Probing the Catalytic Mechanism of the Insulin Receptor Kinase with a Tetrafluorotyrosine-containing Peptide Substrate*

Received for publication, April 25, 2000, and in revised form, June 22, 2000
Published, JBC Papers in Press, June 26, 2000, DOI 10.1074/jbc.M003524200

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The interaction of a synthetic tetrafluorotyrosyl peptide substrate with the activated tyrosine kinase domain of the insulin receptor was studied by steady-state kinetics and x-ray crystallography. The pH-rate profiles indicate that the neutral phenol, rather than the chemically more reactive phenoxy ion, is required for enzyme-catalyzed phosphorylation. The pKα of the tetrafluorotyrosyl hydroxyl is elevated 2 pH units on the enzyme compared with solution, whereas the phenoxide anion species behaves as a weak competitive inhibitor of the tyrosine kinase. A structure of the binary enzyme-substrate complex shows the tetrafluorotyrosyl OEt group at hydrogen bonding distances from the side chains of Asp1132 and Arg1136, consistent with elevation of the pKα. These findings strongly support a reaction mechanism favoring a dissociative transition state.

Protein kinases are enzymes that catalyze transfer of the γ-phosphoryl group from ATP to the alcoholic side chains of amino acid residues on their substrate proteins and peptides. They play important roles in signaling and have been subject to intensive cell biologic, enzymologic, structural, and kinetic scrutiny. Elucidation of the chemical reaction mechanisms of these enzymes is proving to be important in defining the details of their regulation and for designing inhibitors.

The reaction mechanism of phosphoryl transfer reactions can favor either an associative or dissociative pathway (Fig. 1). In the extremes, these are distinguished by dependence on versus independence from nucleophilicity of the phosphoryl acceptor, respectively, distances between the γ-phosphorus atom and the attacking and leaving group oxygens, and other factors (1–7). The associative pathway would benefit from proton abstraction prior to formation of the transition state so that a more reactive oxygen anion is formed, whereas that proton is retained in the dissociative transition state. Earlier kinetic and crystallographic studies of protein kinases, especially the cAMP-dependent protein kinase, demonstrated the importance of a conserved aspartyl residue that is essential for the enzyme-catalyzed reaction (8–10). The location of this Asp116 side chain in the cAMP-dependent protein kinase crystal structure was close enough to the serine hydroxyl that it could create a more reactive nucleophile by removal of the seryl or threonyl proton, in accordance with an associative mechanism (8, 9), but other kinetic evidence was interpreted as favoring a dissociative pathway (5, 11, 12, 14). Never the less, similar positioning of the conserved aspartyl side chain in other protein kinase structures has been offered as further supporting evidence for proton abstraction, thus generating a more reactive entering oxygen as a basis for catalysis (e.g. see Ref. 15). On the other hand, as discussed by Mildvan (2), distances as short as 2.7 Å between the entering oxygen and the γ-phosphorus could be estimated from modeling studies (16) and as long as 5.3 Å from NMR (17); the latter indicates a reaction pathway with a more dissociative character. Furthermore, the mechanism of phosphoryl transfer in chemical reactions of phosphate monoesters, as models for enzyme-catalyzed reactions, appears to be dissociative with relatively little contribution of nucleophilicity to the transition state (18, 19). On the premise that chemical and enzymatic reactions differ in stabilization but not character of the transition state (20–26), it would seem that protein kinases should follow a more dissociative pathway.

For protein tyrosine kinases, the ability to vary nucleophilicity by aromatic ring substitution on tyrosine facilitated a chemical analysis of the phosphoryl transfer reaction catalyzed by C-terminal Src kinase (Csk).1 Analyzing the pH-independent kinetic parameters for a series of substituted tyrosine derivatives with pKα’s ranging from 5.2 to 10, it was shown that kcat and kcat/Km were relatively independent of nucleophilicity of the attacking phenolic residue, yielding a very small Bronsted nucleophile coefficient (0 < b < 0.1) (6). The pH-rate profiles also demonstrated that the chemically more reactive phenoxide anion species was enzymatically less reactive than the neutral phenol species. Further studies with Csk revealed that the reverse reaction, where the fluorotyrosyl peptide is the leaving group, gave a Bronsted leaving group coefficient (bν) of −0.3 (3). This supports protonation of the phenolic OH in both directions. Thus, evidence from the forward and reverse reactions together suggest that the character of the transition state for Csk is mostly dissociative (26). Fluorotyrosyl analogs have also been used with the insulin receptor (IR) tyrosine kinase (27, 28), but the opposite conclusion had been reached in those studies; i.e. the transition state

1 The abbreviations used are: Csk, C-terminal Src kinase; HPLC, high performance liquid chromatography; IR, insulin receptor; IRKΔ3, cytoplasmic kinase domain of the IR; IRK3P, tris-phosphorylated core kinase domain of the human insulin receptor; IRK, insulin receptor kinase; Fmoc, N-9-fluorenlymethoxycarbonyl.

* This work was supported by Grant DK50074 from the National Institutes of Health (NIH) (to R. A. K.), Grant DK52916 from NIH (to S. R. H.), by the Winston Foundation (to K. K.), and by the Burroughs Welcome Fund and NIH Grant CA74305 (to P. A. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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for phosphoryl transfer seemed to be associative. Therefore, the analysis reported here was undertaken to specifically address this discrepancy between Csk and IR, making use of the following three advances since the earlier work of Graves and co-workers (27, 29): 1) There is now a better understanding of the substrate-specificity determinants for the insulin receptor (29–32), which was a factor in design of the peptide substrates. 2) The reconstituted cytoplasmic kinase domain of the insulin receptor (IRKD) can be expressed as a highly purified protein whose autophosphorylation state can be fixed experimentally (31–34). 3) The ternary complex of the catalytic core with a tyrosyl peptide substrate and MgATP analog has been determined, which establishes a distance between O\text{P}_2 of the reactive Tyr and the γ-phosphorous atom of the adenine nucleotide (32). Our results with the autophosphorylated and maximally activated IRKD support a dissociative mechanism for this protein kinase, show an increase in \(k_{\text{diss}}\) of the enzyme-bound fluorotyrosyl group compared with aqueous solution, and demonstrate that this fluorotyrosyl side chain can form hydrogen bonds virtually identical to those between the unmodified tyrosyl residue of the substrate and the catalytic core of the kinase.

**EXPERIMENTAL PROCEDURES**

**General**—Dithiothreitol (Sigma Ultra), the disodium salt of ATP (from equine muscle, catalog number A-5394), and bovine serum albumin (radioimmunoassay grade) were purchased from Sigma; hydrogenated Triton X-100 (protein grade) was from Calbiochem; EDTA was from Fluka; Tris acetate, Tris base, Tris HCl, and electrophoresis reagents were from Roche Molecular Biochemicals; 1,3-bis[tris(hydroxymethyl)-methylamino]propane (bis-tris propane) was from Sigma; Tris acetate, Tris base, Tris HCl, and electrophoresis reagents were from Fluka; Tris acetate, Tris base, Tris HCl, and electrophoresis reagents were from Roche Molecular Biochemicals; magnesium acetate (MgAc\(_2\), enzyme grade) was from Fisher. Insect cell culture media and fetal bovine serum were from Life Technologies, Inc.

**Peptide Synthesis**—Tetrafluorotyrosine was enzymatically prepared with tyrosine phenol-lyase as described previously and converted to the corresponding Fmoc derivative (6). Fmoc-tetrafluorotyrosine was subsequently incorporated in place of tyrosine in the previously described 18-residue IRKD substrate peptide, IRS727 (35), with the amino acid sequence KKKKLPGTGDYMNMSVPGD (36) modified from the rat sequence (residues 718–735, with Gly 718 and Leu 720 changed to Thr and Ser, respectively) was prepared by Fmoc synthesis and purified by HPLC, as described previously (36). Peptides had free amino and carboxyl termini and included the consensus sequence DIExYMMX which interacts at the P-1 to P+3 subsites on the catalytic core of the kinase have been established (32); Tyr is the P0 residue. Synthetic peptides and adenine nucleotides were dissolved in 50 mM TrisAc, pH 7.0, the pH value was readjusted to 7.0 with the addition of dilute acetic acid and/or NaOH, and the concentrations were adjusted to the desired values for stock solutions, which were determined using the following extinction coefficients: ATP, \(\epsilon = 15,300 \text{ cm}^{-1} \text{ M}^{-1} \text{ at } 259\text{ nm}\); IRS727 and IRS939, \(\epsilon = 1300 \text{ cm}^{-1} \text{ M}^{-1} \text{ at } 280\text{ nm}\). These stocks were kept at –20 °C under frequent use or at –80 °C for storage. Integrity of the reagents was checked periodically by reverse phase HPLC (peptides) or by thin layer chromatography (nucleotides). The \(k_{\text{diss}}\) of the peptide group in fluoro-IRS727 was determined spectrophotometrically at 280 nm, at a peptide concentration of 0.25 mM.

**IRKD Expression and Purification**—Baculovirus-encoding amino acid residues 953–1355 of the insulin receptor's cytoplasmic kinase domain was used to express the IRKD in High FiveTM cells (Invitrogen). Enzyme purification, autophosphorylation, and storage were essentially as described previously (38). The purified protein was quantified spectrophotometrically at 280 nm (\(\epsilon = 40,200 \text{ cm}^{-1} \text{ M}^{-1}\) for the unphosphorylated IRKD, and \(\epsilon = 35,200 \text{ cm}^{-1} \text{ M}^{-1}\) for the autophosphorylated IRKD). The autophosphorylation stoichiometry for the IRKD was 5.4 mol phosphate/mol enzyme, and the enzyme was determined to be maximally activated (specific activity of 800 mol phospho-IRS939/min/molar IRKD at 1 mM ATP, 20 mM MgAc\(_2\)). The absence of an unphosphorylated or monophosphorylated activation was determined by the presence of 20% bis and 80% tris phosphorylation of the activation loop were established in the purified phosphoprotein by endoproteinase Lys-C digestion and HPLC mapping (not shown).

**Peptide Phosphorylation Assays**—Steady-state kinetic parameters were determined with the autophosphorylated IRKD as the enzyme, using an HPLC-based assay (36). Quantification of the apo and phosphopeptide were done by peak area integration with Chrome Perfect Software (Justice Innovations, Palo Alto, CA). Dead-end inhibition studies were done over 0.0–0.63 mM fluoro-IRS727, constant 0.08 mM ATP, and variable IRS939 (0.04–0.2 mM). Reactions were performed at room temperature in 50 mM bis-tris propane, 5 mM dithiothreitol, 0.05% bovine serum albumin \((w/v)\); 2–8 mM phospho-IRKD, and 20 mM MgAc\(_2\) and at the pH values indicated in the figure legends. The pH of stock solutions of peptide, ATP, dithiothreitol, and bovine serum albumin solutions were adjusted accordingly. The reactions were initiated with the addition of peptide substrate. Kinetic parameters were determined from the best global fits of the data. The pH dependence of the observed rate constant \(k_{\text{cat,obs}}\) were fit assuming a single proton dissociation.

**X-ray Crystallographic Studies**—Expression and purification of the tris-phosphorylated core kinase domain of the human insulin receptor (IRK3P, residues 978–1283) has been described previously (32). Crystals (150–200 \(\mu\)m) belong to the trigonal space group P3\(_2\)2\(_1\)2\(_1\) with unit cell dimensions \(a = 125.8\) Å, \(b = 125.8\) Å, \(c = 71.8\) Å when frozen, and 2.0 \(\AA\) and 2.0 \(\AA\), respectively. Crystals belong to the trigonal space group P3\(_2\)2\(_1\)2\(_1\) with unit cell dimensions \(a = 125.8\) Å, \(c = 71.8\) Å when frozen.

**Results and Discussion**

**Comparison of IR and IRKD Phosphorylation**

**FIG.1. Associative and dissociative reaction mechanisms for phosphoryl transfer from ATP to the phenolic hydroxyl of tyrosine.** The upper pathway depicts an associative transition state in which the entering and leaving groups are closer to the γ-phosphorous atom and where the axial bond number to the entering O\text{P}_2 could be as high as one, versus a dissociative transition state, shown in the lower pathway, in which these groups are farther apart, and the axial bond number could be as low as zero.

\[\text{R-}O\text{P}_{\text{ADP}}\text{+ O}_{\text{P}}\text{ADP} \Rightarrow \text{R-}O\text{P}_2\text{ADP} \text{+ ADP}\]

This dissociative reaction is assumed to be rate limiting, in which the entering and leaving groups are closer to the γ-phosphorous atom and where the axial bond number to the entering O\text{P}_2 could be as high as one, versus a dissociative transition state, shown in the lower pathway, in which these groups are farther apart, and the axial bond number could be as low as zero.
nitrogen stream at −160 °C. Because of only ~80% completeness of the synchrotron data, the data were merged with the rotating anode data, giving an overall R_{sym} of 8.3%, an overall I/I_{ref} of 24.4, and an overall completeness of 91% for data between 30.0 and 2.6 Å. Data were processed using DENZO and SCALPFACK (40). A molecular replacement solution was found with AMoRe (41) using Protein Data Bank entry 1H3 (32) as a search model. Rigid-body, positional, and B-factor refinement were carried out using CNS (42). Model building was performed using O (43). The final model (2464 atoms) has a crystallographic R value of 24% (free R value of 31%) with root mean square deviations of 0.009 Å for bonds and 1.5° for angles (30.0–2.6 Å).

RESULTS

The insulin receptor is not a soluble kinase; it is a hetero-meric transmembrane protein whose intracellular kinase activity is regulated by extracellular insulin binding (44). To perform these studies in a manner similar to those done with the soluble enzyme Csk, we have utilized the highly purified and activated (autophosphorylated) cytoplasmic portion of the IR. The IRKD was previously shown to be a good model for the holomeric receptor’s basal and activated states, and these features are retained by the conserved catalytic core used in crystallization (33, 45, 46). The tetrafluoro tyrosyl peptide substrate (fluoro-IRS727) was chosen for these studies, because the unmodified form (IRS727) was cocry stallized with the catalytic core of the IRKD (32). The peptide-inhibition studies were done using IRS939 as the substrate because of signal to noise considerations in the peptide phosphorylation assays.

Kinetic Analysis—Kinase assays of IRS727 using the IRKD revealed somewhat different kinetic parameters than those reported previously for the full-length IR (35). This was possibly because of differences in assay procedures and kinase or reagent purity. Here, global fitting of the data from our steady-state assays gave the following kinetic parameters at pH 7: k_{cat} = 14 ± 2 s^{-1}, and K_m = 0.18 ± 0.02 mM IRS727. Analysis of fluoro-IRS727 showed that it was apparently a poorer substrate than IRS727 at pH 7, with k_{cat} = 0.5 ± 0.1 s^{-1}, and K_m = 0.21 ± 0.05 mM. Given the observed rate reduction, and previous claims that no phosphorylation was detected with a different fluoro tyrosyl peptide (27, 28), structural identity of the phosphorylated fluoro-IRS727 product was confirmed rigorously by mass spectrometric analysis after HPLC purification. In addition, >95% conversion to phosphorylated fluoro-IRS727 was observed after prolonged incubation with activated IRKD, determined by the HPLC-based assay (not shown).

To assess further the significance of these findings, pH-rate profiles were performed with peptide substrates for IRKD. Preliminary experiments confirmed that IRKD enzymatic activity versus time appeared stable between pH values of 6 and 10. In this range, phosphorylation of IRS727 showed little change in k_{cat} (Fig. 2) or k_{cat}/K_m (not shown) with increasing pH values. In contrast, fluoro-IRS727 showed a marked 18-fold decrease in k_{cat} with increasing pH values; the highest activity was observed at pH 6.0, and a pK_a = 7.6 was calculated for the basic limb (Fig. 2). These results suggest that a group present in fluoro-IRS727 but not in IRS727 requires protonation for enzymatic processing. This group is likely to be the tetrafluoro-tyrosine side chain. The pK_a = 7.6 is higher than the pK_a = 5.7 measured spectrophotometrically for the fluoro-IRS727 in aqueous solution (Fig. 2, inset). This implies that the tetrafluoro-tyrosine’s pK_a is higher when the substrate is bound to the enzyme. At pH 7.0, protonated fluoro-IRS727 in solution is only 5% of the total, calculated from the Henderson-Hasselbach equation and the pK_a of 5.7 of the free peptide. The value k_{cat}/K_m, which reflects the protonation equilibrium of the free substrate, should be 20-fold greater than the observed value, because only the neutral phenolic is the substrate. With this correction it can be calculated that this tetrafluoro tyrosyl peptide is only 2-fold less efficient as a substrate than the tyrosyl peptide. To determine whether the phenolate form of the peptide would bind to the enzyme, we measured the inhibitory properties of fluoro-IRS727 at pH 9, where the tetrafluorophenolic group would be fully deprotonated in free solution, and the peptide would be minimally active as substrate (k_{cat} = 0.04 s^{-1}) compared with the varied peptide substrate IRS939 (k_{cat} = 12 ± 2 s^{-1}; K_m = 0.040 ± 0.002 mM). Fluoro-IRS727 is a linear competitive inhibitor against IRS939, with K_i = 0.59 ± 0.14 mM determined from a global fit of the data (Fig. 3). Therefore the phenolate form of fluoro-IRS727 binds weakly to the kinase.

Crystal Structure of the Binary Complex—To gain better insight into the interaction of the tetrafluoro tyrosyl group with active site residues in the kinase, the crystal structure of the fluoro-IRS727 complexed with the IRKD core was determined at 2.6Å resolution (Fig. 4). When compared with the ternary complex of the autophosphorylated core with the unmodified IRS727 and Mg-5'-adenyl imidodiphosphate (32), the P0 residue (the reactive tyrosine) of the substrate peptide and key residues in the active site with which it makes contact are virtually unchanged (root mean square deviation of 0.2 Å for the side-chain and main-chain atoms of residues Asp^{1132} and Arg^{1136} and the P0 tetrafluorotyrosine). Importantly, the distance between the phenolic hydroxyl and Asp^{1132} is nearly the same in the fluoro-IRS727 complex (2.8 Å) as in the ternary complex (2.7 Å), indicating that a hydrogen bond is made between these groups in both cases. In addition, the distance between the phenolic O_d and the N_d of Arg^{1136} is unperturbed, and the hydrogen bond between N_d of Arg^{1136} and O_d of Asp^{1132} is also maintained. Together these describe a triangle of hydrogen bonding interactions (Fig. 4). This structure is compatible with the significant elevation of the phenolic pK_a in the complex compared with free solution.

DISCUSSION

The transition states in phosphoryl transfer reactions catalyzed by enzymes have been the subject of lively discourse over the past several years (1–7, 18–25, 47). Mounting evidence suggests that these enzymes affect catalysis by enhancing but not fundamentally altering the non-enzymatic mechanisms.

![Fig. 2. The pH dependence of k_{cat} for fluoro-IRS727. The log(k_{cat}) was plotted versus the reaction pH values for IRS727 (squares) and for fluoro-IRS727 (circles). The inset shows the spectrophotometric titration of fluoro-IRS727 (triangles). The dotted lines are the best theoretical fit for these data, assuming a single ionizable group in both cases.](http://example.com/fig2.png)

It was not possible to precisely measure the k_{cat}/K_m dependence on pH because of inadequate signal to noise ratios and the tendency of activated IRKD to autodephosphorylate at high enzyme concentration, which would have been employed to increase net product formation and improve the signal.
Dissociative Transition State of the Insulin Receptor Kinase

Fig. 3. Competitive inhibition by fluoro-IRS727. Inhibition of IRS939 phosphorylation was determined at pH 9.0, and the data are presented as a double-reciprocal plot. Reaction conditions are given under “Experimental Procedures.” The concentrations of fluoro-IRS727 were 0 mM (solid circles), 0.32 mM (open circles), and 0.63 mM (inverted solid triangles).

Among the experimental approaches most consistently applied to chemical and enzymatic reactions has been linear free energy relationships to probe the mechanistic role of nucleophilicity (1, 18). Measurements of $\beta_{\text{nuc}} \leq 0.3$ for non-enzymatic phosphoryl transfer reactions involving phosphate monoesters suggested only a small impact of nucleophilicity in formation of the transition state and therefore favored a dissociative mechanism (1, 13, 18, 48–50). Extending this conclusion to protein kinases would suggest that proton abstraction, to generate the chemically more reactive anionic oxygen, should not be a significant feature of enzyme-catalyzed phosphoryl transfer.

This hypothesis can be tested directly using substrates for protein tyrosine kinases, which offer the unusual opportunity to alter nucleophilicity of the entering oxygen in an enzymatic phosphoryl transfer reaction by modification of the aromatic ring in the substrate (6, 25, 26). Systematic substitution of hydrogens with fluorine confers a broad range of $K_p$ effects and allows Bronsted coefficients to be determined. This approach has provided compelling evidence to support a dissociative transition state for the reaction catalyzed by Csk (6, 26) but an associative transition state for the reaction catalyzed by IR (27, 28). There were two important concerns not addressed specifically in the study on Csk that are addressed here and that allowed us to re-evaluate the basis for an apparent discrepancy between Csk and IRK catalytic mechanisms. First, because the fluorine atomic radius is only 0.2 Å greater than the hydrogen atomic radius, these substitutions should not impose steric constraints on orientation of the P0 residue in the active site. That has been confirmed by comparison of two structures, showing the virtual absence of any positional difference between the enzyme-bound P0 tetrafluortyrosine, reported here, and the enzyme-bound P0 tyrosine reported previously by Hubbard (32). Indeed, this shows that positioning of the phosphoryl-acceptor oxygen occurs even in the absence of MgATP (or analog), at least in a ground state binary complex. This structural observation (Fig. 4) allows greater confidence in the significance of kinetic studies using fluorne-substituted tyrosyl peptides with respect to the phosphoryl transfer mechanism of the natural tyrosine-containing substrates.

Second, it was presumed that the $K_p$ of the fluorotyrosyl derivatives may be altered in the enzyme-bound state (27). The $k_{\text{cat}}$ versus pH profile likely represents the $K_p$ of the phenolic group when the substrate is bound to the enzyme (in the ternary complex). It is apparently elevated 1.5–2 pH units when compared with aqueous solution (Fig. 2). The increased $K_p$ in the bound state is also supported by the x-ray structure, which shows a hydrogen bond present between Asp1132 and the tetrafluortyrosine phenol. These crystals were grown at pH 8.5, and in order for the neutral phenol to be the major species in the complex at this pH we would expect a higher $K_p$ in this complex (perhaps 2–3 units greater than for the free peptide). These crystals lack an ATP analog that could affect the $K_p$ of the phenolic group. Additionally, the $K_p$ in the solid state may be harder to assess given the presence of large quantities of polyethylene glycol and other factors that might govern crystallization. However, the physical basis for $K_p$ elevation observed in these kinetic experiments could be the electrostatic repulsion that would result from simultaneous deprotonation of the substrate-hydroxyl and the conserved catalytic aspartyl side chain, whose proximity has been established for IRK (see Ref. 32 and Fig. 4).

The elevated $K_p$ may appear surprising at first glance, because the enzymatic reaction ultimately catalyzes proton removal concomitant with phosphoryl transfer. However, we can estimate $\beta_{\text{nuc}} \approx 0.1$ by taking into account the fact that $k_{\text{cat}}/K_p$ should represent all steps prior to the first irreversible step and that $k_{\text{cat}}/K_p$ of fluoro-IRS727 (solution $K_p = 5.7$) is only 2-fold less than IRS727 (the tyrosyl group in solution will have a $K_p = 10$). This small value is in good agreement with the $\beta_{\text{nuc}}$ determined for protein tyrosine kinase Csk using a broader range of fluorotyrosyl derivatives (6, 26). Therefore, the reaction mechanism for IRK favors a dissociative pathway such that facilitation of proton removal early in the reaction coordinate is unnecessary, and apparently unfavorable, as discussed above. Our finding that the phenolate form of fluoro-IRS727 was a weak competitive inhibitor and not a substrate further supports this view. It seems clear, therefore, that a neutral phenolic group of the P0 residue is required for enzymatic processing by IRK, as with Csk, and the transition states for IRK and Csk are likely to be similar.

This and other published evidence (1–3) suggests the protein-tyrosine and serine-threonine kinases, as do other enzymes involved in phosphoryl transfer of phosphate monoesters, likely favor dissociative transition states.
Establishing the nature of the protein kinase transition states provides geometric and functional constraints for understanding the roles of individual residues in regulation of kinase activity, the factors that govern protein substrate selectivity, and the design of transition state analogs as selective inhibitors.

REFERENCES