overnight. Membranes were dropped into liquid nitrogen for 30 s, dried, and incubated at 30°C with the chromogenic substrate X-GAL [J. Breeden and K. Nasmyth, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 643 (1985)]. Color development was clearly visible after 30 min with the MLH1-PMS1 pairwise combination and a positive control, the yeast protein kinase HRR25 [M. F. Hoekstra *et al*, *Science* **253**, 1031 (1991)] in combination with the HIT1 protein; the other samples were monitored over the next 12 hours. Quantitative assays revealed 5.7 U of β-galactosidase activity for the MLH1-PMS1 combination, 252 U of activity for the positive control, and less than 1 U of activity for all other combinations.

19. The *MLH1* gene was subcloned into an *ADE2*-encoding PG-1 plasmid, which directs high-level constitutive expression in yeast [M. Shena, D. Picard, K. Yamamoto, *Methods Enzymol.* **194**, 389 (1991)]. This construct was transformed into a diploid strain containing the pairwise combination pGAD-MSH2–pBTM-PMS1.
20. For production of MBP fusion matrices, the *MLH1*, *PMS1*, and *MSH2* genes were cloned into the polylinker of the pMAL-c2 vector (New England Biolabs). Luria broth (500 ml) containing glucose (1 g) and ampicillin (50 mg) was inoculated with 5 ml of an overnight culture of cells expressing the fusion proteins. A crude extract containing MBP fusion proteins was prepared according to the manufacturer's instructions (New England Biolabs). Amylose resin was poured into a 0.7 cm by 15 cm column, and each column was washed with 8 volumes of buffer [20 mM tris HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol (DTT)]. The crude extract was then loaded onto the column, followed by washing with 10 to 12 volumes of buffer.
21. T. A. Prolla, Q. Pang, E. Alani, R. D. Kolodner, R. M. Liskay, data not shown.
22. Plasmids pPY97 and pMQ269 were used to construct homoduplex probes and heteroduplex probes containing a G-T mismatch. The pPY97 plasmid contains the wild-type bacteriophage P22 *mnt* gene [R. T. Sauer *et al.*, *J. Mol. Biol.* **168**, 699 (1983)], and pMQ269 has a T → C substitution at position 40. About 50 pmol of two 20-bp oligonucleotides complementary to the *mnt* gene, and 108 bp apart, were treated with T4 kinase (BRL) in the presence of excess ATP. In the polymerase chain reaction (PCR), one of the primers was phosphorylated with T4 kinase and the other was unmodified, depending on which template plasmid (pPY97 or pMQ269) was used. This protocol generates a pair of PCR products, each of which contains a different strand containing a 5′ terminal phosphate group. For heteroduplex construction, the double-stranded PCR products generated from pPY97 and pMQ269 were extracted with phenol, precipitated with ethanol, and treated with 5 U of lambda exonuclease (BRL) to degrade the phosphorylated strand of each PCR product. We then generated heteroduplex DNA by annealing single-stranded products from pPY97 and pMQ269 in 1 mM tris HCl (pH 7.5) and 100 mM NaCl. Homoduplex and heteroduplex DNA were labeled with T4 kinase and [γ-^{32}P]ATP. For gel retardation assays, MLH1, PMS1, and MSH2 were initially prepared as MBP fusion proteins and were eluted from amylose resin with column buffer containing 10 mM maltose. Factor Xa (New England Biolabs) was added to a final concentration of 20 μg/ml, and the mixture was incubated at 4°C for 16 hours. The fusion protein cleavage mixture was absorbed on a hydroxyapatite column, and the maltose-free proteins were eluted with 0.5 M sodium phosphate. Proteins were then passed through a second amylose column. The proteins in the flow-through fractions were found to be mostly free of the MBP domain, as determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stored at −20°C.
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30. We thank M. G. Marinus for plasmids pPY97 and pMQ269, M. Hoekstra for the HRR25 and HIT1 positive control plasmids, and M. Thayer and M. Hoekstra for critical comments on the manuscript. Supported by an NSF graduate fellowship (T.A.P.), a Merck fellowship from the Life Sciences Research Foundation (E.A.), NSF grant MCB 9314116 and NIH grants GM 45413 and GM 322741 (R.M.L.), and NIH grant HG00305/GM50006 (R.D.K.).

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A Specific Inhibitor of the Epidermal Growth Factor Receptor Tyrosine Kinase

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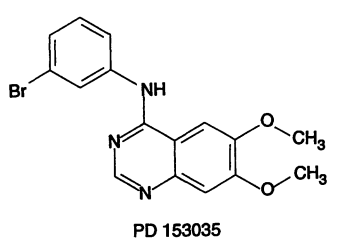
A small molecule called PD 153035 inhibited the epidermal growth factor (EGF) receptor tyrosine kinase with a 5-pM inhibition constant. The inhibitor was specific for the EGF receptor tyrosine kinase and inhibited other purified tyrosine kinases only at micromolar or higher concentrations. PD 153035 rapidly suppressed autophosphorylation of the EGF receptor at low nanomolar concentrations in fibroblasts or in human epidermoid carcinoma cells and selectively blocked EGF-mediated cellular processes including mitogenesis, early gene expression, and oncogenic transformation. PD 153035 demonstrates an increase in potency over that of other tyrosine kinase inhibitors of four to five orders of magnitude for inhibition of isolated EGF receptor tyrosine kinase and three to four orders of magnitude for inhibition of cellular phosphorylation.

The EGF receptor is a 170-kD plasma membrane glycoprotein possessing an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain that exhibits protein tyrosine kinase activity (*1*). Ligand binding to the EGF receptor results in activation of the kinase activity and leads to autophosphorylation on at least five tyrosines located in the COOH-terminal tail region (*2*). These initial events are followed by tyrosine phosphorylation of various protein substrates, leading to a myriad of signaling and cellular activities (*3*).

Overexpression of the EGF receptor or its ligands, EGF and transforming growth factor–α, can produce a neoplastic phenotype in cells (*4*) and transgenic mice (*5*). Monoclonal antibodies or antisera that block the function of the EGF receptor cause tumor regression in mice bearing human A-431 epidermoid, SW948 colorectal, or nasopharyngeal carcinomas (*6*). A correlation between the amount of EGF receptor expression in clinical tumor isolates and poor prognosis or short survival time has been established in patients with breast cancer (*7*), squamous cell carcinoma of the lung and oral cavity (*8*), bladder carcinoma (*9*), and esophageal cancer (*10*). For these reasons, inhibitors of the EGF receptor tyrosine kinase are potentially useful as chemotherapeutic agents for the treatment of cancer (*11*).

The molecule PD 153035 was synthesized as one of a series of compounds evaluated as tyrosine kinase inhibitors (Fig. 1). PD 153035 inhibited the EGF receptor tyrosine kinase by 50% at a concentration of 29 ± 5.1 pM (Fig. 2). Because PD 153035 inhibits the reaction at concentrations comparable to those of the enzyme itself, conventional steady-state Michaelis-Menten analysis to determine the inhibition constants (K_i) is inappropriate. The K_i value, therefore, was calculated by nonlinear regression analysis of the equations developed by Morrison (*12*) for evaluation of tight binding inhibitors and was estimated at 5.2 ± 1.2 pM (n = 4). Other less potent analogs of PD 153035, with which steady-state analysis was possible, showed competitive inhibition with respect to adenosine



PD 153035

Fig. 1. Chemical structure of PD 153035.

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triphosphate (ATP) (*13*), which suggests that PD 153035 might also be competitive with ATP. Erbstatin, genistein, and a sulfonylbenzoyl-nitrostyrene (*14*) were tested in the same assay with PD 153035 and had median inhibition concentration (IC_{50}) values of 367 ± 27, 1179 ± 254, and 546 ± 191 nM, respectively, which indicates that these commonly cited inhibitors of tyrosine kinase were between four and five orders of magnitude less potent than PD 153035.

The inhibitory potency of PD 153035 was determined against a panel of six different isolated recombinant protein tyrosine kinases (*15*). PD 153035 had little effect on the platelet-derived growth factor (PDGF) receptor, the fibroblast growth factor (FGF) receptor, the colony stimulating factor–1 (CSF-1) receptor, the insulin receptor, or on src tyrosine kinases at concentrations as high as 50 μM. The only other tyrosine kinase that was appreciably inhibited by PD 153035 was $p185^{erbB2}$, which is also a member of the EGF family of receptors and had an IC_{50} of 2.3 μM.

The potency and selectivity of PD 153035 were also apparent in its effects on viable cells. In Swiss 3T3 fibroblasts treated with various concentrations of PD 153035 and then stimulated with EGF, PDGF, or basic FGF (bFGF), PD 153035 suppressed tyrosine phosphorylation induced with EGF by 50% at a concentration of approximately 15 nM, whereas phosphorylation induced by bFGF or PDGF was affected only if concentrations were over 10 μM (Fig. 3). The EGF receptor was immunoprecipitated from A-431 human epidermoid carcinoma cells that were treated with various concentrations of PD 153035, then stimulated with EGF. Autophosphorylation of the receptor was inhibited by 50% at a PD 153035 concentration of 14 nM (Fig. 4). The inhibition of phosphorylation was rapid and occurred virtually as soon as the cells were exposed to PD 153035, whereas the tight binding nature of this compound prolonged inhibition after the compound was removed from the cells (Fig. 5).

PD 153035 is a very specific inhibitor of EGF-dependent cellular processes. EGF-dependent mitogenesis was inhibited 50% by PD 153035 at a concentration of 0.08 μM, whereas PDGF-, bFGF-, and serum-dependent mitogenesis were inhibited only if concentrations were over 5 μM (*16*). PD 153035 also abolished EGF-dependent expression of c-*jun* mRNA in Swiss 3T3 fi-

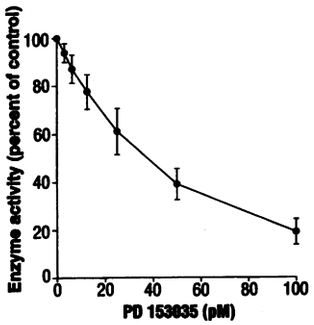

Fig. 2. Inhibition of EGF receptor tyrosine kinase by PD 153035. The EGF receptor was isolated from A-431 human epidermoid carcinoma cells by immunoaffinity chromatography (*18*). Enzyme reactions were done in a total volume of 0.1 ml containing 25 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 2 mM $MnCl_2$, 50 μM sodium vanadate, 0.5 to 1.0 ng of enzyme (which also contained enough EGF to make the final concentrations 2 μg/ml), 10 μM ATP containing 1 μCi of [^{32}P]ATP, varying concentrations of PD 153035, and 200 μM of a substrate peptide based on a portion of phospholipase C-γ1 having the sequence Lys-His-Lys-Lys-Leu-Ala-Glu-Gly-Ser-Ala-Tyr^{472}-Glu-Glu-Val. The reaction was initiated by addition of the ATP. After 10 min at room temperature, the reaction was terminated by addition of 2 ml of 75 mM phosphoric acid, and the solution was passed through a 2.5-cm phosphocellulose filter disk that bound the peptide. The filter was washed five times with 75 mM phosphoric acid and placed in a vial with 5 ml of scintillation fluid. The uninhibited control activity produced approximately 100,000 cpm. The error bars represent SEM (n = 4).

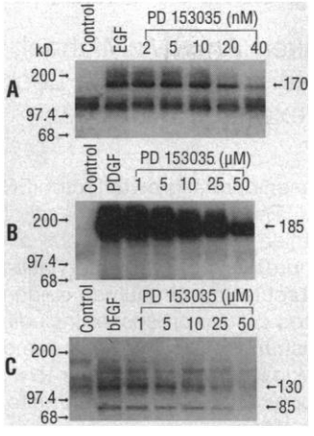

Fig. 3. Effect of PD 153035 on tyrosine phosphorylation in Swiss 3T3 fibroblasts induced by EGF (**A**), PDGF (**B**), or bFGF (**C**). Cells were grown to confluency in six-well plates and incubated in serum-free medium for 18 hours. The cells were exposed to various concentrations of PD 153035 for 2 hours and then to EGF, PDGF, or bFGF (20 ng/ml) for 5 min. Whole-cell extracts were made by scraping the cells into 0.2 ml of boiling Laemmli buffer. The extracts were transferred to a microfuge tube and heated to 100°C for 5 min. A portion (35 μl) of the whole-cell extract was loaded onto a polyacrylamide gel (4 to 20%). Proteins in the gel were electrophoretically transferred to nitrocellulose, and the membrane was washed once in 10 mM tris (pH 7.2), 150 mM NaCl, and 0.01% azide (TNA) and blocked overnight in TNA containing bovine serum albumin (5%) and ovalbumin (1%) (blocking buffer). The membrane was blotted for 2 hours with an antibody to phosphotyrosine (Upstate Biotechnology; 1 μg/ml in blocking buffer) and then washed twice in TNA, twice in TNA containing 0.05% Tween-20 and 0.05% Nonidet P-40, and finally twice in TNA. The membranes were then incubated for 2 hours in blocking buffer containing 0.1 μCi/ml of ^{125}I-labeled protein A and then washed again as above. After the blots were dry, they were loaded into a film cassette and exposed to x-ray film for 1 to 7 days.

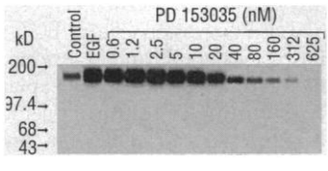

Fig. 4. Effect of PD 153035 on EGF receptor phosphorylation in A-431 human epidermoid carcinoma cells. Cells were grown to 100% confluency in 100-mm petri dishes and incubated without serum for 18 hours. The monolayers were exposed to various concentrations of the drug for 2 hours and then treated with EGF (20 ng/ml) for 5 min. The medium was removed and the monolayer was scraped into 1 ml of ice-cold lysis buffer (*19*). The lysate was transferred to a microfuge tube, placed on ice for 15 min, and centrifuged 5 min at 10,000*g*. The supernatant was transferred to a clean microfuge tube, and antibody to the EGF receptor (5 μg) was added. The tubes were rotated for 2 hours at 4°C, after which 25 μl of protein A–Sepharose was added and rotation was continued for at least 2 hours. The protein A–Sepharose was washed five times with 50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, and 0.02% sodium azide. The precipitates were resuspended with 30 μl of Laemmli buffer, heated to 100°C for 5 min, and centrifuged to obtain the supernatant. The entire supernatant was electrophoresed, transferred, and blotted for phosphotyrosine as described (Fig. 3).

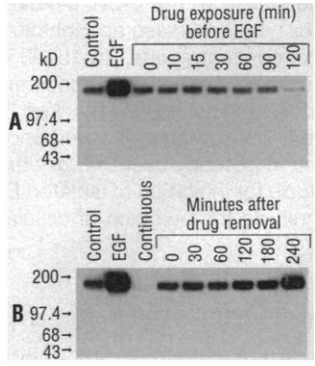

Fig. 5. Time course of the inhibition of EGF receptor autophosphorylation (**A**) or of reversal of inhibition (**B**) by PD 153035 in A-431 human epidermoid cells. Cells were grown to confluency in 100-mm plates, and growth was arrested in serum-free medium for 18 hours. In (A), the cells were exposed to PD 153035 (1 μM), and EGF was added at varying times after the inhibitor. In (B), the cells were exposed to 1 μM PD 153035 for 2 hours, after which the drug was removed by washing of the cells twice with drug-free medium. EGF was then added at various times for 5 min. Immunoprecipitations and protein immunoblots with antibodies to phosphotyrosine were done as described (Figs. 3 and 4).

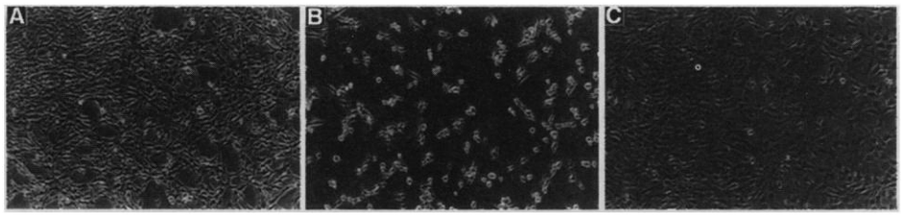

Fig. 6. Reversion, in the presence of EGF and PD 153035 (0.3 μM), of Swiss 3T3 cells that were transformed by overexpression of the human EGF receptor. (**A**) Control. (**B**) EGF (100 ng/ml). (**C**) EGF (100 ng/ml) and PD 153035 (0.3 μM).

broblasts at 0.05 μM but had no effect on early gene expression in response to the other growth factors at concentrations as high as 1 μM (*17*). EGF-dependent expression of c-*jun* mRNA in the A-431 cells was inhibited at a concentration of 0.16 μM (*17*). Finally, PD 153035 reverted the transformed morphology of fibroblasts transfected with human EGF receptor (*20*) in the presence of EGF (100 ng/ml). Exposure to 0.3 μM PD 153035 for 24 hours caused the cells to flatten and resemble the nontransformed phenotype (Fig. 6).

These studies have disclosed an inhibitor of the EGF receptor tyrosine kinase of high potency and specificity. The properties of PD 153035 demonstrate that inhibition of a purified tyrosine kinase by molecules of low molecular weight is feasible at picomolar concentrations and that enough structural diversity exists among different tyrosine kinases to allow inhibitors to discriminate among closely related enzymes.

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15. DNA coding for the soluble intracellular domains containing the active kinase of the PDGF receptor (full-length gene provided by L. T. Williams, Univ. of California, San Francisco), the CSF receptor (full-length gene provided by C. J. Sherr, St. Jude Hospital, Memphis, TN), and *erbB2* were cloned into the baculovirus transfer vector pETL. Baculoviruses containing the sequences for v-*src* and the intracellular domain of the insulin receptor were from R. Jove (Univ. of Michigan, Ann Arbor) and D. Lebwohl (Memorial Sloan-Kettering Cancer Center, New York), respectively. Enzyme assays contained 25 mM Hepes buffer (pH 7.4), 150 mM $NaCl_2$, 10 mM $MnCl_2$, 10 μM [γ-^{32}P]ATP, a random polymer of glutamic acid and tyrosine (4:1; 250 μg/ml), and 100 to 500 ng of enzyme. The incorporation of ^{32}P into the polypeptide was determined by precipitation with trichloroacetic acid. Values represent the mean of two experiments.
16. Cells were grown to confluency in 24-well plates containing a mixture of 50% Dulbecco's modified Eagle's medium (DMEM) and 50% F12 medium (Gibco), plus fetal bovine serum (10%), and then deprived of serum for 18 hours. The cells were then exposed to various concentrations of PD 153035 for 2 hours, after which different growth factors were added to each well. The cells were incubated for 24 hours at 37°C and then [^{3}H]thymidine incorporation into DNA over the course of 2 hours was determined by precipitation with trichloroacetic acid. Values represent the mean ± SEM of three separate determinations.
17. Swiss 3T3 fibroblasts or A-431 human epidermoid carcinoma cells were grown to confluency in 100-mm plates, made serum-free for 18 hours, treated for 2 hours with various concentrations of PD 153035 or of solvent control, and stimulated with EGF, PDGF, or bFGF (20 ng/ml) or with serum (10%) for 45 min. Total RNA was isolated by means of the RNAzol B test (Tel Test, Friendswood, TX) and 20 μg were electrophoresed on a 1% agarose gel. The RNA was transferred to hybond N (Amersham) and blotted with a 40-mer probe to c-*jun* (Oncogene Science) that had previously been end-labeled with [^{32}P]ATP.
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Fc Receptors Initiate the Arthus Reaction: Redefining the Inflammatory Cascade

Diana L. Sylvestre and Jeffrey V. Ravetch

Antibody-antigen complexes initiate the inflammatory response and are central to the pathogenesis of tissue injury. The classical model for this immunopathological cascade, the Arthus reaction, was reinvestigated with a murine strain deficient in Fc receptor expression. Despite normal inflammatory responses to other stimuli, the inflammatory response to immune complexes was markedly attenuated. These results suggest that immune complex–triggered inflammation is initiated by cell bound Fc receptors and is then amplified by cellular mediators and activated complement. These results redefine the inflammatory cascade and may offer other approaches for the study and treatment of immunological injury.

Immune complex deposition in such diseases as rheumatoid arthritis, systemic lupus erythematosus, and immune vasculitis is the pathogenic factor that triggers the inflammatory cascade, leading to tissue damage and the ensuing morbidity and mortality. The experimental model of immune complex–mediated pathogenesis described by Maurice Arthus in 1903 (*1*) was originally characterized by the edema, hemorrhage, and neutrophil infiltration resulting from intradermal injections of horse serum into sensitized rabbits. Because of its ease and reproducibility, the experimental variant most commonly used now is the reverse passive Arthus reaction: antibody is injected into the skin and antigen injected intravenously, resulting in the local formation of immune complexes and its sequelae (*2*). Experiments with this reaction support the currently accepted model of inflammation in which antibodies bind their antigen-forming immune complexes, which in turn bind and activate complement by means of the "classical pathway" (*2*, *3*). This initiation phase of the response generates chemotactic peptides that cause neutrophil invasion and activation, with subsequent degranulation and release of inflammatory mediators. Depletion studies have confirmed the requirements for immune com-

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