Arch. Pharm. Res. Vol.19, No.6, pp. 490-496, 1996

Antitumor Activities of Hypericin as a Protein Tyrosine Kinase Blocker

Kwang-Sup Kil[1], Young-Na Yum[1], Seung-Hoon Seo[2] and Kyung-Tae Lee[2]

[1]Department of Pathology, Institute of Toxicology, Korea FDA, Seoul 122-020, Korea and [2]College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

(Received September 12, 1996)

Naphtodianthrone hypericin produced a potent antitumor activity *in vitro* against several tumor cells. However, it did not show any cytotoxicity on normal cells such as *Macaccus rheus* monkey kidney cells (MA-104) and primary cultured rat hepatocytes up to 500 μM concentration. Hypericin added to A431 human epidermoid carcinoma cell membrane inhibited the autophosphorylation of the epidermal growth factor (EGF) receptor and the tyrosine phosphorylation of RR-SRC peptide catalyzed by an EGF-receptor. Similarly, treatment of the A431 cells with hypericin inhibited the tyrosine phosphorylation of EGF-dependent endogenous EGF-receptor by western blotting analysis. Hypericin also inhibited the T cell PTK, $P56^{lck}$, in a dose-dependent fashion with an IC_{50}=5 μM. The tyrosine phosphorylation on RR-SRC peptide and EGF-induced receptor autophosphorylation, either *in vitro* or in intact cells was inhibited by hypericin at the same concentration as that in A431 cell proliferation. These data suggest that hypericin directly inhibits EGF-receptor and $P56^{lck}$ PTK activity *in vitro* and can mediate such action *in vivo*.

Key words : Hypericin, Protein tyrosine kinase, $P56^{lck}$, Epidermal growth factor, Cytotoxicity, RR-SRC peptide

INTRODUCTION

Phosphorylation of proteins on tyrosine residues is a key biochemical reaction that mediates a large variety of cellular signals (Hunter and Cooper, 1985; Yarden and Ullrich, 1988), including the control of the cell cycle and cell differentiation. Many of cellular plasma membrane receptors, such as the receptors for epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin, possess an integral, intracellular, tyrosine kinase moiety that is activated upon the binding of its specific ligand to the extracellular domain of the receptor (Ullrich and Schlessinger, 1990; Yarden and Ullrich, 1988). The involvement of abnormally high protein tyrosine kinase (PTK) activity with a large variety of growth related disease state has already been established (Bishop, 1987). In many cases, the enhanced PTK activity of oncogene products or the overexpression of their normal counterparts was found to be essential for their transforming activity (Bishop, 1987; Ross, 1986; Ullrich and Schlessinger, 1990). Hyperproliferation of cells leading to nonmalignant growth is also often associated with enhanced PTK activity, for example, the enhanced PTK activity of PDGF receptor which results from its exposure to sustained levels of PDGF seen in atherosclerosis and restenosis (Ross, 1986). The overexpression of PTK oncoproteins, which results in enhanced kinase activity, can also alter the developmental pattern of cell types into which they have been introduced (Basler and Haften, 1988; Filvaroff *et al.*, 1990; Weissman and Aaronson, 1985).

The involvement of PTKs in a wide range of disease states has thus lead to the general concept that development of PTK blockers is a reasonable approach to combat hyperproliferative conditions which result from enhanced activity of PTKs (Levitzki, 1990; Yaish *et al.*, 1988). Several active compounds have been isolated from bio-source [e.g. erbstatin (Umezawa and Imoto, 1991), herbimycin A (Fukazawa *et al.*, 1991), staurosporin (Secrist *et al.*, 1990), flavonoids (Akiyama *et al.*, 1987) and many more have been chemically synthesized [e.g., tyrphostins (Levizki *et al.*, 1991), thiazolidine-diones (Geissler *et al.*, 1990)]. Consequently, it was established that compounds like herbimycin A (Murakami *et al.*, 1988), tyrphostins (Lyall et al., 1989) and thiazolidine-diones (Geissler *et al.*, 1990) all inhibit the growth of cultures cells by their specific in-

Correspondence to: Kyung-Tae Lee, College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

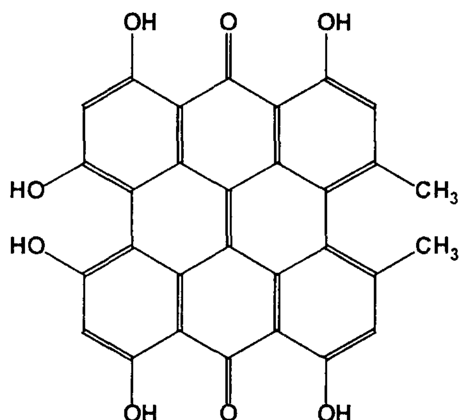

Fig. 1. Structural features of hypericin

terference with PTK activities.

Among the anthranoids, hypericin is aromatic polycyclic dione and derived from plants of the *Hypericum* family (Fig. 1). It has recently been reported to be highly effective in preventing viral-induced manifestations that follow a variety of retroviral infections both *in vivo* and *in vitro* (Meruleo *et al.*, 1988; Lavie *et al.*, 1989). This compound probably interferes with viral infection and/or spread by direct inactivation of the virus or by preventing virus shedding, budding, or assembly at the cell membrane (Tang *et al.*, 1990).

This paper reports the strong antitumor activity *in vitro* and inhibition of the PTK activity of the EGF-receptor and $P56^{lck}$ by hypericin at submicromolar concentrations.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Dulbeco's modified eagle's medium (DMEM), penicillin-streptomycin, trysin-EDTA, aprotinin, leupeptin, phenylmethylsulfonylfloride (PMSF), hypericin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), diaminobenzidine tetrachloride(DAB), dithiothreitol (DTT), trichloroacetic acid (TCA), anti-phosphotyrosine antibody and hypericin were obtained from Sigma. RR-SRC peptide was from BRL. Phosphocellulose filter was from Whatman. $[\gamma\text{-}^{32}P]$ ATP, hyperfilm β-max, anti-mouse-Ig perdidase-linked $F(ab')_2$ fragment were from Amersham. A431 human epidermoid carcinoma cells (ATCC CTL 1555), SNU-1 human stomach cancer and SNU-C4 human colon cancer cells were obtained from Korean Cell Line Bank. MA-104 *Macaccus rheus* monkey kidney cells was kindly provided from Dr. D. H. Kim (Kyung Hee University, Seoul). $HepG_2$ and MH_1C_1 hepatoma cells were from Dept. Endocrynology (Kyung Hee Medical Center, Seoul).

Cell culture

A431 cells were grown at 37°C in DMEM supplemented with 10% FBS, 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml). MH_1C_1 cells were grown in Ham's F10 medium, supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin. P 388, L-1210, SNU-1, SNU-C4, $HepG_2$ and MA-104 cells were grown in RPMI medium supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin. Above cells were grown in a humidified atmosphere of 95% air 5% CO_2 and the cells were seeded in plates after three passages.

Isolation and culture of rat hepatocytes

Rat hepatocytes were obtained from male Sprague-Dawley CD rats (200-350 g) by use of the two-step collagenase perfusion method as described by Lee *et al.* (1994).

Rat hepatocytes were seeded at a density of 1×10^5 cells in 96 well plates in 100 μl culture medium containing 10% FBS. The medium was a mixture of 75% (v/v) MEM and 25% (v/v) Medium 199 supplemented with 10 μg bovine insulin per ml.

Preparation of crude A431 membranes

A431 cell crude membrane extracts were prepared basically as described in Thom *et al.* (1977). Briefly, the cells were trypsinized, washed several times with PBS, concentrated by centrifugation and lysed by a hypotonic EDTA/borate buffer pH 10.2 supplemented with PMSF (1 mM), aprotinin (2 μg/ml) and leupeptin (2 μg/ml). After passing through a nylon gauze, the filterate was centrifuged at 500 g at 4°C for 5 min and the supernatant was further centrifuged at 100,000 g at 4°C for 30 min . The pellet (membrane fraction) was suspended in membrane buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol supplemented with 1 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin) and stored at -70°C for until use. Protein analysis was performed by Bradford method using bovine serum albumin as a standard (Kim, 1995).

Antiproliferative assay

Assays were performed essentially as described previously (Denizot and Lang, 1986 and Baek *et al.*, 1996). Cells were seeded into microtiter plates and incubated overnight. Hypericin dissolved in dimethylsulfoxide (DMSO) was added in serial dilution (the final DMSO concentrations in all assays did not exceed 1%). Twenty four hours after seeding, 100 μl new media or containing above hypericins at the indicated concentrations were added and the plates were incubated for 48 hrs. Cells were washed once

before adding 50 μl FBS-free medium containing 5 mg/ml (MTT) concentration. After 4 hrs incubation at 37°C, the medium was discarded and formazan blue formed into the cells was replaced by adding 100 μl DMSO. Optical density was measured at 570 nm. All experiments were performed after exposure of light at 1 cm distance with an standard flourescent lamp (T-1203, Sigma Chemical. Co. U.S.A.) for 15 min.

EGF-receptor kinase activity (autophosphorylation)

EGF-receptor autophosphorylation assays were carried out in a final volume of 25 μl containing 2.5 μg crude A431 membrane, 10 μM [γ-^{32}P]ATP (20 mCi/mol) and phosphorylation buffer (1 mM DTT, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 100 μM Na_3V04 and 20 mM Hepes pH 7.5). The membrane was activated with EGF (1 μg/ml) for 10 min at 0°C before being incubated with appropriate amounts of hypericin. After 10 min incubation with inhibitor at 37°C, phosphorylation was started by adding [γ-^{32}P]ATP to the mixture at 30°C for 5 min, and the reaction was terminated by heating at 95°C for 5 min in SDS-PAGE denaturation buffer.

EGF-receptor kinase activity (RR-SRC peptide phosphorylation)

The EGF-receptor kinase activity towards an exogenous substrate was assayed by 0.5 mM RR-SRC peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) in a phosphorylation mixture (0.1 mM DTT, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 20 μM EDTA, 25 μg/ml BSA, 0.15% NP-40, 100 μM Na_3V0_4, 1 μCi [γ-^{32}P] ATP, 60 μM ATP and 20 mM Hepes pH 7.5) with 1 μg/ml A431 membrane, 20 μl final volume. After incubation at 25°C for 5 min, the reaction was stopped by adding of 20 μl 10% cold TCA and incubation on ice for 10 min. The sample was centrifuged and the supernatant 15 μl was spotted on phosphocellulose disc. After washing 3 times with 1% acetic acid and 2 times with water, the radioactivity incorporated in the RR-SRC peptide was measured by scintillation counting. Blank values were obtained by running the phosphorylation assay without RR-SRC peptide.

Immunoblot analysis

A431 cells were seeded in T-25 tissue culture flasks at 10^5 cells/flask in DMEM with 10% FBS, grown to 80% confluency and then starved for 24 hrs in DMEM containing 0.5% FBS. Hypericin was added and incubated with cells with the various concentration for 2 hrs. EGF (100 ng/ml) was added to cultures which were incubated another 10 min. Cells were scraped from the dish surface into the culture medium, centrifuged at 1000 g for 5 min and washed with 10 ml phosphate buffered saline. Cells were lysed in SDS-PAGE sample buffer and boiled for 5 min. Following SDS-PAGE (8% gels), proteins were transferred onto nitrocellulose filter membrane by electro-dry blotting (Novex system). Membranes were blocked with 5% skim milk for 30 min and then incubated with primary antibody (1 μg/ml in 5% skim milk) at room temperature for 1 h. The membrane was washed 3 times with PBS, incubated for 60 min with anti-mouse IgG in PBS containing 1% BSA. Immunoreactive bands were visualized by immersion in a mixture of 15 ml Tris-HCl/pH 7.4, 0.03% $NiCl_2$, 10 mg diaminobenzene tetrachloride and 15 μl 30% H_20_2. The position of the marker proteins was localized by brief staining with 0.5% ponceau s in acetic acid.

P56^{lck} kinase activity

P56^{lck} was purified and activity was assayed as previously described (Trevillyan *et al.*, 1990) at 30°C in 50 μl reaction volumes containing 25 mM Hepes (pH 7.4), 50 mM $MgCl_2$, 20 μM orthovanadate, 0.05% NP-40, 1 mM RR-SRC peptide substrate and various concentrations of hypericin. Assays were initiated by the addition of 5 μCi [γ-^{32}P]ATP and analyzed as previously reported (Trevillyan *et al.*, 1990).

RESULTS AND DISCUSSION

Antiproliferative Assays

The antiproliferative activities of hypericin against several kinds of tumor and normal cells were examined by MTT test after 2-day treatment. Hypericin produced no cytotoxicity during incubation with normal primary cultured rat hepatocytes and MA-104 monkey kidney cells up to 500 μM concentration, whereas it showed an IC_{50} at concentration of 0.15

Table I. Sensitivity of cultured cell to hypericin

Cell line	Origin		IC_{50}* (μM)
P388	leukemia	mouse	0.15×10^{-3}
L-1210	leukemia	mouse	0.38
SNU-C4	colon cancer	human	0.14
SNU-1	stomach cancer	human	0.34
MH1C1	moris hepatoma	rat	2
HepG2	hepatoma	human	112
A-431	epidermoid carcinoma	human	1.7
MA-104	kidney cell	monkye	>500
Hepatocyte**	liver	rat	>500

*IC_{50} values represent the mean of three independent experiments and were defined as the drug concentrations which resulted in a 50% decrease in cell number as compared with the control cultures in the absence of inhibitor.
**Hepatocytes were primary cultured isolated by two-step collagenase method.

pM-112 μM in the tumor cells. Hypericin clearly expresses a significant specificity in inhibiting P 388 leukemia (IC_{50}=0.15 pM) and L-1210 leukemia (IC_{50}= 0.38 μM) cells. Gastrointestinal tumor cells such as SNU-1 and SNU-C4 cells were inhibited in a similar manner and the IC_{50}'s in those cells were 0.34 and 0. 14 μM, respectively (Table I).

These results raise the possibility that this drug can be used as a chemotherapeutic agent because of the remarkable antiproliferative effect on tumor cells and the lack of toxicity on normal cells *in vitro*.

Inhibition on autophosphorylation of EGF-receptor

To further investigate the mechanism of growth inhibition by hypericin, the effect of hypericin on the activity of the EGF-receptor in A431 cell was examined. First, we tested the effect of this inhibitor on the phosphorylation of the EGF-receptor itself. EGF-receptor autophosphorylation, which occurs under physiological condition, was investigated using plasma membrane preparation from A431 cells. The EGF-receptor with a molecular weight of 170 kDa which indicates phosphorylated form, was identified in these preparations by immunoblot experiments (data not shown). The major radioactive spot on 8% SDS-PAGE gels corresponds to this phosphoprotein (Fig. 2). Fig. 2 shows an apparent increase in EGF receptor phosphorylation by EGF and its dose-dependent inhibition by hypericin on the EGF-stimulated EGF-receptor (170 KDa). IC_{50} was about 2 μM similar to its IC_{50} on cell growth inhibition (Table 1).

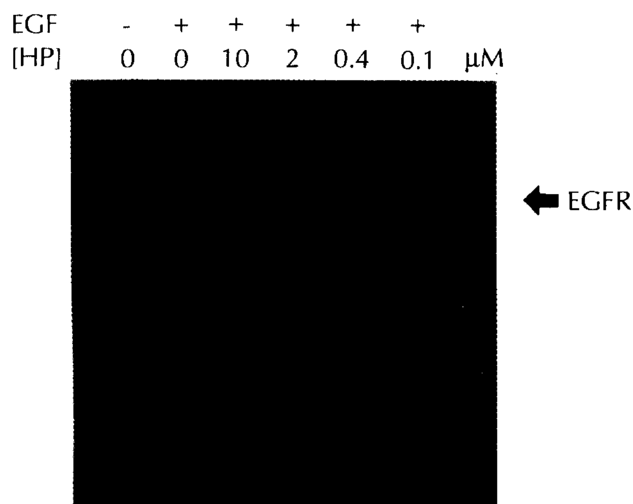

Fig. 2. Effect of hypericin on autophosphorylation of EGF-receptor autophosphorylation *in vitro*. A431 membranes (2.5 μg) were incubated for 10 min with EGF (1 μg/ml) before addition of the indicated concentrations of inhibitor. After 10 min incubation with inhibitor at 37°C, autophosphorylation reaction was started by addition of radiolabeled ATP and terminated by the addition of SDS-PAGE sample buffer. An autoradiogram of the dried SDS gel (8%) after 2 hrs of exposure is shown.

Phosphorylation of EGF-receptor in the absence of EGF (basal phosphorylation) was not detected. Similar result was obtained when immunoprecipitation of A 431 cells with antibody against phosphotyrosine was conducted in the presence of phenyl phosphate.

Inhibition on EGFR-PTK activity with exogenous substrate phosphorylation

We next examined the effect of hypericin on PTK activity of EGF-receptor using RR-SRC peptide as an exogenous substrate. RR-SRC peptide has tyrosine residue instead of serine and threonine, which allows its use in providing tyrosine phosphorylation by EGF-receptor. Hypericin caused a dose-dependent inhibition on the PTK activity when RR-SRC peptide was used. RR-SRC peptide phosphorylation was completely blocked when hypericin was used at a concentration of 20 μM and IC_{50} was about 3 μM.

Autophosphorylation of the EGF-receptor is considered to be the first enzymatic event following ligand binding (Hsuan *et al.*, 1989). The physiological function of this event is incompletely understood. However, it is generally considered as a regulatory mechanism which results in the relief of an inhibitory constraint (Bertics, 1985). EGF autophosphorylation *in vitro* was inhibited by hypericin with an IC_{50} similar to that for inhibition of exogenous substrate phosphorylation in this paper. Since autophosphorylation occurs either by an intramolecular reaction (Ullrich and Schlessinger, 1990) or by trans-phosphorylation within EGF-receptor dimers (Yarden and Ullrich, 1988), the local substrate concentration can not be accurately determined, but may be in the molar range due to proximity effects. Thus, hypericin is efficient inhibitors of both exogenous substrate phosphorylation and EGF-receptor autophosphorylation *in vitro*.

Effect of hypericin on EGF-induced receptor autophosphorylation in A431 intact cells

We further investigated the effect of hypericin on the EGF-dependent phosphorylation of intracellular target proteins in the serum-starved A431 cells. Fig. 3 depicts the experiments in which the EGF-activated phosphorylation of EGF-receptor and its inhibition by hypericin. The effect of hypericin on EGF-induced tyrosine phosphorylation was studied using A431 cell line. EGF was added to serum starved cells which had been treated with various concentrations of sample. EGF-dependent cellular tyrosine phosphorylation was monitored by immunoblotting with phosphotyrosine antibody. EGF-receptor proteins showed a dramatic increase in the phosphorylation in

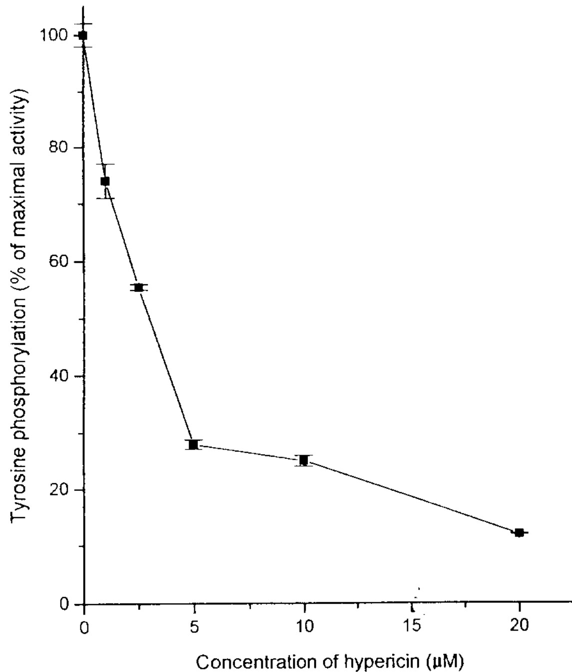

Fig. 3. Inhibition of EGFR-PTK activity with RR-SRC as a substrate by hypericin. EGFR kinase activity was assayed as described under "Experimental Procedure" using 0.5 mM RR-SRC peptide as exogenous substrate. Experiment was repeated three times and the mean±S.D. are shown. Results are expressed as percent of phosphorylation activity of the RR-SRC in control samples.

EGF	-	+	+	+	+	+	
[HP]	0	0	10	2	0.4	0.1	μM

◄ EGFR

Fig. 4. Inhibition of EGF-receptor autphosphorylation by hypericin in intact cells. Serum-starved A431 cells were incubated with indicated concentrations for 2 hrs prior to addition of EGF. After cell lysis and SDS-PAGE of cell extracts, proteins were transferred to nitrocellulose membrane. Immunoblot analysis was performed using anti-phosphotyrosine antibody.

response to EGF. Similar result was obtained when EGF-receptor autophosphorylation was tested (Fig. 2). The inhibitory effect of hypericin on EGF-receptor autophosphorylation was dependent on drug concentration. Similar reults were obtained when the non-physiological peptide substrate RR-SRC was used. The effect of hypericin was very pronounced, reducing the phosphorylation of EGF-receptor with IC_{50} value of 2 μM.

$P56^{lck}$ PTK inhibition

Engagement of the CD3-Ti (TCR) complex by Ag/MHC, lectin mitogen or CD3 mAb initiates the rapid tyrosine phosphorylation of several cellular substrates including TCR-ζ. T cells express several kinds of PTK that potentially could mediate CD3-Ti stimulated protein tyrosine phosphorylation, including c-*fyn* and *lck*. Presently one candidate enzyme mediating the phosphorylation of TCR-ζ is the T cell specific PTK, $P56^{lck}$, which is associated with the T cell surface Ags, CD4 and CD8. Hypericin inhibited immunoaffinity-purified $P56^{lck}$ kinase activity in a dose dependant fashion with an IC_{50} of 5 μM.

Because of the remarkable antiretroviral effects of hypericin observed *in vitro* as well as *in vivo* (Lavie *et al.*, 1989), this compound can be used in retrovial induced disease such as the acquired immnunodeficiency syndrome (AIDS). The causative agent of AIDS is the human immunodefciency virus which upon binding to the cell surface CD4 lymphocyte receptor, enters into the cell and leads to cytopathic effects causing cell death (Lifson *et al.*, 1986). Recently it was shown the HIV-CD4 T-cell receptor interaction induces a rapid tyrosine phosphorylation of different target proteins as well as protein kinase C activation and consequent CD4-receptor phosphorylation (Cohen *et al.*, 1992). These observations strongly suggest that early tyrosine phosphorylations, probably due to the activated receptor associated $PP56^{lck}$ PTK activity, and protein kinase C activation are required for intracelllar transmission of the HIV-induced cytopathic signal. The remarkable inhibitory activity of hypericin towards the EGF-receptor and $P56^{lck}$ PTK shown in this study, could be fundamental in explaining the *in vitro* anti-HIV activity of hypericin (Meruleo *et al.*, 1988), if inhibition of the early HIV-induced tyrosine phosphorylation is required to block the cellular activation process leading to cell death. This intriguing possibility deserves further assesment of the potentially important pharmacological features of hypericin.

Besides the potential use as an anti-HIV drug, its drastic and specific effect on the PTK activity of EGR-receptor suggests its role as an anti-proliferative agent, requiring a further exploration of the mechanistic relationship between PTK activity inhibition and anti-proliferative effect.

ACKNOWLEDGEMENT

The authors thank the Eun-Ah, Jung for technical assistance. This paper was supported in part by the Kyung Hee University, 1995.

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