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INHIBITION OF EPIDERMAL GROWTH FACTOR RECEPTOR TYROSINE KINASE ACTIVITY BY HYPERICIN

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Abstract—The naphthodianthrone hypericin produces a potent and irreversible inhibition of the epidermal growth factor (EGF) receptor tyrosine kinase activity. The inhibition was time and temperature dependent but did not depend on EGF activation. The IC_{50} values obtained were 0.37–8.7 μM with membranes incubated for 30 min at 30° or 10 min at 0°, respectively. Kinetic analyses with poly(Glu,Ala,Tyr) 6:3:1 [poly(GAT)] as an exogenous substrate were in agreement with the irreversible nature of the inhibition. Irradiation for 30 min with fluorescent light caused a dramatic photosensitizing effect and resulted in an IC_{50} value of 44 nM. This effect was due to a type I mechanism, since the exclusion of oxygen did not alter the inhibition curve. The inhibition was inversely proportional to the amounts of membranes used, which probably reflects the non-specific sequestration of hypericin into the lipid bilayer. Ser/Thr protein kinases such as protein kinase A, casein kinase 1 and 2 and the enzyme 5′-nucleotidase, were not inhibited by hypericin not even at high concentrations (>100 μM).

Change in protein phosphorylation constitutes a major mechanism by which extracellular signals are transduced in cellular responses leading to cell proliferation or differentiation [1]. Kinases are generally classified as Ser/Thr-specific or Tyr-specific according to their ability to specifically phosphorylate serine/threonine or tyrosine residues in target proteins. The protein-tyrosine kinase (PTK§) activity was discovered a little more than a decade ago as the transforming activity of Rous sarcoma viral protein pp60*src* [2] and has since been associated with other oncoproteins and with many growth factor receptors [3].

Aberrant phosphorylation by mutations or overexpression of cellular kinases has been implicated in the molecular mechanisms underlying the progressive transformation of a normal cell to a cancer cell [3, 4]. The therapeutic use of specific PTK inhibitors could therefore represent a major breakthrough in the chemotherapy of neoplastic diseases [5]. Furthermore, if specific, these compounds would also be convenient tools for studying the biochemical function of a particular kinase [6].

Since enhanced PTK activity often accompanies cell transformation, e.g. in the case of truncated, overexpressed or overstimulated epidermal growth factor (EGF)-receptors [7, 8] or related membrane receptors [9–11], there has been an intense search for PTK-inhibitors over the last decade. Several active compounds have been isolated from biosources (e.g. erbstatin [12] (Fig. 1), herbimycin A [13], staurosporine [14], flavonoids [15]), and many more have been chemically synthesized (e.g. hydroxycinnamamides [16], tyrphostins [17], thiazolidine-diones [18] and multisubstrate analogues [19, 20]). Consequently, it was established that compounds like herbimycin A [21, 22], tyrphostins [23, 24], thiazolidine-diones [18] and sulphonyl-benzoyl-nitrostyrenes [20] all inhibit the growth of cultured cells by their specific interference with PTK activities.

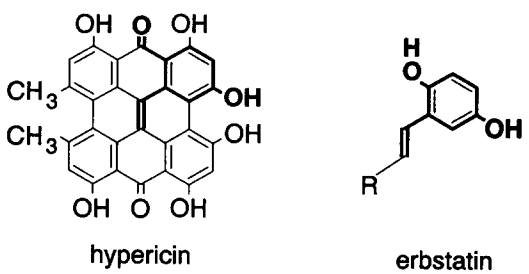

Fig. 1. Structural features of hypericin. The presence of the erbstatin moiety is indicated (R = NHCHO).

Conclusive generalizations on the basic structure of a PTK-inhibitor are difficult to make since active compounds seemingly belong to several different chemical classes. However, the majority of synthetic routes have been based on the idea of mimicking tyrosine in an attempt to find nonphosphorylatable analogues that compete with the PTK substrates.

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§ Abbreviations: PMSF, phenylmethylsulphonylfluoride; poly(GAT), poly(Glu,Ala,Tyr) 6:3:1; EGF, epidermal growth factor; BCA, bicinchonic acid; TCA, trichloroacetic acid; FCS, foetal calf serum; DMSO, dimethylsulphoxide; PBS, phosphate-buffered saline; SDS, sodium dodecylsulphate; DMEM, Dulbecco's modified Eagle's medium; PTK, protein-tyrosine kinase.

Consequently, many inhibitors are substituted hydroxylated aromatic molecules.

An abnormal regulation of the EGF-receptor and its ligand transforming growth factor (TGF-α) could be important in the etiology of skin diseases like psoriasis (see Discussion [25–27]). A dramatic inhibition of the EGF-stimulated receptor PTK was noticed following psoralen/ultraviolet light (PUVA) treatment of A431 cells [28] and this could be relevant to the psoriasis treatment. Intrigued by the observation that dithranol, a widely used anti-psoriatic compound [29] with a hydroxylated anthranoid skeleton, shares a structural similarity with many known PTK inhibitors, the question was raised whether this compound and related structures showed specific PTK inhibitor characteristics.

Among the anthranoids with an interesting activity we selected hypericin, the main constituent of plants of the genus *Hypericum* [30], for further studies. This compound is characterized by a naphthodianthrone structure containing the hydroxylated styrene-skeleton of erbstatin (Fig. 1). It features a photodynamic effect [31] responsible for inducing hypericism in cattle ingesting large amounts of *Hypericum* on pastures [32], but no toxic effects have been observed in laboratory mice or in humans, where the compound is clinically used as an antidepressive agent [33, 34]. Much attention has recently been paid to the intriguing anti-viral activity of hypericin *in vivo* [35, 36] as well as *in vitro* [35–42]. An inhibition of HIV-1 reverse transcriptase [37] and protein kinase C [43] have been implicated in the *in vitro* effect, but on the whole, the molecular mechanism of the antiviral activity of hypericin is poorly understood.

This paper reports the inhibition of the PTK activity of the EGF-receptor by hypericin at submicromolar concentrations. Our observations suggest that the inhibition is irreversible but varies with the incubation time, the temperature, the membrane concentration and the incidence of light.

MATERIALS AND METHODS

Materials. Mouse EGF was purchased from Boehringer (Mannheim, Germany). Casein kinase 1 (CK-1, sp. act. 50 nmol Pi/min/mg) and casein kinase 2 (CK-2, sp. act. 1 μmol Pi/min/mg) were prepared according to Meggio *et al.* [44] with an extra final ion-exchange chromatography purification step [Mono S HR5/5 column and Mono Q HR5/5 column (Pharmacia, Uppsala, Sweden) in the case of CK-I and CK-II, respectively]. Protein kinase A (bovine, catalytic subunit, sp. act. 41 pM U/μg), 5′-nucleotidase (sp. act. 230 U/mg, from *Crotalus* venom), histone (type II-AS), poly(Glu,Ala,Tyr) 6:3:1 [poly(GAT)] (20,000–50,000) and protease inhibitors [phenylmethylsulphonylfluoride (PMSF), aprotinin, leupeptin, pepstatin] were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Hypericin (TLC grade, purity > 99%) (Carl Roth GmgH and Co., Karlsruhe, Germany) and quercetin (Sigma) were dissolved in dimethylsulphoxide (DMSO) (Janssen Chimica) and stored at −20° in dark conditions. In these conditions hypericin proved to be stable for at least 2 months. [γ-^{32}P]ATP (110 TBq/mmol) was from Amersham International (Amersham, U.K.). The monoclonal anti-human EGF receptor immunoglobulin (IgG) raised against a synthetic peptide representing residues 985–996 of the human EGF-receptor (internal domain) was used in immunoblot experiments (Boehringer). Protein quantification was performed using the bicinchonic acid (BCA) method (Pierce) with bovine serum albumin (Boehringer) as standard.

Preparation of crude A431 membranes. A431 human epidermoid carcinoma cells (ATCC CRL 1555) were grown at 37° in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/L glucose, Gibco, Paisley, U.K.) supplemented with 10% foetal calf serum (FCS) (Seralab), 2 mM L-glutamine (Gibco), penicillin (100 IU/mL), streptomycin (100 IU/mL) and nystatin (50 U/mL) in a humidified 5% CO_2 atmosphere. A431 cell crude membrane extracts were prepared basically as described previously [45] Briefly, the cells were trypsinized, washed several times with phosphate-buffered saline (PBS), concentrated by centrifugation and lysed by a 20 mM borate buffer pH 10.2 supplemented with 0.2 mM EDTA, 1 mM PMSF, aprotinin (2 μg/mL), and leupeptin (2 μg/mL). After passing through a nylon gauze, the filtrate was centrifuged at 25,000 *g* for 30 min at 4°. The pellet (crude membrane fraction) was suspended (*ca.* 2 μg/μL) in membrane buffer [50 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$ supplemented with 1 mM PMSF, aprotinin (2 μg/mL) and leupeptin (2 μg/mL)] and stored at −80° for a maximum of 2 weeks before use.

EGF-receptor kinase activity (autophosphorylation). EGF-receptor autophosphorylation assays [46] were carried out in a final volume of 25 μL containing (if not specified otherwise) 2.5 μg crude A431 membrane, 10 μM [γ-^{32}P]ATP (185 kBq) and phosphorylation buffer [1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 100 μM sodium vanadate and 20 mM Hepes pH 7.5]. The final DMSO concentration was 4% which only slightly increased the PTK activity of the EGF-receptor. The membranes were activated with EGF (1 μg/mL) for 20 min at 0° before being incubated with appropriate amounts of inhibitor or DMSO (control) for certain time periods at 0° or 30°. Phosphorylation was started by adding [γ-^{32}P]ATP to the mixture for 8 min at 0°, and the reaction was terminated by heating at 95° for 4 min in sodium dodecylsulphate–polyacrilamide gel electrophoresis (SDS–PAGE) denaturation buffer. Unless specified otherwise, the whole procedure was performed under standard laboratory illumination. The 170 kDa EGF-receptor protein detected by autoradiography on 7.5% SDS–polyacrylamide gels was cut out and quantified by liquid scintillation counting. In some experiments 0.25% Triton X-100 was added to the phosphorylation buffer followed by solubilization of the EGF-receptor and removal of insoluble material by centrifugation at 4° (14,000 *g*, 5 min). Phosphorylation assays were then performed on the supernatant fractions as described above.

Light experiments were performed by incubating membranes with different concentrations of hypericin for 30 min at 0° while irradiated at 1-cm distance with a standard fluorescent lamp (LF-20W/2, 4300°K,

Acec). Anaerobic phosphorylation reactions were performed under nitrogen after degassing all solutions and membrane preparations under reduced pressure.

EGF-receptor kinase activity [poly(GAT) phosphorylation]. The EGF-receptor kinase activity towards an exogenous substrate was assayed by including different concentrations of poly(GAT) in the phosphorylation mixture (2.5 μg crude A431 membrane, 50 μL final volume). After 20 min at 30° the sample was centrifuged (14,000 *g*, 40 sec) and the poly(GAT) in 30 μL of the supernatant was precipitated with ice-cold 20% trichloroacetic acid (TCA) in the presence of 200 μg bovine serum albumin. After washing three times with 20% TCA the radioactivity incorporated in poly(GAT) was measured by liquid scintillation counting. Blank values were obtained by running the phosphorylation assay without poly(GAT). They amounted to 24.3% (SD 2.3) of the total activity. Blank values without membranes amounted to *ca.* 4%.

Other enzymic reactions. Casein kinase 1 and casein kinase 2 were assayed at 37° for 4 min as described [44] in a reaction mixture (final volume 50 μL) consisting of 50 mM Tris pH 7.4, 12 mM $MgCl_2$, 150 mM NaCl, 100 μM [γ-^{32}P]ATP (3.7 kBq), casein (2 mg/mL). 5'-Nucleotidase was assayed at 37° for 10 min as described [47] in a reaction mixture (final volume 1 mL) containing 0.1 M Tris pH 8.5, 10 mM $MgCl_2$, 10 mM 5'-cAMP. Protein kinase A activity was determined at 37° for 10 min as described [48] with omission of the cAMP activation step in a reaction mixture (final volume 50 μL) containing 50 mM KH_2PO_4 pH 6.8, 10 mM $MgCl_2$, histone IIA-S (2 μg/mL), 1 mM [γ-^{32}P]ATP (37 kBq). All reactions were performed after incubating the samples for 10 min at 37° with different concentrations of hypericin, or DMSO (control).

Phosphoamino acid analysis. The phosphoamino acid content of the *in vitro* autophosphorylated EGF-receptor present in crude A431 membranes and separated from contaminating phosphorylated proteins by 7.5% SDS–PAGE was determined after partial acid hydrolysis according to Cooper *et al.* [49].

Statistical analysis. Each experiment was repeated three times and the mean and the standard deviation (SD) were calculated. All inhibition and kinetic data were mathematically analysed running a computer program for curve fitting and non-linear regression (Inplot). Two fits of data were statistically compared by analysing separately (two sets of data) or simultaneously (pooled data) the overall values for the sum of squares (SS) followed by F ratio calculation [50].

RESULTS

Inhibition of the autophosphorylation of the EGF-receptor

The effect of hypericin and quercetin on the autophosphorylation of the EGF-receptor were investigated, using a crude membrane preparation of A431 cells. Immunoblot experiments identified the EGF-receptor as a 170 kDa protein in these preparations (not shown). This phosphoprotein corresponds to the major radioactive spot on 7.5% SDS–polyacrylamide gels (Fig. 2). Subsequent phosphoamino acid analysis of the autophosphorylated EGF-receptor revealed the presence of phosphotyrosine only.

Hypericin causes a dose- and time-dependent inhibition of the EGF-stimulated autophosphorylation of the EGF-receptor (Figs 2 and 3A). The IC_{50} value (50 percent of inhibition in comparison with control experiments) decreases from 8.7 to 0.46 μM for a 10 and 30 min incubation period, respectively, (Fig. 3A). A longer incubation (60 min) with hypericin did not alter the inhibition curve anymore (significance of difference between two fits: $P > 0.05$) (not shown). Incubation of the membranes with hypericin at 30° for either 10 or 30 min resulted in IC_{50} values of 1.0 μM (data not shown) and 0.37 μM, respectively. For comparison, treatment of A431 membranes with quercetin, a known PTK-inhibitor [15], resulted in an inhibition (IC_{50}: 21 μM) (Fig. 3A), which is not time or temperature dependent.

Preliminary experiments showed that the basal autophosphorylation activity in the absence of hypericin is enhanced 2.3-fold by EGF (Fig. 2), which is consistent with previous data [51]. It can be seen (Fig. 2) that the inhibitory effect of hypericin on the EGF-receptor associated tyrosine kinase activity also affected the basal EGF-receptor autophosphorylation. Moreover, when membranes

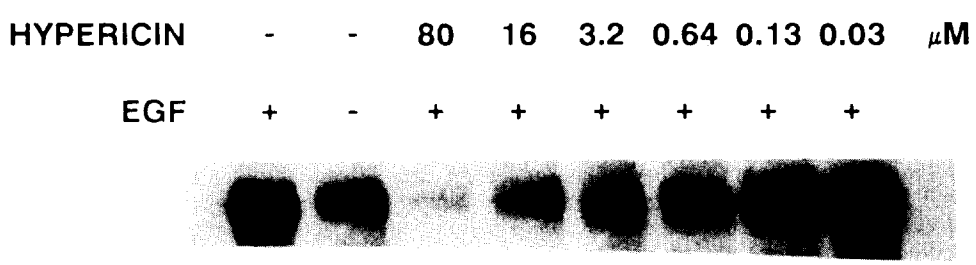

Fig. 2. Autoradiograph showing the effect of various concentrations of hypericin on the EGF stimulated autophosphorylation of the EGF-receptor after a 30 min incubation period at 0°, as detected in a 7.5% SDS–polyacrylamide gel as the major phosphoprotein (170 kDa).

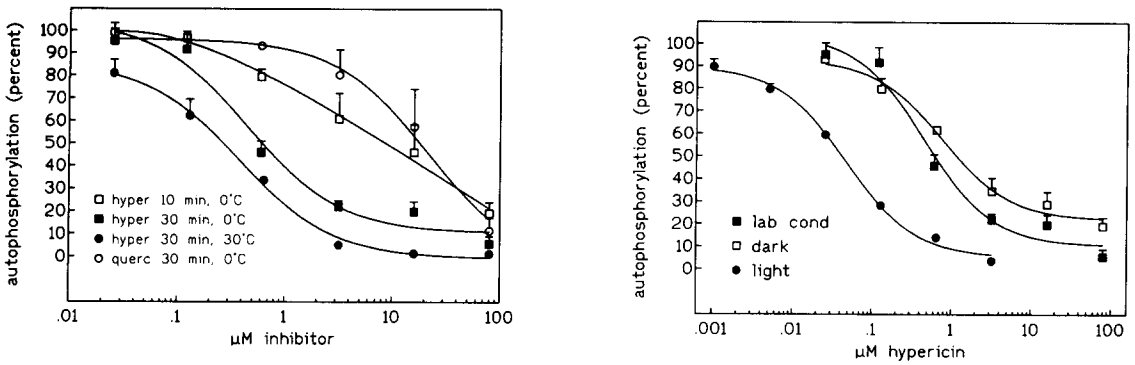

Fig. 3. The autophosphorylation of the EGF-receptor was investigated after EGF stimulation (1 μg/mL) of 2.5 μg crude A431 membranes at 0° for 20 min in the presence of different amounts of inhibitor or DMSO (control). Autophosphorylation of the EGF-receptor was determined as described in Materials and Methods. Each experiment was repeated three times and the mean ± SD are shown. Results are expressed as per cent of autophosphorylation activity of the EGF-receptor in control samples. (A) The incubations with the inhibitors quercetin (quer) or hypericin (hyper) were performed under standard laboratory light conditions for different periods at 0° or 30° as indicated. (B) During the incubation period (30 min, 0°) with hypericin the samples were irradiated with fluorescent light or kept in the dark. For comparison the inhibition curve obtained after incubating the samples with hypericin for 30 min at 0° under standard laboratory light condition is shown.

were not activated with EGF prior to an incubation period with different concentrations of hypericin (30 min, 0°), no difference was seen in the inhibition pattern shown in Fig. 3A ($P > 0.05$) (data not shown). It is inferred that the inhibition exhibited by hypericin is not dependent upon EGF stimulation.

A gradual chemical conversion of hypericin to a more potent inhibitory molecule would explain the time-dependent inhibition. Since, preincubating hypericin in phosphorylation buffer for different periods of time before adding the membranes did not generate a more active inhibitor species, it was concluded that the inhibition depends upon the time of contact between inhibitor and membranes.

Photosensitizing effects of hypericin

Since hypericin possesses strong photosensitizing characteristics [52–54], the inhibition of the EGF-receptor autophosphorylation by hypericin was examined after irradiating the samples for 30 min at 0° with fluorescent light. Under these circumstances the IC_{50} value for hypericin dramatically decreased to 44 nM (Fig. 3B). Assays performed after incubating crude A431 membranes for 30 min at 0° with hypericin in dark incubation conditions produced an IC_{50} of 0.75 μM which was not statistically different from results obtained with standard laboratory illumination ($P > 0.05$) (Fig. 3B). The relative importance of type I (free radical intermediates) or type II (involving activated oxygen) mechanisms in the observed photosensitization was investigated performing experiments under anaerobic conditions. Irradiation under these anaerobic conditions produced the same sensitization as observed in the presence of oxygen and no difference was noticed in the IC_{50} value ($P > 0.05$) (data not shown). This suggests that the photosensitizing effect of hypericin depends upon the formation of hypericin radicals (type I mechanism) leading to an enhanced inhibition of the autophosphorylation of the EGF-receptor.

Irreversibility of the inhibitory effect of hypericin

In order to test the reversibility of the PTK inhibition, membranes were incubated with hypericin or quercetin, followed by a double wash with phosphorylation buffer. In a control experiment (Fig. 4A: no washes) the inhibition exhibited by hypericin (16 μM) and quercetin (80 μM) were comparable, whereas after a double wash (Fig. 4B) the PTK activity of the EGF-receptor was restored almost completely in the case quercetin was used as inhibitor. The effect of hypericin however was still present.

In the course of the experiments it was noticed that solubilization of the membrane preparations with Triton X-100 prior to incubation with hypericin abolished completely the inhibitory activity (Fig. 5). Interestingly, in the reverse order, i.e. membranes incubated with hypericin followed by a Triton X-100 solubilization, a full inhibition was preserved (Fig. 5).

In order to document further the irreversible mechanism of inhibition of the PTK activity of the EGF-receptor by hypericin, its exogenous activity towards the PTK substrate poly(GAT) was assayed at various concentrations of both poly(GAT) and ATP in the presence of hypericin. Hypericin exerted a complex inhibition on the phosphorylation of the exogenous substrate poly(GAT): small amounts of inhibitor suggested a mixed competition whereas a non-competitive inhibition is observed at higher hypericin concentrations (results not shown). The K_m for poly(GAT) was found to be 83 μM. Hypericin acted as a non-competitive inhibitor when ATP was

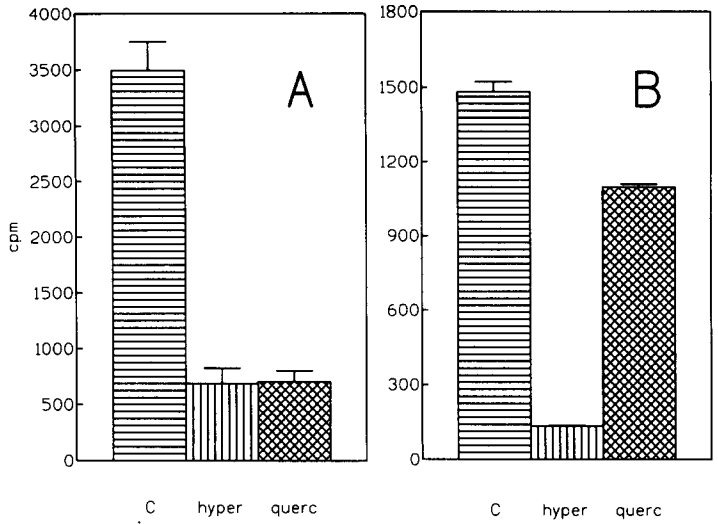

Fig. 4. Autophosphorylation of the EGF-receptor, measured as in Fig. 3, in the presence of either 16 μM hypericin (hyper), 80 μM quercetin (querc) or vehicle (C). The following two conditions were used: (A) membranes were incubated for 30 min at 0° with the inhibitors before autophosphorylation (B) after incubation (30 min, 0°), the inhibitors were washed out by pelleting the membranes twice in a microcentrifuge (10,000 g, 5 min) followed by resuspension in fresh phosphorylation buffer before autophosphorylation. Each experiment was repeated three times and the mean ± SD are shown.

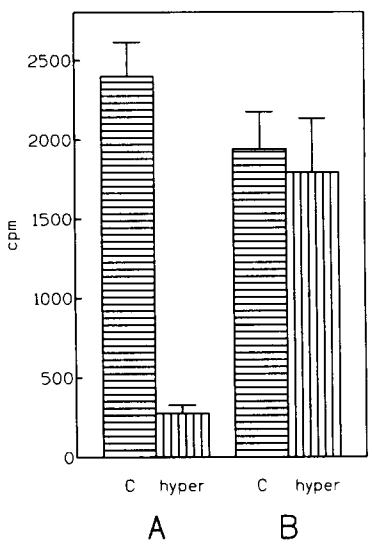

Fig. 5. Autophosphorylation of the EGF-receptor, measured as in Fig. 3, in the presence of 16 μM hypericin (hyper) or vehicle (C). The following two conditions were used: (A) after incubation with hypericin, the membranes were homogenized in phosphorylation buffer supplemented with 0.25% Triton X-100, centrifuged (5 min, 10,000 g) and the supernatant fraction used for autophosphorylation of the EGF-receptor (B) the membranes were homogenized in phosphorylation buffer supplemented with 0.25% Triton X-100 and centrifuged. The supernatant fraction was incubated with hypericin (30 min 0°) before autophosphorylation. Each experiment was repeated three times and the mean ± SD are shown.

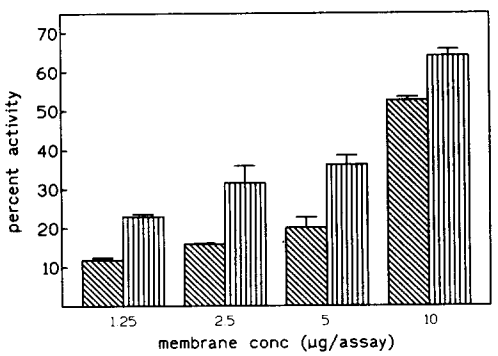

Fig. 6. Autophosphorylation activity of the EGF-receptor determined after incubating different amounts of membranes (1.25, 2.5, 5 and 10 μg) with 16 μM hypericin (hatched, diagonal) or 3.2 μM hypericin (hatched, vertical) for 30 min at 0° as in Fig. 3. Values are expressed as per cent of control values. Each experiment was repeated three times and the mean ± SD are shown.

used as the variable substrate. The K_m for ATP was 16 μM.

Effect of membrane concentration

As can be seen in Fig. 6, the inhibition of the EGF-receptor autophosphorylation by hypericin (3.2 and 16 μM) was inversely proportional to the amounts of crude A431 membranes used. The inclusion of excess proteins such as bovine serum albumin (0.01–0.15%) in the experiments did not interfere with the inhibition by hypericin (not

Table 1. Fifty per cent of inhibition values (IC_{50}) of hypericin determined in the present study or previously reported for different enzymatic activities

Enzyme	IC_{50} (μM)	Light condition	Ref.
EGF-receptor (b)	0.37	Standard	(a)
	0.044	Fluorescent light	(a)
Casein kinase I	>100	Standard	(a)
Casein kinase II	>100	Standard	(a)
5'-Nucleotidase	>100	Standard	(a)
Protein kinase A (c)	>100	Standard	(a)
Protein kinase A	>80	Standard	[43]
Protein kinase C (d)	3.4	Standard	[43]
MLCK (e)	>40	Standard	[43]
Monoaminoxidase A (b)	68	Standard	[56]
Monoaminoxidase B (b)	420	Standard	[56]
Reverse transcriptase (f)	0.77	Standard	[37]
DNA polymerase	14.7	Standard	[37]
Succinoxidase	14	Standard	[52]
	2.4	Fluorescent light	[52]

If not mentioned in the literature data are presumed to have been determined under standard laboratory illumination. (a) Present study, (b) irreversible inhibition, (c) catalytic subunit, (d) regulatory domain is inhibited, (e) myosin light chain kinase, (f) other authors [35, 36, 38] did not find an inhibition.

shown). Considering the lipophilic nature of hypericin, the membrane effects are probably due to a decrease in the soluble inhibitor concentration by an aspecific accumulation in the lipid bilayer.

Effect of hypericin on other enzymes

The specificity of the hypericin inhibition was explored by testing the sensitivity to inhibition of three Ser/Thr kinases (casein kinase 1, casein kinase 2 and protein kinase A) and one nucleotidase (5'-nucleotidase, from snake venom). None of these enzymes were inhibited even at high concentrations ($IC_{50} > 100\ \mu M$) of hypericin as shown in Table I, which also includes the known literature data on hypericin inhibitions.

DISCUSSION

This study reports a dramatic and dose-dependent inhibition of the EGF-receptor PTK autophosphorylation *in vitro* by the anthranoid hypericin. Significantly, quercetin, a compound with known PTK-inhibitory activity [15], showed no time dependence and did not irreversibly inhibit the autophosphorylation. Moreover, when assayed in the presence of the exogenous substrate poly(GAT), hypericin showed a non-competitive inhibition towards ATP and exerted inhibition towards the phosphate acceptor, showing a non-competitive inhibition at high hypericin concentrations. This is in agreement with the inhibitory activity of an irreversible inhibitor since a non-competitive behaviour is expected when the concentration of active enzyme in solution is decreased by irreversible binding to the inhibitor, influencing the velocity of the reaction without affecting the K_m value [55].

Unexpectedly, when the membrane preparations were homogenized in phosphorylation buffer supplemented with Triton X-100 before hypericin incubation, the PTK activity of EGF-receptors was no longer inhibited. Conversely, an incubation of hypericin with the EGF-receptor prior to homogenization did not abrogate the inhibition. Since it is reasonable to assume that the detergent would disrupt the interaction of hypericin molecules hydrophobically bound to the EGF-receptor, it is inferred that the irreversible binding of hypericin is of a covalent nature. The abrogation of the inhibitory effect by homogenizing membranes in the presence of Triton X-100 before incubation with hypericin is poorly understood. It is possible that the lipophilic molecule hypericin surrounds itself by detergent molecules in an aqueous environment, thereby preventing the interaction with the receptor. However, it is known that hypericin localizes selectively in membranes when incubated with cultured cells, probably by the presence of a lipid bilayer [52, 53]. Consequently, the integrity of the membrane structure could also be very important for a full inhibitory effect: it is assumed that the sequestration of the compound in the membrane leads to an enhanced inhibition by favouring the interaction with the EGF-receptor. Of interest, the accumulation in membranes has also been implicated in the mechanism of the anti-retroviral activity of hypericin [38, 39].

The present work illustrates the important effect of fluorescent light on the inhibition of the EGF-receptor autophosphorylation by hypericin. When compared with standard light conditions the calculated IC_{50} value was 10-fold lower after irradiation. This extends previously observed photosensitizing effects on the biological activities of the compound [31, 40, 41, 52–54]. Photosensitization is described as a process initiated by photon excitation of the ground state of the sensitizer (S) to the excited singlet state ($^1S^*$). After spin inversion, the generated excited triplet state ($^3S^*$) is converted by a hydrogen

or electron transfer from a substrate to a radical ($SH^{\cdot}$) or a radical anion ($S^{\cdot -}$), respectively (type I mechanism). Conversely, in the presence of oxygen the triplet state of the sensitizer might generate highly active singlet state oxygen ($^1O_2$), the main product of the type II mechanism [32, 52]. In general, the photosensitizing effects of hypericin, e.g. oxidation of organic molecules [31], toxicity [53, 54], enzyme inhibition [52] or antiviral activity [40, 41], are described as type II reactions, since in most cases a substantial contribution of singlet oxygen could be proven. However, since experiments performed either in anaerobic or aerobic conditions gave similar results, we did not find any evidence for a type II dependent photosensitizing mechanism.

Due to its antiretroviral effects observed *in vitro* as well as *in vivo* and the lack of toxicity and mice and humans [35], hypericin is under investigation as a therapeutic tool against retroviral induced diseases such as the acquired immunodeficiency syndrome (AIDS) [35–42]. The causative agent of AIDS is the human immunodeficiency virus (HIV) which upon binding to the cell surface CD4 lymphocyte receptor, enters into the cell and leads to cytopathic effects causing cell death [57]. Recently it was shown the HIV-CD4 T-cell-receptor interaction induces a rapid tyrosine phosphorylation of different target proteins [58] as well as protein kinase C activation and consequent CD4-receptor phosphorylation [59]. These observations strongly suggest that early tyrosine phosphorylations, probably due to the activated receptor associated $pp56^{lkc}$ PTK activity [60], and protein kinase C activation are required for intracellular transmission of the HIV-induced cytopathic signal. Therefore, the potent inhibitory activity of hypericin towards the PTK activity of the EGF-receptor shown in this study, could become important in explaining the reported *in vitro* anti-HIV activity of hypericin [37, 40]. Besides, since inhibitors of PTK activity are commensurate with potential antineoplastic activity, the molecule deserves further exploration to fully assess its potential important pharmacological features.

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