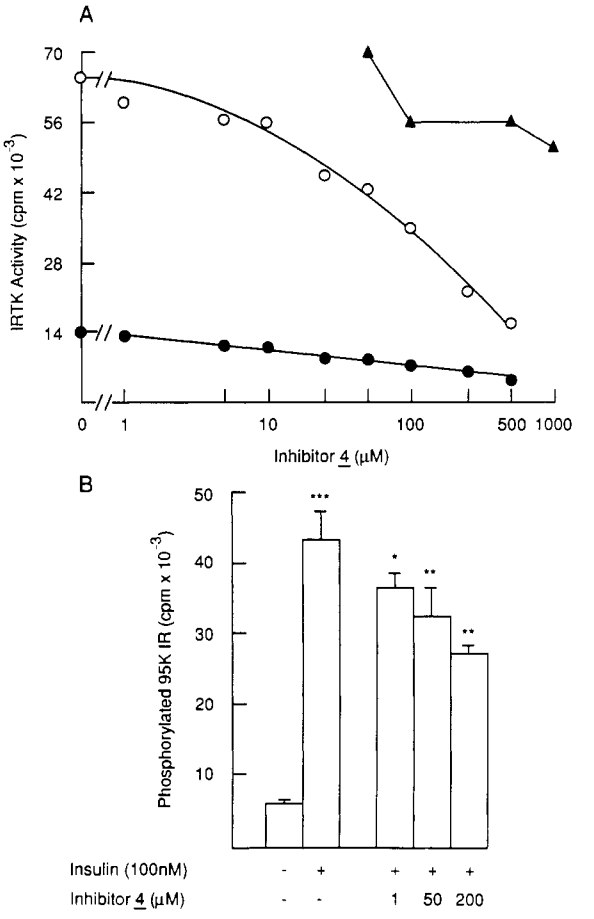

FIGURE 2: (A) Effect of inhibitor **4** on basal and insulin-stimulated IRTK activity. Portions of wheat germ purified human placental insulin receptor (8 fmol of insulin binding activity) were incubated in the absence (●) and presence (○) of insulin for 15 min at 23 °C. $MnCl_2$ (2 mM) and $MgCl_2$ (12 mM) were then added followed by [γ-^{32}P]ATP (50 μM). Increasing (0–500 μM) concentrations of inhibitor **4** were then added. After 15 min at 23 °C, AII (2 mM) was added and the incubation terminated after 15 min at 23 °C as described under Materials and Methods. Also shown are the effects of prodrug **11** (▲) on insulin-stimulated IRTK activity. (B) Effect of inhibitor **4** on insulin receptor 95-kDa β-subunit autophosphorylation. The phosphorylation state of partially purified human placental insulin receptor was determined with Ab 1142–1159 as described under Materials and Methods. Concentrations of inhibitor **4** up to 200 μM were studied. Not significant at (*) $P = 0.05$, (**) $P < 0.05$, and (***) $P < 0.001$ (compared to basal activity).

inhibitor **4** decreased with increasing concentrations of metal–ATP, suggesting cooperative binding between inhibitor and metal–ATP substrate. A replot of the slopes vs [metal–ATP]$^{-1}$ (not shown) yielded a straight line (r = 0.983) that did not pass through the origin, indicating noncompetitive inhibition relative to ATP [this was checked by plotting the data according to the method of Cornish-Bowden (1974)]. A K_m value for metal–ATP of 48 μM was calculated. From the equation intercept = $1/V_mK_i$ (Dixon plot), a K_i for inhibitor **4** of 95 μM was calculated. This inhibition was specific for tyrosine kinase since concentrations of inhibitor **4** as high as 1.0 mM were without effect on the activity of cAMP-dependent protein kinase. Also, concentrations of inhibitor **4** up to 420 μM had no inhibitory effect on the activity of protein kinase C.

Antibodies were raised against two peptide sequences from the human insulin receptor β-subunit. The antibody to peptide 1315–1330 immunoprecipitated both the phosphorylated and nonphosphorylated forms of the insulin receptor. The antibody raised to peptide 1142–1159 has properties similar to those

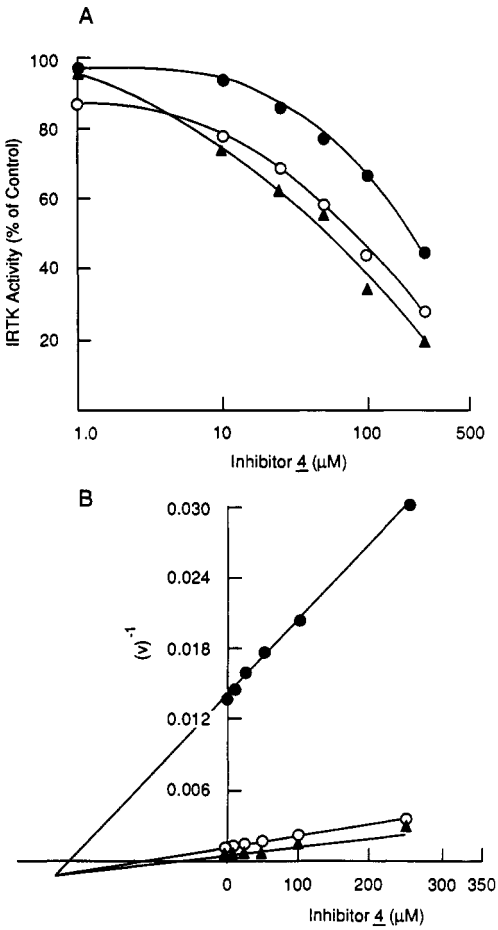

FIGURE 3: (A) Inhibition of insulin-stimulated IRTK activity by inhibitor **4** with the concentration of AII substrate held constant at 2.5 mM and the concentration of metal–ATP at 5 (●), 50 (○), and 500 μM (▲). The data are presented as percent of control (activity in the absence of compound **4** at each ATP concentration). (B) Dixon (1953) plot of data derived from (A).

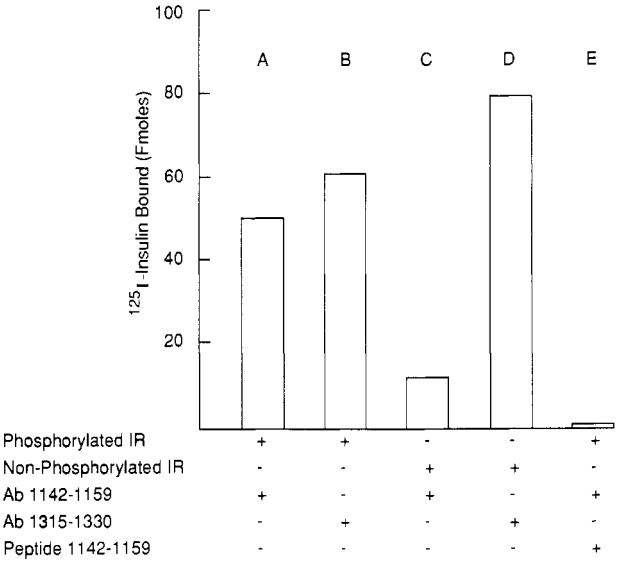

FIGURE 4: Immunoprecipitation of insulin receptor. Human placental insulin receptor (70 fmol of insulin binding activity) was phosphorylated with unlabeled ATP as described previously (Begum et al., 1985) (A, B, and E). Control (nonphosphorylated) reaction tubes contained 90 fmol of insulin receptor (C and D). Ab 1142–1159 (A, C, and E) and Ab 1315–1330 (B and D) were added at final dilutions of 1:100. Peptide 1142–1159 was at 50:1. After a 16-h incubation at 4 °C, ^{125}I-insulin binding to the precipitated receptor was performed as previously described (Herrera & Rosen, 1986).

of the P2 antibody described by Herrera and Rosen (1986) in that it only immunoprecipitates the receptor after prior incubation with insulin and ATP. Thus, this antibody is specific for the phosphorylated form of the insulin receptor β-subunit. Ab 1142–1159 immunoprecipitates 82% of the in vitro phosphorylated receptor that Ab 1315–1330 immunoprecipitates (Figure 4, panels A and B). Conversely, for the in vitro nonphosphorylated receptor, Ab 1142–1159 immunoprecipitated only 15% of the receptor that Ab 1315–1330 immunoprecipitated (Figure 4, panels C and D). Again, these data indicate that Ab 1142–1159 can selectively immunoprecipitate the phosphorylated insulin receptor. Finally, incubation of the phosphorylated insulin receptor with excess peptide 1142–1159 completely inhibited immunoprecipitation by Ab 1142–1159 (Figure 4, panel E), demonstrating specificity of the antibody.

(hIR)CHO cells, which have been stably transfected with human placental insulin receptor, were preincubated for 1 h at 37 °C in the presence of increasing (0–500 μM) concentrations of prodrug **11**. After a 10-min incubation with insulin (100 nM), the cells were solubilized, and the insulin receptor was purified by wheat germ affinity chromatography. The ability of the isolated receptor to phosphorylate AII substrate in the absence of added insulin was inhibited in a concentration-dependent manner (Figure 5A) by the treatment of the cells with the prodrug. The state of phosphorylation of these same receptors was then determined with Ab 1142–1159 (Figure 5B). A concentration-dependent decrease in β-subunit autophosphorylation in intact (hIR)CHO cells was found. Thus, the decrease in AII phosphorylation was likely due to a decrease in β-subunit autophosphorylation since inhibitor **4** was probably removed during receptor preparation. A time-dependent inhibition of (hIR)CHO cell insulin receptor autophosphorylation was found in the presence of two concentrations of prodrug **11** (Figure 5C). These data demonstrate the ability of this prodrug to enter the cell with subsequent cleavage, in a time-dependent manner, to yield the active parent inhibitor **4**. The amount of phosphorylated receptor, as measured with Ab 1142–1159, remained constant over time in both basal (no insulin) and insulin-stimulated cells in the absence of inhibitor (data not shown). Similarly, the total receptor concentration over the course of these experiments remained constant, as determined with Ab 1315–1330, thus eliminating the possibility that the observed IRTK inhibition was caused by a reduction in receptor concentration.

Figure 6A shows the effect of 1-h pretreatment with prodrug **11** (0–500 μM) on the subsequent insulin-stimulated (10^{-7} M) uptake of 2-deoxyglucose uptake in (hIR)CHO cells. Half-maximal inhibition of 2-deoxyglucose uptake occurred at about 10 μM, whereas inhibitor **4**, at a concentration of 1000 μM, had little effect. (Insulin-stimulated 2-deoxyglucose uptake was still at ~80% of its control value in the presence of 1000 μM inhibitor **4**.)

The ability of prodrugs **8–11** to inhibit glucose oxidation in isolated rat adipocytes was examined. Incubation of rat adipocytes with prodrug **11** and insulin for 90 min inhibited glucose oxidation (relative to no prodrug) in a concentration-dependent manner (Figure 6B), exhibiting an IC_{50} of about 10 μM. In contrast, the parent inhibitor **4** had no effect on insulin-stimulated glucose oxidation at concentrations up to 2000 μM. Prodrugs **8–10** were considerably less potent than prodrug **11** in inhibiting insulin-stimulated glucose oxidation under these conditions, all exhibiting IC_{50}'s greater than 100 μM.

The data shown in Table I indicate that prodrug **11** does not affect insulin-stimulated [^{3}H]thymidine uptake into

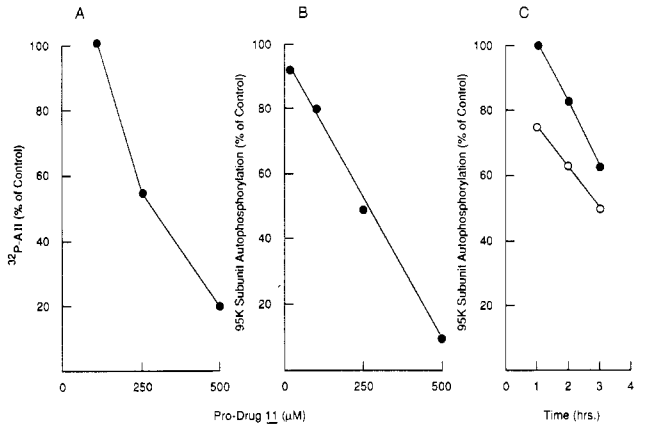

FIGURE 5: (A) Phosphorylation of AII by soluble (hIR)CHO cell membranes. Cells (~10^6/plate) were preincubated with increasing (0–500 μM) prodrug **11** for 1 h at 37 °C. Insulin (100 nM) was then added and the incubation continued for 10 min at 37 °C. The cells were then scraped and solubilized for 2 h at 4 °C in a buffer containing (at final concentrations) 50 mM Hepes, pH 7.5, 1% Triton X-100, and 500 μM sodium orthovanadate. After centrifugation (100000*g*, 15 min, 4 °C), the soluble receptor (0.50 mL) was mixed for 30 min at 4 °C with 200 μL of settled wheat germ–agarose. Following centrifugation (1 min, 4 °C), the agarose was washed twice with 50 mM Hepes, pH 7.5, containing 150 mM NaCl, 0.10% Triton X-100, and 500 μM sodium orthovanadate. The insulin receptor was eluted by mixing (30 min, 4 °C) the wheat germ–agarose with buffer containing 0.30 M *N*-acetyl-D-glucosamine. The supernatant was then used to measure IRTK-catalyzed phosphorylation of peptide (AII) substrate as described under Materials and Methods except that insulin addition was omitted. (B) Phosphorylation state of the 95-kDa insulin receptor β-subunit in (hIR)CHO cells. Wheat germ purified insulin receptor from (hIR)CHO cells used in (A) was assayed for phosphorylated insulin receptor as described under Materials and Methods. (C) Time-dependent inhibition of β-subunit autophosphorylation. CHO cells were preincubated with prodrug **11** at 20 μM (●) and at 100 μM (○) for 1, 2, or 3 h at 37 °C. Insulin (10^{-7} M) was then added and the incubation continued for 10 min at 37 °C. The cells were then scraped and solubilized for 2 h at 4 °C in a buffer containing 1% Triton X-100, 50 mM Hepes, pH 7.4, and 500 μM sodium orthovandate. After centrifugation (100000*g*, 15 min, 4 °C), the soluble receptor was diluted to 0.50% Triton X-100. Precipitation with Ab 1142–1159 and ^{125}I-insulin binding were performed as described in the legend to Figure 4.

Table I: Effect of Inhibitor **4** and Prodrug **11** on Insulin-Stimulated [^{3}H]Thymidine Uptake into (hIR)CHO Cells[a]

compd (μM)	[^{3}H]thymidine uptake (cpm ± SD)	% of control
inhibitor **4**		
0.10	1043 ± 73	101
1.00	1041 ± 167	101
10	1112 ± 235	107
100	1117 ± 27	108
prodrug **11**		
0.10	1130 ± 151	109
1.00	1003 ± 145	97
10	1210 ± 100	117
100	1024 ± 131	99

[a] Near-confluent cells were incubated for 24 h in complete media except that fetal calf serum was replaced with BSA (0.5%). The cells were then preincubated with the indicated concentrations of inhibitor **4** or prodrug **11** for 1 h at 37 °C. Insulin (10^{-7} M) was added and the incubation continued overnight at 37 °C. [^{3}H]Thymidine (0.50 μCi/well) was added, and the cells were incubated for 2 h at 37 °C. [^{3}H]-Thymidine uptake was determined as described by Gherzi et al. (1987). Data are expressed as the mean ± SD. Insulin stimulated activity 3.85-fold over basal levels (1036 ± 193 vs 269 ± 28).

(hIR)CHO cells, suggesting that the mitogenic effect of insulin is not modulated by the tyrosine kinase activity associated with the insulin receptor. This result agrees with the conclusions of Debant et al. (1988) in which CHO cells mutated at tyrosine residues 1162 and 1163 lacked an insulin-stimulated effect on

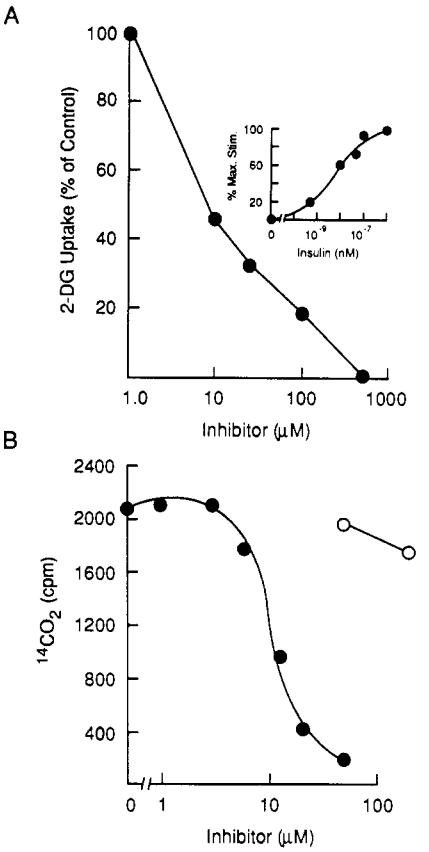

FIGURE 6: (A) Effect of prodrug **11** on insulin-stimulated [^{3}H]-2-deoxyglucose uptake into (hIR)CHO cells. Cells were preincubated with prodrug **11** (●) for 1 h at 37 °C; then, [^{3}H]-2-deoxyglucose uptake was measured as previously described (Cascieri et al., 1986). Data are expressed as the percent of stimulation at 10^{-7} M insulin. (Inset) Stimulation of [^{3}H]-2-deoxyglucose uptake as a function of insulin concentration. (B) Concentration-dependent inhibition of insulin-stimulated glucose oxidation. Isolated rat adipocytes (5 × 10^4 cells/mL) were incubated with insulin (1.7 × 10^{-10} M) and increasing concentrations of prodrug **11** (●) or inhibitor **4** (○) for 90 min at 23 °C. $^{14}CO_2$ production was measured as described by Gliemann (1965).

glycogen synthesis and glucose transport, yet retained an unaltered insulin-stimulated response for DNA synthesis. The data in Table I also indicate that since [^{3}H]thymidine uptake is not altered by concentrations of prodrug **11** as high as 100 μM, there does not appear to be toxic effects by prodrug **11** on these cells.

The intracellular esterase-catalyzed cleavage of prodrug **11** yields formaldehyde and parent inhibitor **4** in a molar ratio of 3:1. To examine possible effects of formaldehyde on the tyrosine kinase activity, we assayed the kinase activity of wheat germ purified insulin receptor toward a synthetic peptide (AII) in the presence of formaldehyde at concentrations as high as 500 μM. We found no effect on insulin-stimulated IRTK activity (data not shown). In addition, we found no effect of formaldehyde (up to 100 μM) on insulin-stimulated glucose oxidation in isolated rat adipocytes (data not shown).

Discussion

We have described the synthesis and biological effects of a specific tyrosine kinase inhibitor and have shown that it inhibits insulin stimulation of glucose uptake and oxidation. At the time our investigations were initiated, there were no suitable IRTK inhibitors known to be noncompetitive with ATP and selective for tyrosine kinases. It was, therefore, necessary to design an inhibitor for the IRTK beginning only with the knowledge that the substrates are proteins or peptides

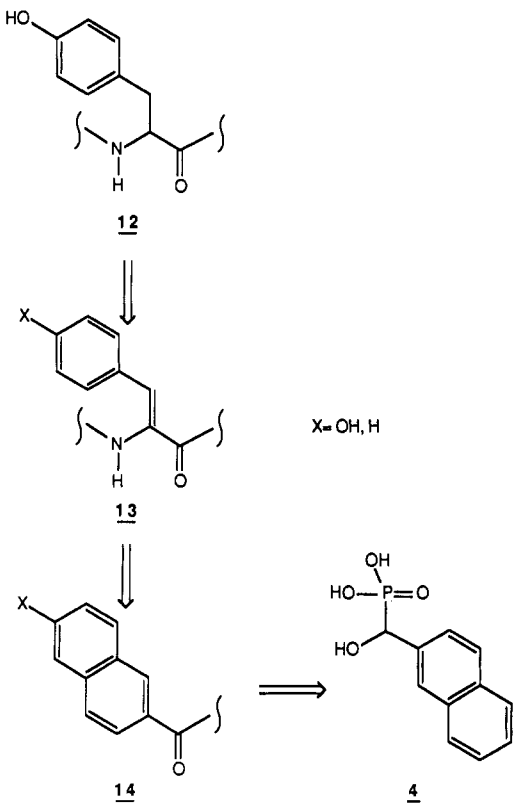

FIGURE 7: Conceptual genesis of inhibitor.

(the autophosphorylation sequence had not yet been identified) and with current proposals for the enzymatic mechanism of kinases (Knowles, 1980; Bramson et al., 1984). It was assumed that the phosphate transfer from ATP to the tyrosyl hydroxyl follows a symmetrical, in-line associative process. It was further assumed that a general base is involved in removing the hydroxyl proton (possibly as Asp or Glu side chain) and that a magnesium atom and two cationic enzyme side chains (possibly Arg and/or Lys) interact with the oxygens of the transferring phosphate.

An α-hydroxymethyl phosphonate moiety was conceived as a functional group arrangement that could provide a hydroxyl group for interaction with the general base and phosphonate oxygens for interaction with the magnesium atom and enzyme cationic side chains. The α-hydroxymethyl phosphonate unit was then incorporated into various peptides as a replacement for the tyrosine hydroxyl group. Although this approach led to a number of inhibitors of the IRTK (results to be reported elsewhere), they were not capable of penetrating cells.

Wong and Goldberg (1984) reported that substitution of dehydro-Phe for Tyr in the substrate sequence of [Val^5]angiotensin II resulted in an inhibitor of the oncogene-derived tyrosine kinase pp60$^{v\text{-}src}$. On the basis of the possibility that the preferred conformation for substrates and inhibitors might be similar for the pp60$^{v\text{-}src}$ tyrosine kinase and IRTK, we utilized this information to design a nonpeptidal framework onto which we could append the α-hydroxy phosphonate unit. Initially, it was desirable to investigate very simple nonpeptidal analogues since they offered the highest probability of cell membrane permeability.

The conceptual process used in arriving at a naphthalene substitution for the peptide region of the inhibitors is outlined in Figure 7. It was thought that dehydro-Phe might function as a conformationally restricted, nonphosphorylatable Tyr analogue in the pp60$^{v\text{-}src}$ inhibitor, i.e., **12** to **13** (X = OH) to **13** (X = H). The planar structure of dehydro-Phe (Ajo et al., 1981) is geometrically similar to that of a naphthalene. When the naphthalene structure is overlaid onto dehydro-Phe, the 2-position of the naphthalene becomes analogous to the para position of dehydro-Phe, i.e., **13** to **14**. Appending the α-hydroxymethyl phosphonate to the 2-position of the naphthalene and further simplification of the peptide replacement by deleting the carboxy extension gave inhibitor **4**.

Inhibitor **4** was found to inhibit insulin-stimulated IRTK trans- (IC_{50} = 100 μM) and autophosphorylation (IC_{50} = 200 μM) activity in a concentration-dependent manner (Figure 2) and was specific for tyrosine, rather than serine, kinases. This specificity may be due to the resemblance of the naphthalene region of the inhibitor to the substrate tyrosine, a similarity not shared by serine-containing substrates. Unlike the inhibition of IRTK by amiloride (Davis & Czech, 1985), inhibitor **4** was noncompetitive with respect to ATP and, in fact, seems to bind cooperatively (Figure 3). This finding is interesting because the phosphonate group might have been expected to occupy a position within the active site similar to that of the γ-phosphate of ATP, resulting in competitive binding vs ATP. One possible explanation is that the active site can accommodate four phosphate (or equivalent) groups. The inhibition of adenylate kinase by Ap_5A (Lienhard & Secemski, 1973) and the presence of open and closed active site conformations in enzymes like hexokinase (Anderson et al., 1979) are precedent for the ability of the active site to accommodate this extra phosphate. Analogous to these related enzymes, the IRTK substrates may form a binary Michaelis complex in which the substrate tyrosine side chain and the ATP γ-phosphate are initially separated, thereby allowing the insertion of the additional phosphate equivalent present in inhibitor **4**.

Unfortunately, inhibitor **4** does not penetrate cells, as judged by its inability to significantly block 2-deoxyglucose uptake (up to 1 mM **4**, Figure 6A) in (hIR)CHO cells or glucose oxidation (Figure 6B) in isolated rat adipocytes. In order to overcome this remaining deficiency, we extended a prodrug strategy described by Srivastva and Farquhar (1984) to our phosphonates. This strategy involves eliminating the anionic charges on the phosphonate group in inhibitor **4** by esterification with (acyloxy)alkyl groups. The resulting prodrug ester can then be absorbed through the cell membrane via passive absorption and subsequently release the parent inhibitor **4** by intracellular esterase-catalyzed cleavage of the acyloxy group followed by the spontaneous loss of an aldehyde. In the case of inhibitor **4**, we chose to explore two (acyloxy)methyl groups offering different physical properties, stability to buffer, and rate of release by esterases. The prodrugs **8**–**11** (Figure 1) were prepared to test this strategy for obtaining cell penetration.

Of the four prodrugs investigated, prodrug **11** was found to be the most active in blocking glucose oxidation and was therefore chosen for further studies. Our data (Figure 5B) demonstrate that prodrug **11** inhibited insulin-stimulated autophosphorylation of the 95-kDa insulin receptor β-subunit in intact (hIR)CHO cells. This reduced level of autophosphorylation resulted in the isolation of a less active (absence of added insulin) IRTK from these cells (Figure 5A). The data also indicate that prodrug **11** is able to enter the cell and inhibit insulin-stimulated β-subunit autophosphorylation in a time-dependent manner, suggesting the gradual release and/or uptake of the parent inhibitor **4** (Figure 5C). The insulin-stimulated uptake of 2-deoxyglucose into (hIR)CHO cells and the oxidation of glucose to CO_2 in isolated rat adipocytes was inhibited by prodrug **11** in a concentration-de-

pendent manner (Figure 6), with half-maximal inhibition occurring at about 10 μM.

Interestingly, higher concentrations of prodrug **11** were required to inhibit β-subunit autophosphorylation (IC_{50} = 250 μM; Figure 5B) than 2-deoxyglucose uptake (IC_{50} = 10 μM; Figure 6A) in (hIR)CHO cells after a 1-h incubation with the prodrug. This difference may indicate that a 50% stimulation of glucose uptake occurs when the autophosphorylation level is significantly below 50% or may be a consequence of running the two studies on different cell preparations. This observation will require additional studies, under carefully controlled conditions, in order to gain a quantitative correlation of the results.

Prior to initiating these studies, we determined that inhibitor **4** and its prodrug derivative **11** were without effect on insulin binding. Thus, the observed inhibition of IRTK activity and insulin action occurred at a step beyond that of the initial interaction of insulin with its cell surface receptor.

The prodrugs generate 1 equiv of formaldehyde for each (acyloxy)methyl group that is hydrolyzed. However, formaldehyde was shown not to be the cause of the observed prodrug activity because (a) no inhibition of in vitro IRTK activity was observed at high concentrations of formaldehyde and (b) no significant effect by formaldehyde was observed on glucose oxidation in isolated rat adipocytes.

During the course of our studies, the natural product erbstatin was reported to be a tyrosine kinase inhibitor (Umezawa et al., 1986). Erbstatin was shown to inhibit the epidermal growth factor receptor tyrosine kinase (EGFTK) and the growth of human epidermoid carcinoma (A431) cells and IMC carcinoma cells. We have synthesized this compound (Hangauer, 1986) and found that it is somewhat less potent than inhibitor **4** against the IRTK transphosphorylation activity (IC_{50} = 150 μM) under the assay conditions described under Materials and Methods.[2] On the other hand, inhibitor **4** appears to be a weaker inhibitor of the EGFTK transphosphorylation activity (IC_{50} = 250 μM vs 2.0 mM [Val^5]-AII) than the IRTK transphosphorylation activity (IC_{50} = 100 μM vs 2.0 mM AII). Recently, Yaish et al. (1988) reported erbstatin analogues that exhibit strong selectivity for inhibition of the EGFTK vs the IRTK. The results reported herein, taken together with those reported by Yaish et al. (1988), support the notion that selective inhibitors of individual tyrosine kinases can be obtained.

Like erbstatin, inhibitor **4** possesses antiproliferative activity against a variety of tumor cell lines when administered as a prodrug.[3] Of the four prodrugs tested (**8**–**11**), the most potent antiproliferative activity was obtained with **11** whereas the parent drug **4** was ineffective (correlating with the results reported herein against the IRTK in intact cells), presumably due to its inability to penetrate cells. In addition, the nonhydrolyzable ester analogue **3** (Figure 1) was inactive as an antiproliferative agent.[3] Although inhibitor **4** is highly selective for tyrosine kinases vs serine kinases, its selectivity among the tyrosine kinases appears to be modest. Therefore, inhibitor **4** and its prodrugs may be effective tools for studying the role of tyrosine kinases in other cell activities. The ability of **4** to inhibit the EGFTK and perhaps other tyrosine kinases involved in tumor cell proliferation is presumably the reason for its antiproliferative activity against a broad spectrum of tumor cell lines.

In conclusion, our data show that inhibitor **4** is an inhibitor of the IRTK trans- and autophosphorylation activity and indicates that prodrug **11** can penetrate cells, subsequently releasing the parent inhibitor **4**. The development of prodrug **11**, along with the selective antibodies to the phosphorylated and nonphosphorylated insulin receptor β-subunit, enabled us to provide further evidence supporting the necessity of IRTK activity for insulin stimulation of glucose uptake and oxidation.

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Supplementary Material Available

Details for the synthesis and characterization of inhibitors and prodrugs along with their antiproliferative activities against various tumor cell lines (4 pages). Ordering information is given on any current masthead page.

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[2] The experimental procedure for assays against the EGFTK is essentially identical with that used for IRTK except that the concentration of EGF in the assay was 1.0 μM.

[3] Developmental Therapeutic Program, National Cancer Institute, Bethesda, MD (unpublished results). The antiproliferative activity against various tumor cell lines is included in the supplementary material.

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