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PICEATANNOL (3,4,3',5'-TETRAHYDROXY-TRANS-STILBENE) IS A NATURALLY OCCURRING PROTEIN-TYROSINE KINASE INHIBITOR

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Summary-Piceatannol (3,4,3'5'-tetrahydroxy-*trans*-stilbene), a plant secondary natural product that had previously been identified as an antileukemic principle, has been shown to be an inhibitor of protein-tyrosine kinase activity. Piceatannol inhibits the purified thymocyte protein-tyrosine kinase, p40, by competing for the peptide or protein substrate binding site (K_i = 15 μM). Piceatannol also inhibits the activity of the $p56^{lck}$ protein-tyrosine kinase measured either in LSTRA cell membranes or in intact cells. In contrast, piceatannol does not inhibit the activity of the cAMP-dependent protein kinase.

Protein-tyrosine kinases are potentially important drug targets due to their roles as positive regulators of cell proliferation (for reviews, see 1,2). A search for naturally occurring compounds with inhibitory activity toward protein-tyrosine kinases could yield new antitumor agents as well as new biochemical reagents for the study of protein-tyrosine phosphorylation. The identification of such compounds should also yield important structural information regarding the types of compounds that can interact with tyrosine kinases and thus provide a basis for the design of further, more potent inhibitors. In a search for such inhibitors, we examined several natural products that previously had been demonstrated to have some antitumor activity in *in vitro* and/or *in vivo* screens. We report here that one such compound, piceatannol, which had previously been identified as the antileukemic principle in seeds of *Euphorbia lagascae* (3), can serve as a protein-tyrosine kinase inhibitor.

MATERIALS AND METHODS

Piceatannol (3,4,3'5'-tetrahydroxy-*trans*-stilbene) was synthesized as described previously (4). The protein-tyrosine kinase, p40, was isolated from bovine thymus (5). The activity of p40 was measured using the tyrosine-containing peptide angiotensin I as a substrate (5), except that assays contained 8% dimethylsulfoxide, which was used as a carrier for piceatannol. The catalytic subunit of cAMP-dependent protein kinase was isolated from bovine heart by Method I of Bechtel *et al.* (6). The activity of the enzyme was measured using the substrate Leu-Arg-Arg-Ala-Ser-Leu-Gly (0.5 mM) in reactions containing 25 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 100 μM [γ-^{32}P]ATP and 8% dimethylsulfoxide.

LSTRA cells were cultured as described (7). The activity of $p56^{lck}$ was measured in isolated membranes from LSTRA cells (8) using angiotensin I as a substrate. To measure the effect of agents on the phosphorylation of proteins in LSTRA cells, the cells (1 X 10^6 cells/ml, 1ml) were cultured for 2 h in the presence of the indicated concentrations of

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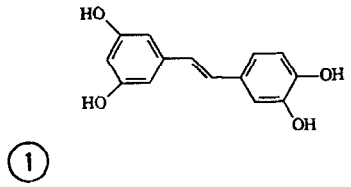
piceatannol. Cells were harvested and washed in phosphate-buffered saline containing 2 mM sodium orthovanadate. Cells were lysed in buffer containing 1.5 % Triton X-100 as described (9) except all solutions contained 2 mM sodium orthovanadate. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Phosphotyrosine-containing proteins were detected by immunoreactivity with anti-phosphotyrosine antibodies and visualized using alkaline phosphatase-conjugated second antibody. Primary antibodies were affinity purified on phosphotyrosine-agarose from serum prepared against the *abl*-gene product expressed in *E. coli*. The immune serum was generously provided by Dr. B. Gallis of Immunex Corp.

RESULTS AND DISCUSSION

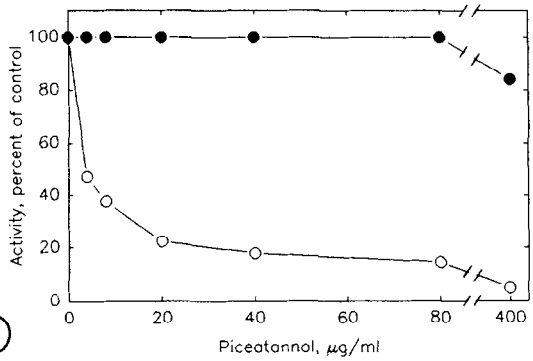
Piceatannol (Fig. 1) is a bioactive stilbene that has been isolated from several plant sources and has been prepared synthetically. A synthetic sample of piceatannol was tested for its ability to inhibit the activity of the p40 protein-tyrosine kinase isolated from bovine thymus (5). As shown in Fig. 2, the phosphorylation of angiotensin I on tyrosine was inhibited by increasing concentrations of piceatannol.

To examine the mechanism of the interaction of piceatannol with p40, the effects of varying concentrations of inhibitor on the kinetics of peptide phosphorylation were examined. As shown in Fig. 3, piceatannol was a competitive inhibitor of p40 with respect to angiotensin I (K_i = 15 μM) and noncompetitive with respect to ATP. These data indicate that piceatannol inhibits that activity of p40 by binding at the protein substrate-binding site. The mode of action of piceatannol differs from that of the naturally occurring flavanoids, many of which are also potent inhibitors of protein-tyrosine kinases (10-13). Those flavanoids that inhibit the activity of the p40 protein-tyrosine kinase (13) and other protein-tyrosine kinases (10-12) compete with ATP for binding to the nucleotide-binding site. The mode of action is similar, however, to that observed for synthetic analogs (tryphostins) (14,15) of erbstatin (16) and derivatives of 4-hydroxycinnamamide (17), which all share in common a structural similarity to tyrosine.

Since it is likely that piceatannol bears a structural resemblance to the tyrosine residue present on a protein or peptide substrate, it might reasonably be expected that piceatannol



(1)



(2)

Figure 1. Piceatannol.

Figure 2. Inhibition of the p40 protein-tyrosine kinase (○) and the catalytic subunit of cAMP-dependent protein kinase (●) by increasing concentrations of piceatannol.

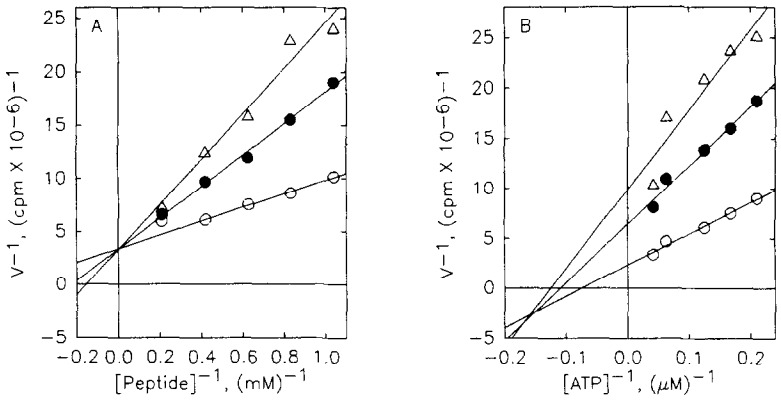


Figure 3. Kinetic analysis of the interactions of piceatannol with the p40 protein-tyrosine kinase. **A**, effect of increasing concentrations of angiotensin I on the inhibition of p40 by piceatannol (0 (○), 4 (●) or 8 (△) μg/ml). **B**, effect of increasing concentrations of [γ-^{32}P]ATP on the inhibition of p40 by piceatannol (0 (○), 4 (●) or 8 (△) μg/ml).

would show little inhibitory activity toward protein kinases with a substrate specificity for serine or threonine. To examine the specificity of this interaction, piceatannol was tested for its ability to inhibit the activity of the catalytic subunit of the cAMP-dependent protein kinase, a protein-serine kinase. As shown in Fig. 2, piceatannol had little effect on the phosphorylation of a synthetic peptide by the catalytic subunit.

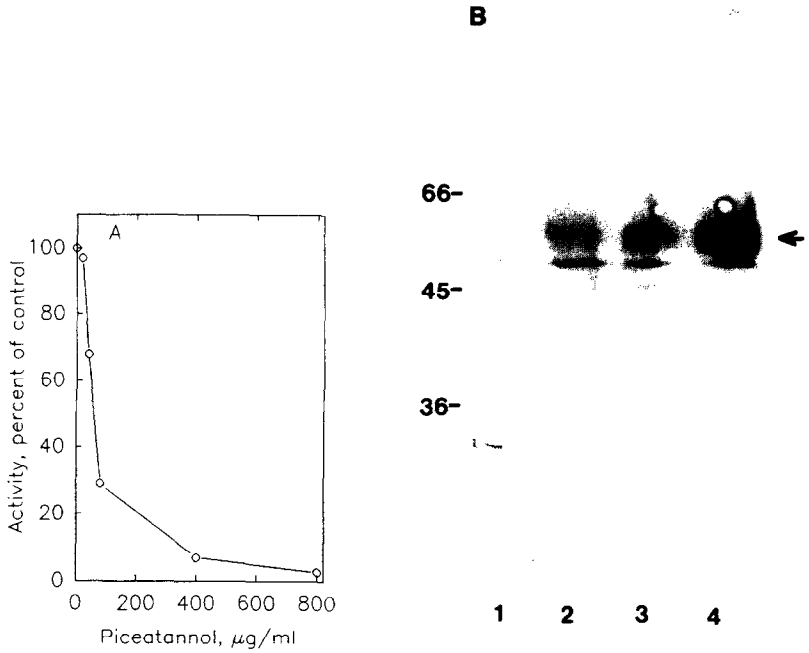


Figure 4. Inhibition of protein-tyrosine phosphorylation in LSTRA cells by piceatannol. **A**, effect of increasing concentrations of piceatannol on the phosphorylation of angiotensin I by membranes prepared from LSTRA cells. **B**, effect of piceatannol on the phosphorylation of p56^{lck} in intact LSTRA cells. LSTRA cells were cultured in the presence of increasing concentrations of piceatannol as described in MATERIALS AND METHODS. Phosphotyrosine-containing proteins were detected on Western blots using affinity-purified anti-phosphotyrosine antibodies.

An effective inhibitor of protein-tyrosine kinases would be one that could penetrate lipid bilayers to inhibit the activity of intracellular tyrosine kinase domains. Certain tyrphostins, for example, can inhibit the tyrosine kinase activity of the EGF-receptor in cultured cells (20). Since we are interested in protein-tyrosine kinases present in lymphoid cells, we tested the ability of piceatannol to inhibit the phosphorylation of proteins in LSTRA cells, which overexpress a membrane-associated protein-tyrosine kinase known as $p56^{lck}$ (18,19). These cells contain elevated levels of phosphotyrosine (21) and the major intracellular phosphotyrosine-containing protein is $p56^{lck}$ itself. We first verified that $p56^{lck}$ was also a target for piceatannol *in vitro* (Fig. 4A). LSTRA cells were then incubated with varying concentrations of piceatannol. Proteins present in a detergent-soluble fraction were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies. As shown in Fig. 4, increasing concentrations of piceatannol resulted in a decrease in the levels of phosphotyrosine found on $p56^{lck}$. These results suggest that piceatannol can inhibit the activity of certain protein-tyrosine kinases in intact cells.

The identification of piceatannol as an inhibitor provides important information regarding the types of compounds that are capable of interacting at the protein substrate-binding sites of protein-tyrosine kinases. Efforts are currently underway to design structural analogs of piceatannol for evaluation as protein-tyrosine kinase inhibitors and antitumor agents.

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