[CANCER RESEARCH 64, 236–242, January 1, 2004]

Cyclolignans as Inhibitors of the Insulin-Like Growth Factor-1 Receptor and Malignant Cell Growth

Ada Girnita,[1] Leonard Girnita,[1] Fabrizio del Prete,[3] Armando Bartolazzi,[3] Olle Larsson,[1] and Magnus Axelson[2]

[1]Department of Oncology and Pathology, Cancer Center Karolinska and [2]Department of Clinical Chemistry, Karolinska Hospital, Stockholm, Sweden, and [3]Laboratory of Immunology Regina Elena Cancer Institute and Department of Pathology, Sant Andrea University Hospital, Rome, Italy

ABSTRACT

The insulin-like growth factor-1 receptor (IGF-1R) plays a pivotal role in transformation, growth, and survival of malignant cells, and has emerged as a general and promising target for cancer treatment. However, no fully selective IGF-1R inhibitors have thus far been found. This is explained by the fact that IGF-1R is highly homologous to the insulin receptor, coinhibition of which may cause diabetic response. The receptors are both tyrosine kinases, and their ATP binding sites are identical, implying that ATP inhibitors cannot discriminate between them. Therefore, the current strategy has been to identify compounds interfering with receptor autophosphorylation at the substrate level. In this study we investigated the effects of cyclolignans and related molecules on IGF-1R activity. We report that certain cyclolignans are potent and selective inhibitors of tyrosine phosphorylation of the IGF-1R. Of particular interest was picropodophyllin (PPP), which is almost nontoxic (LD_{50} >500 mg/kg in rodents). PPP efficiently blocked IGF-1R activity, reduced pAkt and phosphorylated extracellular signal regulated kinase 1 and 2 (pErk1/2), induced apoptosis in cultured IGF-1R-positive tumor cells, and caused complete tumor regression in xenografted and allografted mice. PPP did not affect the insulin receptor or compete with ATP in an *in vitro* kinase assay, suggesting that it may inhibit IGF-1R autophosphorylation at the substrate level. This is also in agreement with our molecular model of how the cyclolignans may act on the IGF-1R kinase. Our results open the possibility to use PPP or related compounds with inhibitory effects on IGF-1R as lead compounds in development of anticancer agents.

INTRODUCTION

The insulin-like growth factor-1 receptor (IGF-1R) plays an important role in transformation and proliferation of malignant cells (1–4). The IGF-1R is also important for preventing apoptosis and maintaining the malignant phenotype of tumor cells, and is involved in tumor cell protection against antitumor therapy (1, 2, 5). In contrast, the IGF-1R is not an absolute requirement for normal cell growth (5, 6).

The IGF-1R consists of two identical extracellular α-subunits that are responsible for ligand binding, and two identical β-subunits with a transmembrane domain, an intracellular tyrosine kinase, and COOH-terminal domain (7). The ligand-receptor interaction results in phosphorylation of tyrosine residues in the tyrosine kinase domain (spanning from amino acid 973 to 1229) of the β-subunit. The primary and key sites are the clustered tyrosines at positions 1131, 1135, and 1136 in the activation loop (6, 8). Phosphorylation of these tyrosine residues is necessary for activation of the receptor kinase. After autophosphorylation, the receptor kinase phosphorylates intracellular proteins, like the insulin receptor (IR) substrate-1 and Shc, which enable activation of the phosphatidylinositol 3′-kinase and the mitogen-activated protein kinase signaling pathways, respectively (8).

On the basis of the pivotal role of IGF-1R in malignant cells, it becomes increasingly evident that IGF-1R is a promising target for anticancer therapy. A direct strategy to interfere with IGF-1R activity is to induce selective inhibition of its tyrosine kinase. However, today there are no fully selective inhibitors of IGF-1R available, *i.e.*, a coinhibition of the IR always occurs. Because the ATP binding sites of the IGF-1R and IR kinases are identical (9), ATP inhibitors cannot discriminate between them. However, compounds interfering with the receptor substrate have emerged as a possibility to develop selective inhibitors (10, 11).

We showed recently that the antiestrogen tamoxifen at high concentrations could reduce tyrosine autophosphorylation of IGF-1R in melanoma cell lines (12). In a search for a highly potent IGF-1R inhibitor, we then started investigating the properties of various tamoxifen-like compounds, like phytoestrogens that are described elsewhere (13, 14). We also studied the three-dimensional structures (folding) of short peptides having the amino acid sequence of the IGF-1R tyrosine domain, including the tyrosine residues at positions 1131, 1135, and 1136, constructed by the computer, to find compounds having the ability to mimic the tyrosine residues and thereby interfere with their phosphorylation (substrate phosphorylation). We then discovered when using a 12-amino acid peptide that the hydroxy groups of two of the three key tyrosines, 1135 and 1136, which have to be autophosphorylated in IGF-1R for activation, could be situated as close as ~0.95 nm (9.5 Å) from each other, and that the apparent angle between these tyrosines was ~60° (Fig. 1*A*). Such a short distance for the corresponding tyrosines in the almost identical tyrosine domain of the IR has not been observed. Molecular modeling showed that a molecule consisting of two benzene rings separated by only one carbon atom could mimic the suggested three-dimensional structure of the two tyrosines of IGF-1R, and thereby possibly inhibits their phosphorylation.

We then observed that certain cyclolignans have the required structural properties (Fig. 1*A*), and as will be reported here, they exhibited strong inhibitory effects on phosphorylation of the IGF-1R. Two of these were the stereoisomers podophyllotoxin (PPT) and picropodophyllin (PPP; Fig. 1*A*).

PPT has been shown previously to possess antineoplastic properties, but its general toxicity prevented further use of it as an anticancer drug (15, 16). The cytotoxicity of PPT has been linked to its interaction with β-tubulin, leading to mitotic arrest (17). Extensive structure-function studies revealed that a *trans*-configuration of the lactone-ring as in PPT is required for binding to β-tubulin (Fig. 1*B*). Consistently, its isomer PPP with a *cis*- configuration has no inhibitory effect on microtubules and apparently lacks cytotoxicity (18, 19). After those observations, PPP has received little or no attention. The current interest in PPT derivatives has instead focused on etoposide (a 4′-demethyl-epipodophyllotoxin glucoside derivative; Refs. 20, 21; Fig. 1*B*), which is a DNA topoisomerase II inhibitor. In contrast with etoposide, the cyclolignans PPT and PPP have no inhibitory effect on DNA topoisomerase II, and consequently do not cause any DNA breakage (22). This is because PPT and PPP lack the 4′-hydroxy group (Fig. 1*B*), which is an absolute structural requirement for interfering with the activity of the enzyme (19).

Received 8/13/03; revised 10/13/03; accepted 10/30/03.

Grant Support: The project was supported by grants from the Swedish Cancer Society (O. L.), the Swedish Research Council (M. A.), the Cancer Society in Stockholm (O. L.), the Swedish Children Cancer Society (O. L.) and the Karolinska Institute. F. d. P. and A. B. were supported by the Italian Association for Cancer Research.

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.

Note: A. Girnita and L. Girnita contributed equally to this work.

Requests for reprints: Olle Larsson, Department of Oncology and Pathology, CCK R8: 04, Karolinska Hospital, se-17176, Stockholm, Sweden. E-mail: olle.larsson@onkpat.ki.se or to: Magnus Axelson, Department of Clinical Chemistry, Karolinska Hospital, SE-17176 Stockholm, Sweden. E-mail: magnus.axelson@ks.se.

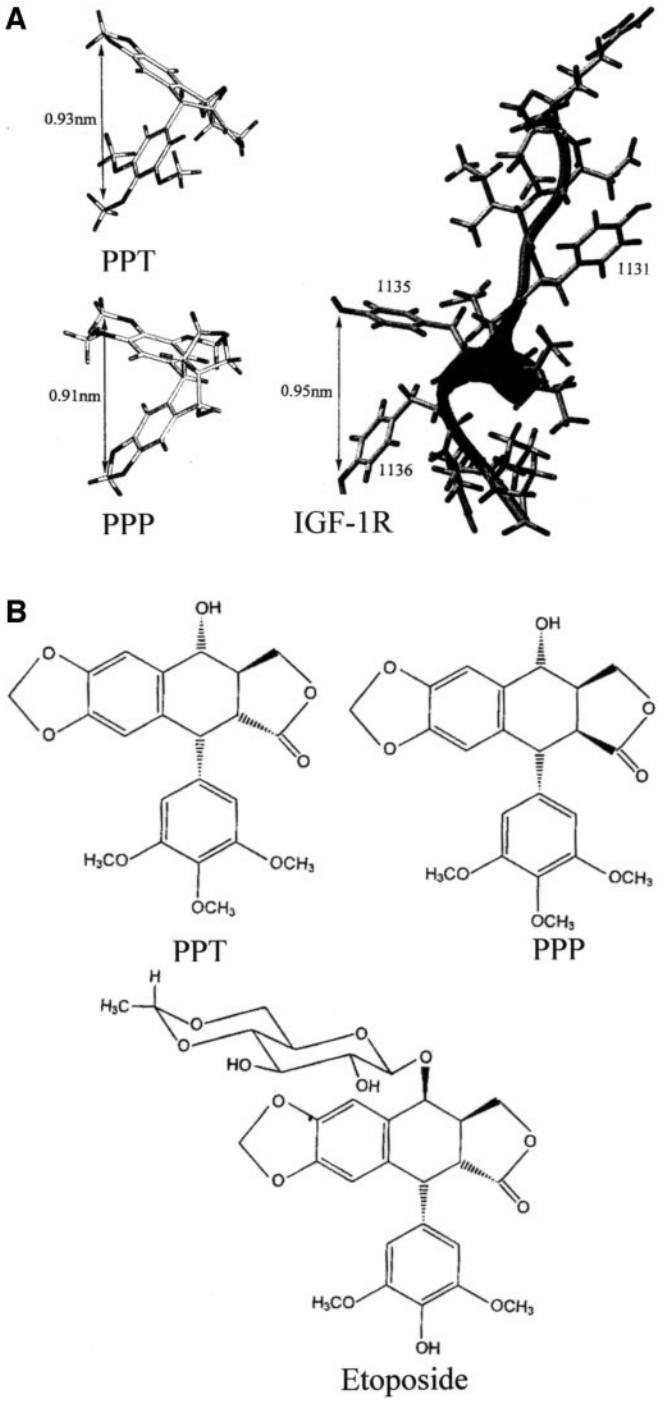

Fig. 1. *A*, the three-dimensional structures of a peptide constructed by a computer from the amino acid sequence 1127–1138 of the insulin-like growth factor (IGF) -1R (activation loop of the tyrosine kinase domain) using the Internal Coordinate Mechanics software compared with the structures of the cyclolignans podophyllotoxin (*PPT*) and picropodophyllin (*PPP*). The tyrosines 1131, 1135, and 1136 are indicated. *B*, the chemical structures of PPT, PPP, and etoposide.

In this study we demonstrate that certain cyclolignans (not etoposide) are highly potent and specific inhibitors of the IGF-1R tyrosine kinase and malignant cell growth.

MATERIALS AND METHODS

Reagents. PPT and deoxypodophyllotoxin (99.9% purity), and PPT-4,6-*O*-benzylidene-β-D-glucopyranoside (AS 3738) were kind gifts from Kurt Leander (Analytecon SA, Pre Jorat, Switzerland). PPP and DPPP (>99.7% purity) were prepared from the former two lignans, respectively, by incubation with sodium acetate in aqueous ethanol (23, 24) followed by recrystallization or high-performance liquid chromatography (no starting material was then detected in the product, *i.e.*, <0.1%). For experimental purposes, the cyclolignans were dissolved in physiological saline (5 μM), ethanol (0.5 mM), or DMSO (0.5 mM) before addition to cell cultures or cell-free solutions. It should be emphasized that PPP and DPPP are relatively nonpolar compounds and must, therefore, be properly dissolved before use. The other phytoestrogens were those used in previous studies (13, 14) or from Sigma (St. Louis, MO). A monoclonal antibody against phosphotyrosine (PY99) and polyclonal antibodies to the α-subunit of IGF-1R (N-20), β-subunit IGF-1R (H-60), epidermal growth factor receptor, platelet-derived growth factor receptor, phosphorylated Akt1 (serine473), Akt1, phosphorylated extracellular signal-regulated kinase (Erk) 1/2, and Erk 1/2 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal antibody against the α-subunit of IGF-1R (αIR-3) and protein A agarose were from Oncogene Science (Manhasset, NY). The anti-IR substrate-1 agarose conjugate antibody was obtained from UBI (Lake Placid, NY).

Cell Cultures. The SK-MEL5, SK-MEL 28, ES1, RD-ES, HEPG2, PC3, JC, and MCF 7 cell lines were from American Type Culture Collection (Rockville, MD). BE, DFB, DFW, C8161, AA, and FM 55 cells were gifts from Rolf Kiessling (Karolinska Hospital, Stockholm, Sweden). The R-, R -v-src, P6, and P12 mouse cell lines were gifts from Renato Baserga (Thomas Jefferson University, Philadelphia, PA). The R- fibroblasts are IGF-1R negative, derived from BALB/3T3 mouse embryo with a targeted disruption of the type 1 receptor for the insulin-like growth factors (25). These cells are anchorage dependent and are, therefore, unable to grow in soft agar and in animals (25). The R -v-src fibroblasts are R- cells transfected with the v-src

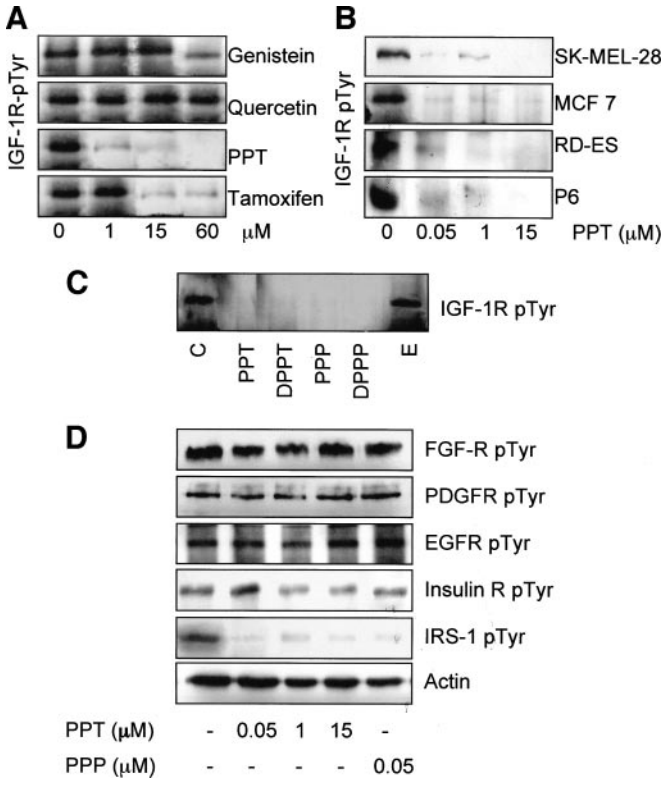

Fig. 2. Effects of podophyllotoxin (*PPT*) and congeners on basal receptor tyrosine phosphorylation (*pTyr*) in intact cells. Cells were incubated with fresh complete medium containing the indicated compounds. Phosphorylation of the indicated receptors was determined by Western blotting. *A*, effects of different concentrations of picropodophyllin (*PPP*) and indicated congeners on insulin-like growth factor (IGF) -1R phosphorylation in FM 55 cells. *B*, effects of PPT on IGF-1R phosphorylation in SK-MEL-28, MCF 7, RD-ES, and P6 cells. *C*, effects of the derivatives deoxypodophyllotoxin, PPP, and DPPP (0.5 μM) on IGF-1R phosphorylation in FM55 cells. The effects of PPT (0.5 μM) and etoposide (*E*; 15 μM) are also shown (*C* = control). *D*, effects of various concentrations of PPT and PPP on phosphorylation of the indicated growth factor receptors and insulin receptor substrate-1 in FM 55 cells. Actin is loading control. The experiments were repeated three times with similar results.

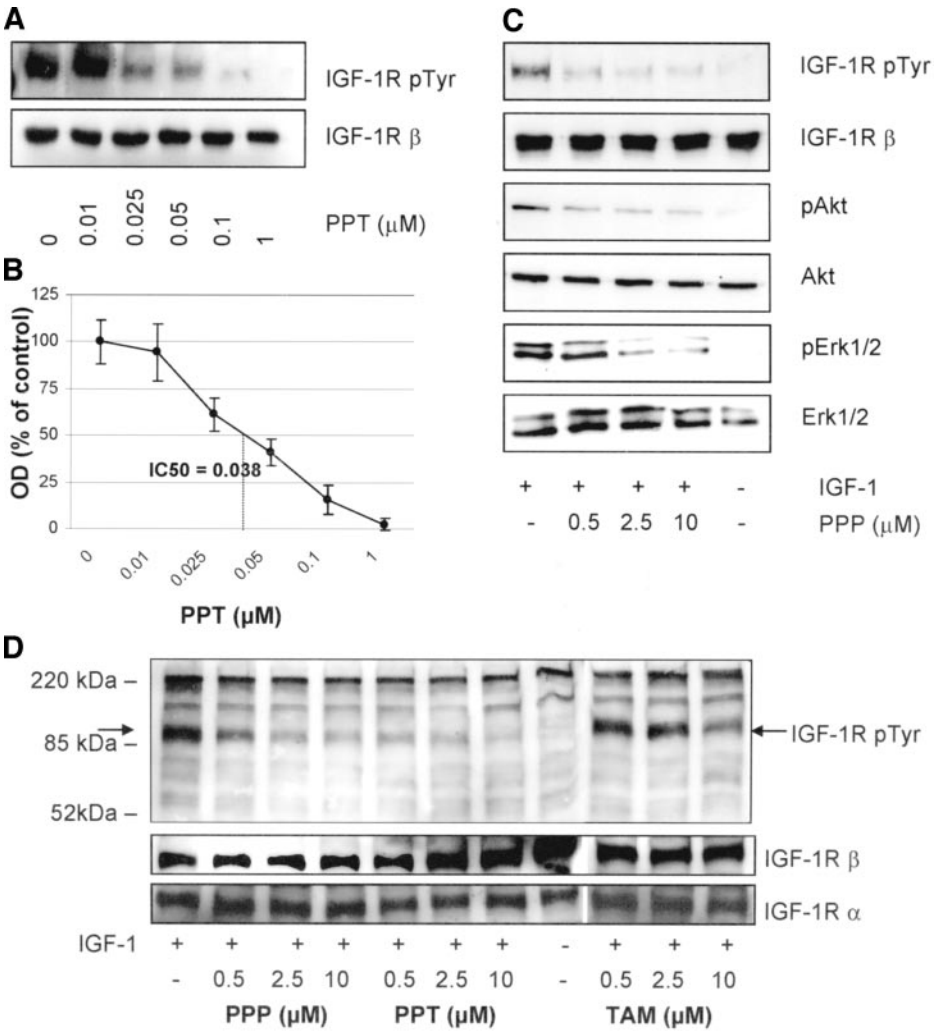

Fig. 3. Dose-response on insulin-like growth factor (IGF) -1R tyrosine phosphorylation (*IGF-1R pTyr*) in intact cells. FM-55 cells were incubated with fresh complete medium containing the indicated concentrations of PPT, after which the levels of IGF-1R phosphorylation were assayed by Western blotting (*A*), and quantified by determining absorbances (*OD*; means and SE of means) of the signals (*B*). The expression of the IGF-1R β-subunit is shown as a loading control in the *bottom panel of A. C*, P6 cells were serum-depleted for 20 h. The cells were then treated with picropodophyllin (*PPP*) at various concentrations for 1 h, and finally stimulated with IGF-1 (20 ng/ml) for 5 min. After purification of IGF-1R with immunoprecipitation, Western blotting was performed for detection of phosphorylated IGF-1R. Phosphorylation of Akt (serine 473; *pAkt*) and Erk1/2 (*pErk1/2*) was assayed directly by Western blotting of the cell lysates. As loading controls the IGF-1R β-subunit, Akt and Erk1/2 were detected. *D*, P6 cells were serum-depleted for 20 h. The cells were then treated with podophyllotoxin (*PPT*), PPP, or tamoxifen at various concentrations for 1 h, and finally stimulated with IGF-1 (100 ng/ml) for 5 min. Western blotting was then performed directly on the cell lysate (without immunoprecipitation) for detection of phosphorylated IGF-1R using the PY antibody. As loading controls both the IGF-1R α- and β-subunit were detected. The experiments were repeated three to five times with similar results.

(being the only single oncogene that can bypass the requirement for a functional IGF-I receptor in anchorage-independent growth), and have IR substrate-1 and Shc constitutively tyrosine phosphorylated (26). The P6 and P12 lines are 3T3 derivative overexpressing the human IGF-1R (25). The cells were cultured in monolayers in standard medium supplemented with 5% (P6) or 10% fetal bovine serum. P6 and R- cell lines were cultured in the presence of G-418 (Promega) and for P12 Hygromicin B.

Tyrosine Phosphorylation of Receptors in Intact Cells. Cells were cultured to subconfluency in 6-cm plates, then fresh medium containing 10% fetal bovine serum and the desired compounds were added for 1 h. The cells were then lysed and subjected to immunoprecipitation using specific receptor antibodies, essentially as described (3). For immunoprecipitation of the IGF-1R the antibodies H-60 or αIR-3 were used. Immunoprecipitates were resolved by SDS PAGE (27), transferred to nitrocellulose membranes (Hybond, Amersham, Uppsala, Sweden), and incubated with antiphosphotyrosine antibody. In a separate experiment IGF-1-induced IGF-1R phosphorylation was assessed. Cells were then serum-depleted for 20 h, treated with inhibitor for 1 h, and finally stimulated with IGF-1 (20 ng/ml) for 5 min before assay. Antibodies to actin (in cell extract) or IGF-1R β-subunit were used as loading controls. After detection the films were scanned for quantifications.

IGF-1R and IR Phosphorylation in Tumors. Samples from fresh-frozen tumors from drug- and solvent-treated mice were cut in pieces and suspended in freshly prepared homogenization buffer as described (28). After centrifugation at 14,000 × *g* for 10 min at 4°C the supernatants were immunoprecipitated for the two receptors, and determination of IGF-1R or IR tyrosine phosphorylation was completed as described above.

***In Vitro* Tyrosine Kinase Assays.** Assay of IGF-1R-catalyzed substrate phosphorylation of pTG, using a 96-well plate tyrosine kinase assay kit (Sigma), was performed essentially as described elsewhere (10, 29). We used recombinant epidermal growth factor receptor, immunoprecipitated IR from HEPG2, immunoprecipitated IGF-1R from P6 cells, and IGF-1R immunodepleted supernatant from P6 (representing "non-IGF-1R tyrosine kinases"). After 30-min treatment of the receptors with the desired compounds in the kinase buffer [50 mM HEPES buffer (pH 7.4), 20 mM $MgCl_2$, 0.1 $MnCl_2$, and 0.2 Na_3VO_4], the kinase reaction was activated by addition of ATP. The phosphorylated polymer substrate was probed with a phosphotyrosine-specific monoclonal antibody conjugated to horseradish peroxidase, clone PT-66. Color was developed with horseradish peroxidase chromogenic substrate *O*-phenylenediamine dihydrochloride and quantitated by spectrophotometry (ELISA reader).

IGF-1R tyrosine autophosphorylation was analyzed by a sandwich ELISA assay, essentially as described (10). Briefly, 96-well plates (Immunolon; Nunc) were coated overnight at 4°C with 1 μg/well of an antibody to IGF-1R β-subunit. The plates were blocked with 1% BSA in PBS Tween for 1 h, and then 80 μg/well of total protein lysate from the P6 cell line was added. As a negative control we used total protein lysate from the R- cell line. The investigated compounds were added in tyrosine kinase buffer without ATP at room temperature for 30 min before kinase activation with ATP. Kinase assay was performed using the Sigma kit (see above). After spectrophotometry the IC_{50} values of inhibitors were determined using the REGRESSION function of Statistica program.

Assay of Cell Growth and Survival *in Vitro*. We performed the determinations using the Cell proliferation kit II (Roche, Inc., Indianapolis, IN), which is based on colorimetric change of the yellow tetrazolium salt 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt in orange formazan dye by the respiratory chain of viable cells (30). All of the standards and experiments were performed in triplicates.

We used a flow cytometry apoptosis detection kit (Clontech, Palo Alto, CA)

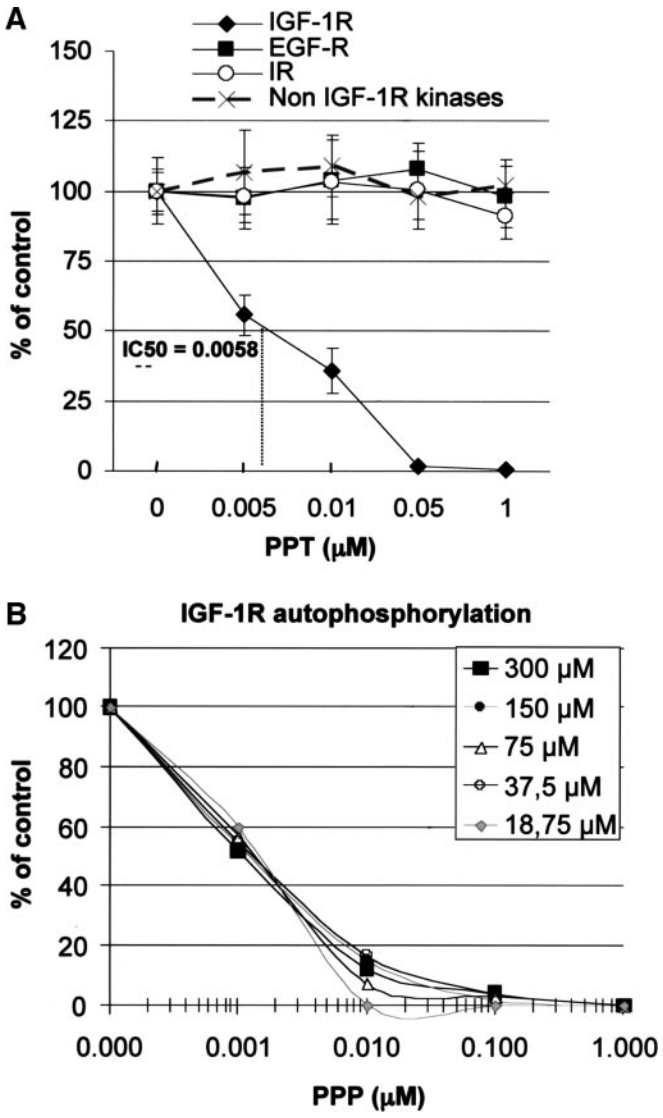

Fig. 4. Effects of cyclolignans on receptor tyrosine kinases *in vitro*. *A*, effects of the indicated concentrations of podophyllotoxin (*PPT*) on insulin-like growth factor (*IGF*) -1R-, insulin receptor (IR)- and epidermal growth factor receptor-catalyzed tyrosine phosphorylation of the poly Tyr Glu (*pTG*) substrate *in vitro*. Non-IGF-1R kinases represent IGF-1R immunodepleted activity. The reactions were quantified by densitometry, and the relative tyrosine kinase activity is shown. Mean values of three experiments are shown; *bars*, ±SE. *B*, dose-response of picropodophyllin (*PPP*) on IGF-1R autophosphorylation *in vitro* at different ATP concentrations. For all indicated values the SEs were <5%. The experiments were repeated two to five times with similar results.

to identify programmed cell death according to the manufacturer's protocol. In brief, cells cultured in six-well plates, were treated with PPP for 6 h, and then harvested and stained with 5 μl Annexin V to detect phosphatidyl serine expression on cells during early apoptotic phases and with 10 μl propidium iodide to exclude dead cells. The reading was done with a FACSCalibur (Becton-Dickinson), and data were analyzed with the Cell Quest program.

***In Vivo* Experiments.** Four to 5-week-old pathogen-free SCID mice were used and housed within plastic isolators in a sterile facility. ES-1, BE, and PC3 cells (all proved to express IGF-1R), or R- v-src (IGF-1R negative) and P12 (overexpressing IGF-1 and IGF-1R), were injected s.c. at 10^7 cells/mice in a 0.2-ml volume of sterile saline solution. Immunocompetent Balb-c mice were injected with 10^7JC murine breast cancer cells per mouse in 0.15-ml volume of sterile saline solution. Experimental treatments with PPP (20 mg/kg/12 h) were performed by daily i.p. injections of the compound in 10 μl volume of DMSO: vegetable oil, 10:1 (v/v). Control mice were treated with the vehicle only. Three animals were treated in each group. Tumor growth was measured every second day using vernier calipers, and the tumor volumes were calculated as a product of length × width × height (31). The mice were carefully observed for the presence of side effects and were sacrificed at the end of the experiments for histological analysis of the lesions. A separate experiment on PPP-treated (systemically and locally) tumor-free mice, including histological analyses of various organs, confirmed previous observations that PPP appears to be nontoxic. All of the experiments were performed according to the ethical guidelines for laboratory animal use and approved by the institutional ethical committee.

RESULTS AND DISCUSSION

Inhibition of Basal IGF-1R Phosphorylation in Intact Cells. Our initial approach to identify IGF-1R inhibitors was to search for compounds being active both on the IGF-1R phosphorylation and cell survival under basal conditions. This means that we used nontransfected malignant cell lines, with verified IGF-1R expression, growing exponentially in 10% fetal bovine serum for both analyses.

In the IGF-1R phosphorylation experiments on intact cells, growing cells were treated with desired compounds for 1 h, after which IGF-1R was precipitated with established specific antibodies. Western blotting was then performed with a phosphotyrosine antibody.

We first investigated the effects of 12 phytoestrogens and related

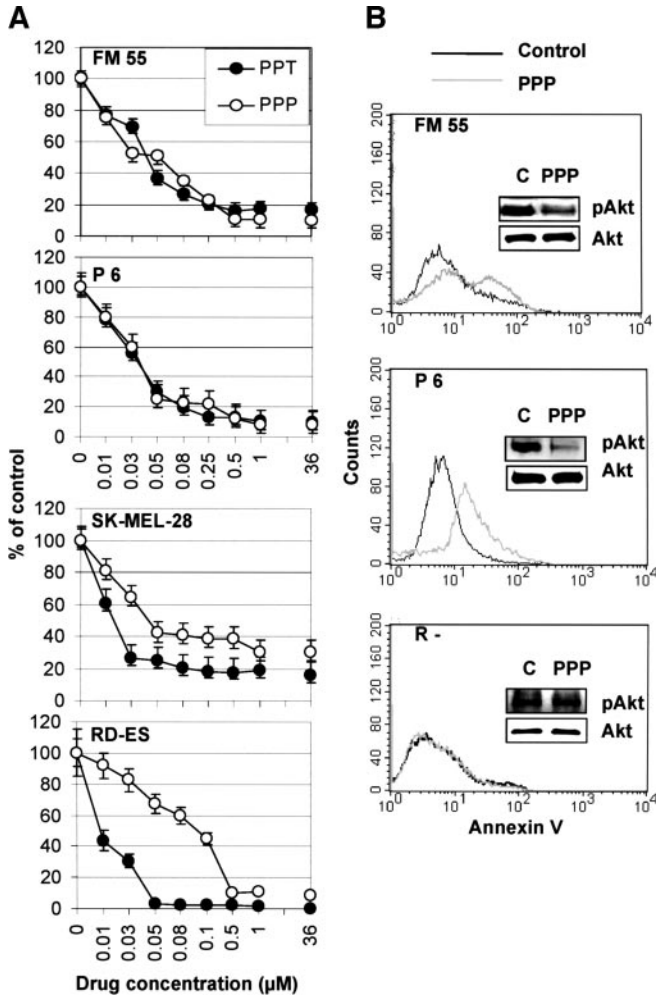

Fig. 5. Effects of IGF-1R inhibitors on malignant cell growth *in vitro*. *A*, dose-response of 48-h treatments with picropodophyllin (*PPP*) and podophyllotoxin (*PPT*) on cell viability of cultures of the FM 55, SK-MEL-28, P6, and RD-ES cells as assayed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt. The cell density at start of the experiments was in all experiments 25,000 cells/well. The values represent means of three experiments; *bars*, ±SE. *B*, FM 55, P6, and IGF-1R negative R- cells were either untreated (control = *C*) or treated with PPP (0.5 μM) for 6 h. Cells were then subjected to simultaneous Annexin V and propidium iodide staining followed by fluorescence-activated cell sorter analysis. In a parallel experiment the same cell lines, treated with PPP (0.5 μM), were analyzed for pAkt (serine473) and Akt (shown in the Annexin V graphs).

Table 1 *Effects of podophyllotoxin (PPT) and picropodophyllin (PPP) on cell viability and basal insulin-like growth factor (IGF)-1R tyrosine phosphorylation in various cell lines*

Cell viability and IGF-1R phosphorylation of intact cells were determined after 48 h and 1 h, respectively. The concentration required for IC_{50} is shown. Each value is based on at least three different experiments. The experiments were repeated three to eight times with similar results.

		PPT (μM)		PPP (μM)	
Origin	Cell line	Viability IC_{50} (48 h)	pIGF-1R IC_{50} (1 h)	Viability IC_{50} (48 h)	pIGF-1R IC_{50} (1 h)
Melanoma	FM 55	<0.05	<0.05	0.06	<0.05
	SK-MEL-28	<0.05	<0.05	<0.05	<0.05
	SK-MEL-5	<0.05	<0.05	nd[a]	nd
	C8161	<0.05	<0.05	<0.05	<0.05
	DFB	0.05	<0.05	0.05	<0.05
	DFW	0.05	<0.05	0.05	<0.05
	AA	<0.05	<0.05	<0.05	nd
Sarcoma	RD-ES	<0.05	<0.05	0.08	<0.05
Breast carcinoma	MCF 7	0.25	<0.05	0.05	<0.05
Prostate carcinoma	PC3	0.1	nd	0.1	nd
Hepatoma	HepG2	>15	—[b]	>15	—[b]
Embryonic mouse fibroblasts	P6	<0.05	<0.05	<0.05	<0.05
	R-	>15	—[b]	>15	—[b]

[a] nd, not determined.
[b] No IGF-1R activity detected.

compounds, including flavones, isoflavones, and lignans, on basal tyrosine phosphorylation of IGF-1R in intact melanoma cells (FM 55). We incubated the cells with the compounds at three different concentrations (1, 15, and 60 μM). Fig. 2*A* shows that genistein and quercetin, representing biologically active isoflavones and flavones, respectively, had only little or no inhibitory effect on IGF-1R phosphorylation. In contrast, the cyclolignan PPT almost completely blocked IGF-1R phosphorylation at all three of the concentrations. Noncyclic lignans, *i.e.*, enterolactone, enterodiol, and matairesinol (13), and other flavones and isoflavones (14) tested were all without effect (data not shown). For comparison, the effect of tamoxifen is also shown in Fig. 2*A*. As reported previously (12), tamoxifen drastically reduced IGF-1R phosphorylation at 15 and 60 μM, but had no effect at 1 μM.

Fig. 2*B* shows that inhibition of IGF-1R phosphorylation by PPT occurred in other tumor cell lines expressing this receptor. Thus, PPT significantly reduced IGF-1R phosphorylation in native malignant cell lines of various origins (the melanoma cell line SK-MEL-28, the breast carcinoma cell line MCF 7, and the sarcoma cell line RD-ES), as well as in mouse fibroblasts with ectopic overexpression of human IGF-1R (line P6).

To investigate the structural requirements for inhibitors of basal IGF-1R phosphorylation, we tested a number of PPT analogues. As mentioned above, noncyclic lignans were not active. However, as illustrated in Fig. 2*C*, we found that the cyclolignans PPP, deoxypodophyllotoxin, and deoxypicropodophyllin were all potent inhibitors of IGF-1R phosphorylation.

The results on the picro derivatives were unexpected, because these compounds have generally been considered to lack biological activity. In contrast, the glucoside derivatives etoposide (Fig. 2*C*) and PPT-4,6-*O*-benzylidene-β-D-glucopyranoside were inactive (data not shown).

Selectivity and Dose Response in Intact Cells. We determined the specificity of PPT and PPP as IGF-1R inhibitors by investigating their effects on tyrosine phosphorylation of other growth factor receptors. Fig. 2*D* shows that basal phosphorylation of the fibroblast growth factor receptor, platelet-derived growth factor receptor, epidermal growth factor receptor, and, notably, the IR, was not affected by PPT or PPP in intact cells even at high concentrations. The latter result was interesting, because the IR is highly homologous to the IGF-1R. As expected, phosphorylation of the IR substrate-1, which is a substrate of the IGF-1R tyrosine kinase, was reduced by PPT and PPP (Fig. 2*D*).

By decreasing the PPT concentrations, we found the IC_{50} value for basal IGF-1R phosphorylation to be as low as 0.04 μM in the intact cells (Fig. 3, *A* and *B*). The expression of IGF-1R, as evaluated by an antibody to the β-subunit, is serving as a loading control (Fig. 3*A*). In Fig. 3*C*, it is shown that PPP efficiently inhibits IGF-1-stimulated IGF-1R, as well as Akt (serine 473) and Erk1/2 phosphorylation. In this experiment the IGF-1R was purified by immunoprecipitation before detection. We also ran a separate experiment on IGF-1-stimulated IGF-1R but excluded the immunoprecipitation step. This experiment involved treatment with PPT and PPP, as well as tamoxifen for comparison. As can be seen, PPT and PPP were essentially equipotent in inhibiting the IGF-1R kinase, whereas tamoxifen was considerably less efficient (Fig. 3*D*). From this experiment, we could also conclude that the expression of neither the α- nor the β-subunit was affected.

Possible Mechanism of Action. We then determined the effects of PPT and PPP on both IGF-1R-catalyzed substrate tyrosine phosphorylation and IGF-1R autophosphorylation in cell-free systems. PPT efficiently decreased the poly-Tyr-Glu (pTG) substrate phosphorylation (IC_{50} value 6 nM; Fig. 4*A*). In contrast, it did not inhibit the substrate phosphorylation of IR and epidermal growth factor receptor tyrosine kinases. Furthermore, we could not detect any inhibitory effects on substrate phosphorylation catalyzed by "non-IGF-1R kinases," which were obtained by immunodepletion of IGF-1R (Fig. 4*A*). PPP produced similar results as PPT (results not shown).

In the next set of cell-free experiments we demonstrated that PPP efficiently inhibited autophosphorylation of the IGF-1R (for details see "Materials and Methods") with an IC_{50} value of ~1 nM (Fig. 4*B*). A similar response was obtained by PPT (data not shown). When various concentrations of ATP (19–300 μM) were tested, the IC_{50} value of PPP remained at 1–2 nM (Fig. 4*B*), suggesting that PPP does not compete with Mg ATP for the ATP binding site. Instead, PPP may interfere with the phosphorylation at the substrate level, which would be in agreement with our molecular model (Fig. 1*A*). Although the amino acid sequence in the activation loop is identical to that of the IR, there are differences outside this core region that may exploit selectivity for an inhibitor (9). Actually, inhibitors targeting the receptor substrate with a relative selectivity for IGF-1R over IR have been reported (10, 11).

Inhibition of Malignant Cell Growth. We studied the effects of PPT and PPP on viability of various tumor cells. Fig. 5*A* shows dose-response curves after incubating melanoma cells (FM 55 and SK-MEL-28), sarcoma cells (RD-ES), and the mouse cell line P6 (overexpressing IGF-1R) with different concentrations of the two cyclolignans for 48 h. As seen, there was a dose-dependent decrease in cell survival up to about 0.05–0.5 μM in all four of the cell lines. Fig. 5*B* shows the effect of PPP on Annexin V binding, as assessed by fluorescence-activated cell sorter analysis. In both P6 and FM 55 cells PPP induced an increase in externalization of phosphatidyl serine

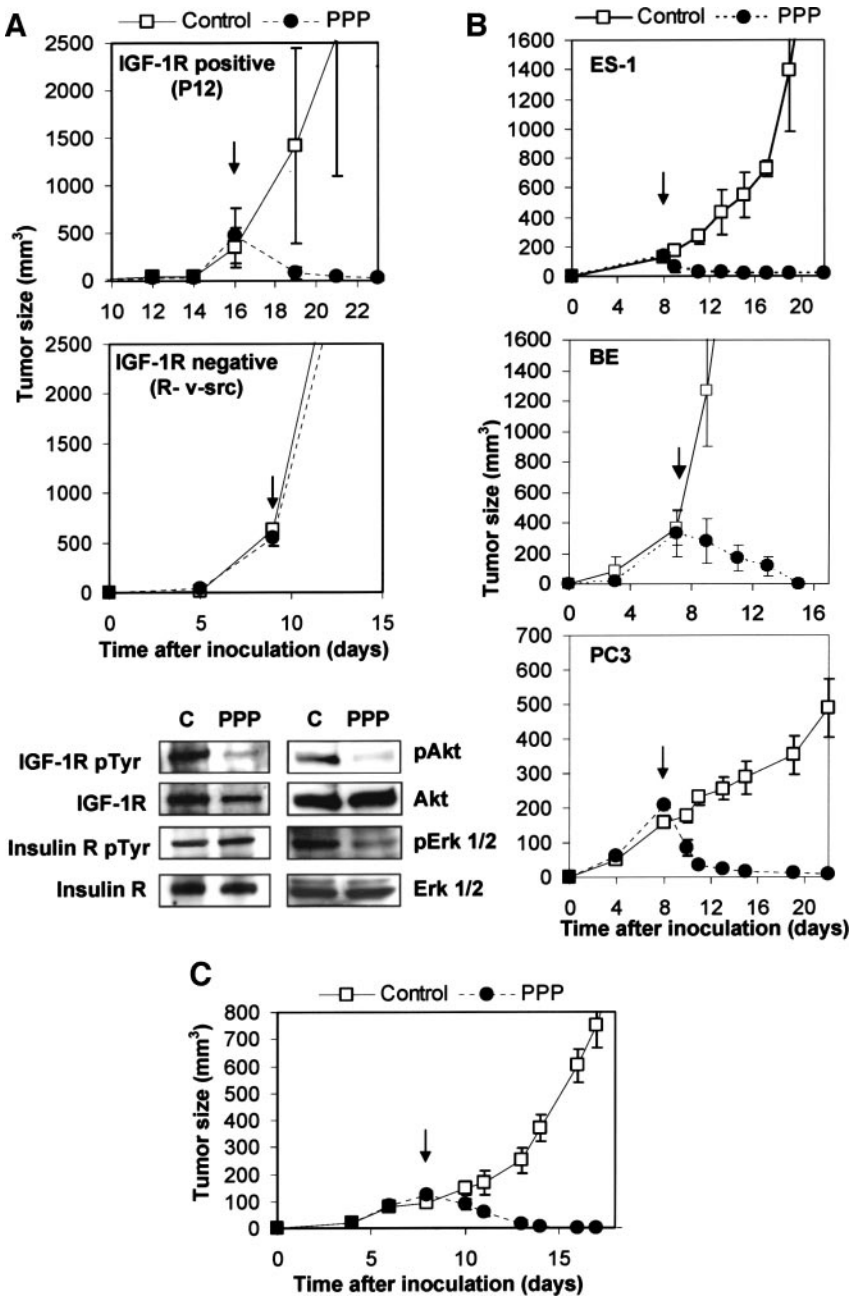

Fig. 6. Effects of insulin-like growth factor (IGF) -1R inhibitors on malignant cell growth *in vivo*. *A*, effect of picropodophyllin (*PPP*) on tumor growth of IGF-1R positive (*P12*) and IGF-1R negative (R- v-src) R- cells allografted in SCID mice (*top panels*). When the tumors were established (on days 16 and 9, respectively), mice were treated with PPP (20 mg/kg/12 h) or drug-free solvent. Tumor growth was measured every second day after start of treatments. In a separate experiment a tumor sample from PPP-treated P12-allografted mouse was taken 4 h after PPP injection, and the effects on phosphorylation of IGF-1R, insulin receptor (IR), Akt, and Erk1/2. *B*, effect of PPP on human cancer growth of ES-1, BE, and PC3 cells xenografted in SCID mice. When the tumors were established (*arrows*), mice were treated with PPP (20 mg/kg/12 h) or drug-free solvent. Three mice were treated in each experiment. Means are shown; *bars*, ±SD. *C*, effect of PPP on tumor growth of murine breast cancer cells (*JC*) allografted in immunocompetent mice. When the tumors were established (*arrow*), mice were treated with PPP (20 mg/kg/12 h) or drug-free solvent. The experiments were repeated 5–10 times with similar results.

from the inner leaflet of the plasma membrane typical for apoptotic cell death, pictured by the increase in Annexin V staining. In contrast, the R- cells (IGF-1R negative) were not affected by PPP. All three of the cell lines were negative for propidium iodide staining (data not shown). It was also confirmed in parallel cell samples that 0.5 μM PPP drastically decreased phosphorylation of Akt, consistent with the crucial role of the phosphatidylinositol 3′-kinase/Akt pathway in IGF-1R-dependent antiapoptosis signaling (2).

The IC_{50} values of PPT and PPP on viability of 13 different cell lines after 48 h of incubation are summarized in Table 1. For comparison, corresponding IC_{50} values for phosphorylation of the IGF-1R are also shown. For 11 of these cell lines, the IC_{50} values of the two lignans ranged from 0.02 to 0.25 μM, and the corresponding IC_{50} values for phosphorylation were of the same order of magnitude (<0.05 μM). In contrast, the IGF-1R negative cells (R-) did not show any response to PPT or PPP. We obtained the same result with the hepatoma cell line HEPG2, but these cells did not exhibit any detectable IGF-1R activity. It may be added that 15 μM of etoposide had no effect on the cell lines listed in Table 1 (data not shown). Taken together, these results suggest that the cytotoxic effect of PPT and PPP on malignant cells is caused by an inactivation of the IGF-1R.

Inhibition of Malignant Cell Growth *in Vivo*. Finally, we investigated the effects of PPP on tumor growth in mice. To first evaluate the selectivity of PPP for IGF-1R *in vivo* we investigated the effect on IGF-1R-positive and IGF-1R-negative tumors. For this purpose P12 (R- with overexpressed human IGF-1 and IGF-1R) and R- v-src (R- with transfected v-src) were established in SCID mice. When the tumors had become established, mice were treated daily with i.p. injections of PPP (20 mg/kg/12 h) or drug-free vehicle for 8–14 days. As shown in Fig. 6*A* PPP blocked growth of IGF-1R-positive tumors and caused complete regression. In contrast, the IGF-1R-negative tumors were not affected and continued growing at a high rate (Fig. 6*A*). These results suggest that PPP selectively inhibits and kills tumor cells expressing the IGF-1R. In the bottom panel of Fig. 6*A*, it is

confirmed that phosphorylation of IGF-1, Akt, and Erk1/2 in a P12 tumor from a PPP-treated mouse is inhibited. In contrast, phosphorylation of IR was not inhibited. We also analyzed serum and urine glucose concentrations in mice treated with PPP. The serum glucose concentration was not increased, rather slightly decreased, and there was no detectable urine glucose (data not shown). These data provide additional evidence that PPP does not inhibit the IR.

In Fig. 6*B* SCID mice xenografted with human ES-1 (Ewing's sarcoma cells), BE (malignant melanoma cells), and PC3 (prostatic carcinoma cells) were treated with PPP. All three of the cell lines had been proven to express IGF-1R activity. Like the case was with the P12 tumors, a complete regression was achieved with all three of the xenografts.

Fig. 6*C* illustrates the effect of PPP on murine breast cancer cell allograft in immunocompetent mice. Treatments were ended after complete tumor regression.

Therapeutic Implications. For anticancer treatment, it is important that an IGF-1R inhibitor does not cross-react with the IR. Coinhibition of the IR would lead to a diabetogenic response *in vivo*, and this very serious side effect cannot obviously be overcome by insulin treatment. Our results show that PPP (at submicromolar concentrations) does not affect IR neither under *in vitro* nor under *in vivo* conditions.

Compared with the notoriously toxic PPT (LD_{50} for rats: 14 mg/kg; Ref. 18), PPP is of particular interest, because it has been shown to be almost nontoxic (LD_{50} in rats: >500 mg/kg; Refs. 17–19) and has even been considered to lack biological activity. Here we show that PPP is a potent inhibitor of IGF-1R and malignant cell growth both in *in vitro* and in animal models, suggesting that PPP, or structurally related compounds, may be used for developing well-tolerated anticancer agents (32, 33).

Our results also show that the administered dose of PPP may be relatively low, although it is probably important that the plasma concentration is high enough to block the IGF-1Rs during the whole treatment time. Daily repeated treatments (as used in this study) or continuous infusion with a nontoxic cyclolignan (or alternative IGF-1R kinase inhibitors) may, therefore, be a good strategy of treatment.

In addition to cancer therapy, IGF-1R inhibitors may be valuable for treatment of other diseases in which IGF-1/IGF-1R is involved in their pathogenesis, like arteriosclerosis, psoriasis, and acromegaly (34–37).

ACKNOWLEDGMENTS

We thank Birgitta Mörk, Kristina Garmark, Margareta Hagelin, and Margareta Rodensjo for excellent technical assistance. Renato Baserga is greatly acknowledged for providing us with IGF-1R positive and negative cell lines.

REFERENCES

1. Baserga, R. The insulin-like growth factor I receptor: a key to tumor growth? Cancer Res., *55:* 249–252, 1995.
2. Baserga, R. The contradictions of the insulin-like growth factor 1 receptor. Oncogene, *19:* 5574–5581, 2000.
3. Girnita, L., Girnita, A., and Larsson, O. Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor. Proc. Natl. Acad. Sci. USA, *100:* 8247–8252, 2003.
4. Girnita, L., Girnita, A., Brodin, B., Xie, Y., Nilsson, G., Dricu, A., Lundeberg, J., Wejde, J., Bartolazzi, A., Wiman, K. G., and Larsson, O. Increased expression of insulin-like growth factor I receptor in malignant cells expressing aberrant p53: functional impact. Cancer Res., *60:* 5278–5283, 2000.
5. Yu, H., and Rohan, T. Role of the insulin-like growth factor family in cancer development and progression. J. Natl. Cancer Inst., *92:* 1472–1489, 2000.
6. LeRoith, D., Werner, H., Beitner-Johnson, D., and Roberts, C. T., Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr. Rev., *16:* 143–163, 1995.
7. Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzel, W., Le Bon, T., Kathuria, S., Chen, E., *et al.* Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J., *5:* 2503–2512, 1986.
8. Navarro, M., and Baserga, R. Limited redundancy of survival signals from the type 1 insulin-like growth factor receptor. Endocrinology, *142:* 1073–1081, 2001.
9. Favelyukis, S., Till, J. H., Hubbard, S. R., and Miller, W. T. Structure and autoregulation of the insulin-like growth factor 1 receptor kinase. Nat. Struct. Biol., *8:* 1058–1063, 2001.
10. Blum, G., Gazit, A., and Levitzki, A. Substrate competitive inhibitors of IGF-1 receptor kinase. Biochemistry, *39:* 15705–15712, 2000.
11. Parang, K., Till, J. H., Ablooglu, A. J., Kohanski, R. A., Hubbard, S. R., and Cole, P. A. Mechanism-based design of a protein kinase inhibitor. Nat. Struct. Biol., *8:* 37–41, 2001.
12. Kanter-Lewensohn, L., Girnita, L., Girnita, A., Dricu, A., Olsson, G., Leech, L., Nilsson, G., Hilding, A., Wejde, J., Brismar, K., and Larsson, O. Tamoxifen-induced cell death in malignant melanoma cells: possible involvement of the insulin-like growth factor-1 (IGF-1) pathway. Mol. Cell. Endocrinol., *165:* 131–137, 2000.
13. Axelson, M., Sjövall, J., Gustafsson, B. E., and Setchell, K. D. Origin of lignans in mammals and identification of a precursor from plants. Nature (Lond.), *298:* 659–660, 1982.
14. Axelson, M., Sjövall, J., Gustafsson, B. E., and Setchell, K. D. Soya–a dietary source of the non-steroidal oestrogen equol in man and animals. J. Endocrinol., *102:* 49–56, 1984.
15. Kelly, M. G., and Hartwell, J. L. The biological effects of and the chemical composition of podophyllin. J. Natl. Cancer Inst., *14:* 967–1011, 1954.
16. Greenspan, E. M., Colsky, J., Schoenbach, E. B., and Sherar, M. J. Response of patients with advanced neoplasms to the intravenous administration of α-peltatin. J. Natl. Cancer Inst., *16:* 1257–1275, 1954.
17. Kelleher, J. K. Tubulin binding affinities of podophyllotoxin and colchicine analogues. Mol. Pharmacol., *13:* 232–241, 1977.
18. Seidlova-Masinova, V., Malinsky, J., and Santavy, F. The biological effects of some podophyllin compounds and their dependence on chemical structure. J. Natl. Cancer Inst., *18:* 359–371, 1957.
19. Ayres, D. C., and Loike, J. D. Lignans. Chemical, biological and clinical properties. Cambridge 121: Cambridge University Press, 1990.
20. Carter, S. K., and Slavik, M. Investigational drugs under study by the United States National Cancer Institute. Cancer Treat. Rev., *3:* 43–60, 1976.
21. Hande, K. R. Etoposide: four decades of development of a topoisomerase II inhibitor. Eur. J. Cancer, *34:* 1514–1521, 1998.
22. Long, B. H., Musial, S. T., and Brattain, M. G. Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP-16–213 and VM-213: A quantitative structure-activity relationship. Biochemistry, *23:* 1183–1188, 1984.
23. Hartwell, J. L., and Schrecker, A. W. The chemistry of podophyllum. Progr. Chem. Org. Nat. Prod., *15:* 83–166, 1958.
24. Buchardt, O., Jensen, R. B., Hansen, H. F., Nielsen, P. E., Andersen, D., and Chinoin, I. Thermal chemistry of podophyllotoxin in ethanol and a comparison of the cytostatic activity of the thermolysis products. J. Pharm. Sci., *75:* 1076–1080, 1986.
25. Rubini, M., Hongo, A., D'Ambrosio, C., and Baserga, R. The IGF-I receptor in mitogenesis and transformation of mouse embryo cells: role of receptor number. Exp. Cell Res., *230:* 284–292, 1997.
26. Valentinis, B., Morrione, A., Taylor, S. J., and Baserga, R. Insulin-like growth factor I receptor signaling in transformation by src oncogenes. Mol. Cell. Biol., *17:* 3744–3754, 1997.
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.
28. Mandal, M., Vadlamudi, R., Nguyen, D., Wang, R. A., Costa, L., Bagheri-Yarmand, R., Mendelsohn, J., and Kumar, R. Growth factors regulate heterogeneous nuclear ribonucleoprotein K expression and function. J. Biol. Chem., *276:* 9699–9704, 2001.
29. Parrizas, M., Gazit, A., Levitzki, A., Wertheimer, E., and LeRoith, D. Specific inhibition of insulin-like growth factor-1 and insulin receptor tyrosine kinase activity and biological function by tyrphostins. Endocrinology, *138:* 1427–1433, 1997.
30. Roehm, N. W., Rodgers, G. H., Hatfield, S. M., and Glasebrook, A. L. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. J. Immunol. Methods, *142:* 257–265, 1991.
31. Laird, A. D., Vajkoczy, P., Shawver, L. K., Thurnher, A., Liang, C., Mohammadi, M., Schlessinger, J., Ullrich, A., Hubbard, S. R., Blake, R. A., Fong, T. A., Strawn, L. M., Sun, L., Tang, C., Hawtin, R., Tang, F., Shenoy, N., Hirth, K. P., McMahon, G., and Cherrington SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. Cancer Res., *60:* 4152–4160, 2000.
32. PCT WO 02/102804 A1 (27.12.2002): New use of specific cyclolignans.
33. PCT WO 02/102805 A1 (27.12.2002): New use of cyclolignans and new cyclolignans.
34. Bayes-Genis, A., Conover, C. A., and Schwartz, R. S. The insulin-like growth factor axis: A review of atherosclerosis and restenosis. Circ. Res., *86:* 125–130, 2000.
35. Patel, V. A., Zhang, Q. J., Siddle, K., Soos, M. A., Goddard, M., Weissberg, P. L., and Bennett, M. R. Defect in insulin-like growth factor-1 survival mechanism in atherosclerotic plaque-derived vascular smooth muscle cells is mediated by reduced surface binding and signaling. Circ. Res., *88:* 895–902, 2001.
36. Wraight, C. J., White, P. J., McKean, S. C., Fogarty, R. D., Venables, D. J., Liepe, I. J., Edmondson, S. R., and Werther, G. A. Reversal of epidermal hyperproliferation in psoriasis by insulin-like growth factor I receptor antisense oligonucleotides. Nat. Biotechnol., *18:* 521–526, 2000.
37. Colao, A., Cuocolo, A., Marzullo, P., Nicolai, E., Ferone, D., Della Morte, A. M., Pivonello, R., Salvatore, M., and Lombardi, G. Is the acromegalic cardiomyopathy reversible? Effect of 5-year normalization of growth hormone and insulin-like growth factor I levels on cardiac performance. J. Clin. Endocrinol. Metab., *86:* 1551–1557, 2001.