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EFFECTS OF ANTHRALIN AND HYPERICIN ON GROWTH FACTOR SIGNALLING AND CELL PROLIFERATION *IN VITRO*

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Abstract—The effect of the anthranoids, anthralin and hypericin, on epidermal growth factor receptor (EGF-R) activation and their degree of specificity was examined. Hypericin, but not anthralin, was found to inhibit binding of [^{125}I]-labelled epidermal growth factor (EGF) to HN5 squamous carcinoma cells that overexpress EGF-R. This effect was a result of a dose- and time-dependent reduction of EGF-R number and affinity. Neither compound directly inhibited EGF-induced tyrosine phosphorylation of the EGF-R in HN5 cells. Although anthralin and hypericin both inhibited the mitogenic effect of EGF in NR6/HER cells (IC_{50}s = 100 nM and 10 μM, respectively), they also had comparable effects on DNA synthesis in response to acidic fibroblast growth factor (aFGF) and platelet-derived growth factor (PDGF). When tested in proliferation assays using cells expressing differing numbers of EGF-R, the growth inhibitory effects of both compounds were independent of EGF-R number. We conclude that, although anthralin and hypericin both inhibit EGF signalling, they do not act specifically on the EGF-R pathway. Moreover, their mechanisms of action do not appear to be comparable.

Key words: hypericin; anthralin; epidermal growth factor-receptor; epidermal growth factor; platelet-derived growth factor; acidic fibroblast growth factor

The anthranoid anthralin (dithranol) is used as a topical treatment for psoriasis, a disease characterised by overproliferation of keratinocytes. Keratinocytes express both the EGF-R† and three of its ligands, TGFα [1], amphiregulin [2], and heparin-binding EGF [3], indicating that growth *in vivo* may be supported by an autocrine mechanism(s). In the psoriatic plaque, there is an upregulation of both EGF-R and TGFα [4, 5] suggesting that overstimulation of the EGF-R pathway may contribute to the pathological state. In support of this, Gottlieb *et al.* [6] have shown that anthralin decreases keratinocyte TGFα expression, inhibits cellular binding of [^{125}I]EGF (but not [^{125}I]IGF-1), and selectively inhibits keratinocyte growth.

Hypericin, a naturally occurring compound extracted from plants of the Hypericum genus, is structurally similar to anthralin (Fig. 1). Hypericin is a photodynamic pigment that produces ROS in the presence of visible light. Although historically it has been used as an antidepressant, hypericin is currently being evaluated as an antiviral agent. It appears to affect viral activity by multiple mechanisms, including inhibition of *de novo* infection of cells, inhibition of virus assembly and budding, and inactivation of virus reverse transcriptase [7, 8]. Recently, De Witte *et al.* [9] have shown that hypericin causes an irreversible time- and dose-dependent inhibition of the EGF-R tyrosine kinase, but is less active against several serine/threonine kinases, in a cell-free system. Because hypericin has structural similarities with the tyrosine kinase inhibitor erbstatin, the authors suggest that it may be a selective tyrosine kinase inhibitor.

Tyrosine kinase activity is a common feature of transmembrane growth factor receptors and other intracellular molecules involved in growth factor signal transduction pathways. Aberrant signalling by these molecules has been implicated in the pathogenesis of neoplasia [10]. In the case of the EGF-R, it has been shown by transfection to cause ligand-dependent transformation when overexpressed [11]. Consistent with this observation, the EGF-R is expressed at high levels in a significant proportion of carcinomas [12] that have also been shown to express ligand [13]. Further, for several tumour types, the presence of EGF-R has been associated with disease recurrence, the presence of metastases, and poor survival [12]. Therefore, agents that specifically antagonise EGF-R signalling pathways are of interest as potential chemotherapeutic drugs. In this study, we have examined the abilities of anthralin and hypericin to (i) modulate EGF-R ligand binding, (ii) inhibit EGF-induced EGF-R tyrosine kinase activity in cultured cells, and (iii) selectively inhibit EGF-R-driven cellular proliferation.

MATERIALS AND METHODS

Materials

Recombinant hEGF and cell culture reagents were purchased from Life Technologies, Paisley, U.K. [^{125}I]EGF was prepared using Iodo-beads (Pierce, Luton, U.K.) according to manufacturers instructions. Partially pure porcine PDGF was purchased from Bioprocessing, Consett, U.K. aFGF was purchased from R&D Systems, Abingdon, U.K. Ultraculture was purchased from Bio-Whittaker, Reading, U.K. A431 cells were ob-

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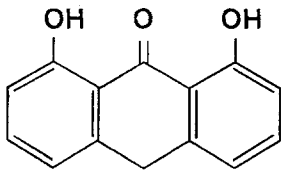
† Abbreviations: EGF, epidermal growth factor; EGF-R, epidermal growth factor-receptor; TGFα, transforming growth factor alpha; IGF-1, insulin-like growth factor-1; PDGF, platelet-derived growth factor; aFGF, acidic fibroblast growth factor; PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; PMSF, phenylmethylsulphonyl fluoride; ECACC, European Collection of Animal Cell Cultures; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

Anthralin



Hypericin

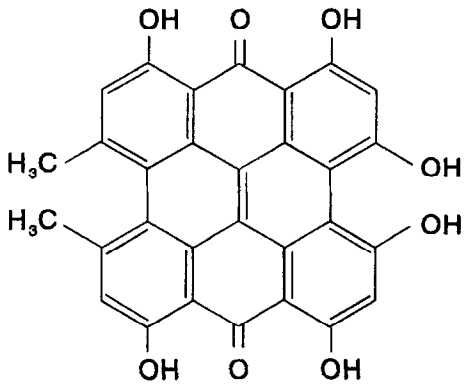


Fig. 1. Structure of anthralin and hypericin.

tained from ECACC, Centre for Applied Microbiology and Research, Salisbury, U.K. LICR-LON-HN5 squamous carcinoma cells [14] were a gift from Prof B. Gusterson, Institute of Cancer Research, Sutton, U.K. NR6 cells and NR6/HER cells were a gift from Dr G. Panayatou, Ludwig Institute for Cancer Research, London. Biotinylated anti-phosphotyrosine antibody (PT-66), anthralin, and hypericin were purchased from Sigma, Poole, U.K. 20 mM stock solutions of the inhibitors in DMSO were stored at −20°C and diluted in assay buffer as required. Dilutions of the vehicle alone had no effect on cell growth or DNA synthesis.

Cell culture

Cells were grown routinely in DMEM containing 10% FBS, 2 mM glutamine, 1 × nonessential amino acids, 10 U/mL penicillin, and 10 μg/mL streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of air containing 5% CO_2.

[^{125}I]EGF binding assay

Radioligand binding assays were performed as previously described [15], with only slight modification. Briefly, HN5 cells were grown to 80% confluence in sterile 96-well plates and then incubated with 10% FBS/DMEM ± anthralin or hypericin at 37°C. The medium was then replaced with DMEM/1% BSA/0.02% sodium azide for 30 min. The cells were then incubated with 10 ng/mL [^{125}I]EGF (sp. act. 0.75 MBq/μg) in DMEM/1% BSA/0.02% azide at 37°C for 2 hr. Under these conditions, there was no internalisation of EGF-R. The radiolabelled cells were then washed with PBS/0.1% BSA. Cells were dried and then dissolved in 0.5 M NaOH for determination of bound ligand by gamma counting. Nonspecific binding of [^{125}I]EGF was measured in the presence of 2 μg/mL unlabelled EGF and was <5% of total binding. To enable ligand binding to be directly related to cell number, a duplicate set of wells was treated as above and then fixed with formol saline for measurement of cell number using the methylene blue protocol described below.

For quantitation of EGF-R number and affinity, HN5 cells were seeded into 24-well tissue culture plates (1×10^5 cells/well) and incubated in medium ± hypericin for 2 hr. The medium was then removed and binding of doubling dilutions of [^{125}I]EGF (0.1–200 ng/mL) was assayed as described above. Nonspecific binding of [^{125}I]EGF was measured in the presence of 5 μg/mL unlabelled EGF and was <2% of total binding. The number and K_d of high- and low-affinity EGF-Rs was derived by Scatchard analysis of bound [^{125}I]EGF.

Measurement of EGF-receptor phosphorylation

HN5 cells were grown to 80% confluence in 10% FBS/DMEM in sterile 96-well plates. The medium was then changed to Ultraculture serum-fee medium and the cells incubated at 37°C for 30 min with medium ± anthralin or hypericin. To induce EGF-R tyrosine phosphorylation, EGF (100 ng/mL) was added to wells for 5 min at 37°C. The cells were then washed with PBS containing phosphatase and protease inhibitors (100 mM NaF, 1 mM $NaVO_4$, and 1 mM PMSF), and solubilized with 100 μL/well of 80 mM Tris pH 6.8 containing 0.1 M DTT, 2% SDS, bromophenol blue, and 10% glycerol. Proteins were separated by SDS-PAGE on a 7.5% acrylamide gel and transferred to a nitrocellulose membrane by electrophoresis. Following blocking of nonspecific binding sites with PBS/3%, skimmed milk, membranes were incubated with biotinylated antiphosphotyrosine antibody (5 μg/mL). Bound antibody was detected with streptavidin biotinylated-HRP and visualised using ECL according to manufacturers instructions (Amersham, U.K.).

Measurement of mitogenic activity

The method used was as previously described [15]. Growth factors and test substances were diluted into mitogenesis assay medium (1:1 PBS and serum free DMEM containing 25 mM HEPES pH 7.5, 2% BSA, 8 μg/mL insulin, and 480 μg/mL transferrin) and added to the cells at appropriate concentrations as indicated in the Figure legend. Where the drugs were added to cells during S phase, they were added in serum free DMEM.

Cell proliferation assay

Methylene blue dye was used to assess the cell density of adherent cell cultures as previously described [15]. Briefly, cells in 10% FBS/DMEM were seeded into sterile 96-well tissue culture plates (5×10^3 cells/well) and allowed to attach for 5 hr. The medium was then removed and replaced with Ultraculture serum free medium ± anthralin or hypericin. The cells were incubated for 4 days; the medium was replaced daily to minimise exhaustion or degradation of the test solution. To measure cell density, the cells were fixed in formol saline (4% formaldehyde, 0.15 M NaCl) and stained with 1% methylene blue in 10 mM $Na_2B_4O_7 \cdot 10H_2O$ pH 8.5, then washed with 10 mM $Na_2B_4O_7 \cdot 10H_2O$ pH 8.5. Dye

was extracted from the cells with 1:1 0.1 M HCl/ethanol. The A_{650nm} of individual wells was determined using a microplate photometer. An A_{650nm} of 1.0 was equivalent to 2.25×10^4 cells/well.

RESULTS

Effect of inhibitors on [^{125}I]EGF binding to HN5 cells

Anthralin has been reported to selectively modify EGF-R levels of keratinocytes in culture [6]. In this study, we compared the effect of anthralin and hypericin on binding of [^{125}I]EGF to the HN5 squamous carcinoma cell line, which expresses high levels of EGF-R. HN5 cells were preincubated with 0.2–50 μM anthralin (1 μM = 0.23 μg/mL) or 0.2–50 μM hypericin (1 μM = 0.5 μg/mL) for 30 min, 2 hr or 21 hr. Although short incubations with anthralin had little effect on subsequent binding of [^{125}I]EGF, after 21 hr exposure to 0.8–12 μM anthralin there was a slight increase in the amount of ligand bound/cell. The only evidence for any reduction in receptor binding was observed after 21 hr exposure to 50 μM anthralin, where ligand binding was reduced by 30% (Fig. 2).

In contrast to the results obtained for anthralin, hypericin was found to cause a dose- and time-dependent decrease in [^{125}I]EGF binding (Fig. 2). The effect of hypericin on EGF-R number and affinity was further investigated in equilibrium binding experiments using Scatchard analysis. As shown in Table 1, pretreatment of cells with hypericin for 2 hr caused a dose-dependent reduction in the number of high- and low-affinity receptors, as well as decreasing their affinity for ligand.

EGF-R tyrosine phosphorylation

Ligand activation of the EGF-R induces rapid receptor autophosphorylation on intracellular tyrosine residues. When HN5 cells are stimulated with EGF and analysed by SDS-PAGE and Western blotting, the major tyrosine-phosphorylated protein detected is the EGF-R. To determine whether the anthranoids affected the EGF-R tyrosine kinase, HN5 cells were pretreated for 30 min with either anthralin or hypericin and then exposed to a high concentration of EGF. By using a relatively short exposure to the drug and assaying activity in the presence of an excess of ligand, we were attempting to minimise the effects of the compounds on ligand binding and to ensure that remaining EGF-Rs had sufficient ligand to fully activate them, even if they were of lower affinity. Under these conditions, a 30 min preincubation with anthralin or hypericin (0.5 μM or 50 μM) was found to have no significant effect on the level of EGF-induced receptor phosphorylation (Fig. 3).

Table 1. Effect of hypericin on EGF-Rs in HN5 cells

	High-affinity receptors		Low-affinity receptors	
	No./cell	K_d (M)	No./cell	K_d (M)
Control	1.8×10^6	9.4×10^{-10}	8.7×10^6	7.8×10^{-9}
+ 12.5 μM hypericin	7.1×10^5	6.1×10^{-9}	1.9×10^6	3.1×10^{-8}
+ 50 μM hypericin	6.1×10^5	7.8×10^{-9}	1.5×10^6	6.9×10^{-8}

HN5 cells were pretreated with hypericin for 2 hr before performing a radioligand binding assay as described in the Materials and Methods. The number of EGF binding sites per cell and their affinities were determined by Scatchard analysis. Results are the mean of duplicate determinations.

Effects on growth factor-induced DNA synthesis

Anthralin and hypericin have both been reported to inhibit cell growth *in vitro* [6, 16], possibly by blocking growth factor signalling pathways. To determine whether or not the compounds inhibited specifically the

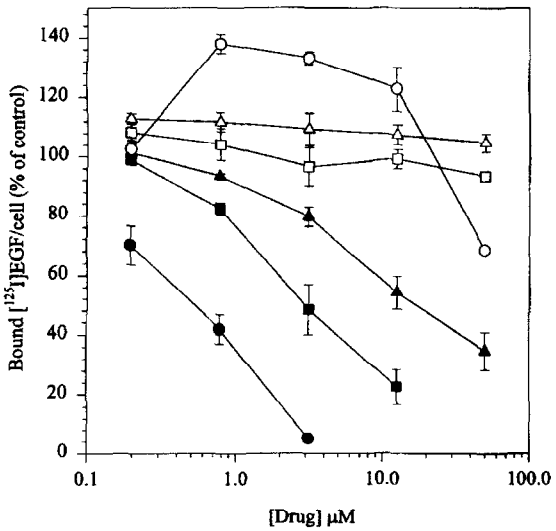


Fig. 2. Effect of compounds on binding of [^{125}I]EGF to HN5 cells. Cells were incubated with anthralin (white symbols) or hypericin (black symbols) for 30 min (triangles), 2 hr (squares), or 21 hr (circles) prior to the addition of [^{125}I]EGF as described in Materials and Methods. Binding of [^{125}I]EGF/cell is expressed as a % of binding to untreated cells. Data points are the mean ± SEM of three individual experiments.

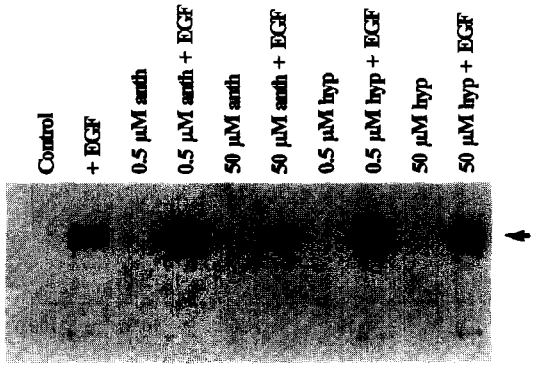


Fig. 3. Effect of anthralin and hypericin on EGF-R tyrosine phosphorylation. Control HN5 cells or cells preincubated with either compound (0.5 or 50 μM) for 30 min were stimulated with 100 ng/mL EGF for 5 min. Cells were then solubilised and analysed by SDS-PAGE and Western blotting as described in Materials and Methods. The blot was incubated with antiphosphotyrosine antibody and the arrow (right) indicates the 170 KDa band corresponding to the EGF-R. The lanes show unstimulated and EGF-stimulated HN5 cells. Treatment with anthralin (anth) or hypericin (hyp) prior to stimulation is as indicated.

EGF-R pathway, quiescent NR6/HER (mouse fibroblasts transfected with the gene for human EGF-R) were stimulated with EGF, PDGF, and aFGF in the presence of dilutions of anthralin or hypericin. Both compounds inhibited EGF-induced DNA synthesis dose-dependently, with anthralin being 100 times more potent than hypericin (IC_{50}s = 100 nM and 10 μM). However, DNA synthesis in response to PDGF and aFGF was similarly inhibited. Figure 4 shows that the dose-response curves for each compound were identical for all three growth factors.

To investigate whether or not the inhibitory effects of the compounds were due to nonspecific effects on thymidine incorporation, cells were incubated with growth factor for 20 hr and anthralin or hypericin were added during the [^{125}I]UdR pulse-labelling phase of the assay. At 20 hr, anthralin had no effect on DNA synthesis induced by EGF, PDGF, or aFGF (results not shown). In contrast, concentrations of hypericin greater than 10 μM caused some inhibition of DNA synthesis, but this effect was less than when added with growth factor at the beginning of the experiment (Fig. 4). It seems likely that the biphasic nature of the hypericin dose-response curve, with an inflection at around 10 μM, reflects the commencement of this secondary effect of hypericin. The dashed line (Fig. 4) shows the dose-response curve for inhibition of EGF-induced DNA synthesis by hypericin after correction for the effect of the compound during the pulse-labelling phase of the assay.

Effects on cell growth

To further evaluate the effect of anthralin or hypericin on EGF-R-induced growth stimulation, we selected four cell lines with different levels of EGF-R expression and examined their growth rate in the presence of the drugs. A431 (squamous carcinoma cell line) and HN5 cells express high levels of EGF-R (approx. 10^6 and 10^7 EGF-R/cell, respectively [14, 17]). NR6 cells lack EGF-R [18], and the NR6/HER cells express moderate levels of human EGF-R. Cells were seeded at a low density on day 0 in 10% FBS/DMEM then grown for 4 days in serum-free medium in the presence of anthralin or hypericin (125 nM-2 μM). HN5 and A431 cells grow in serum-free medium without the requirement for exogenous growth factors, whereas NR6 and NR6/HER cells had sufficient serum-derived growth factors bound to undergo 1–2 doublings in serum-free medium over a four-day period. Both compounds were found to cause dose-dependent inhibition of proliferation of all four cell lines. However, whereas we had observed that anthralin was 100 times more potent than hypericin in the mitogenesis assay, the IC_{50} values for the two compounds differed by less than a factor of 4 for each of the cell lines tested in the proliferation assays (Table 2). Similar results were obtained when the assays were performed in 10% FBS/DMEM (results not shown).

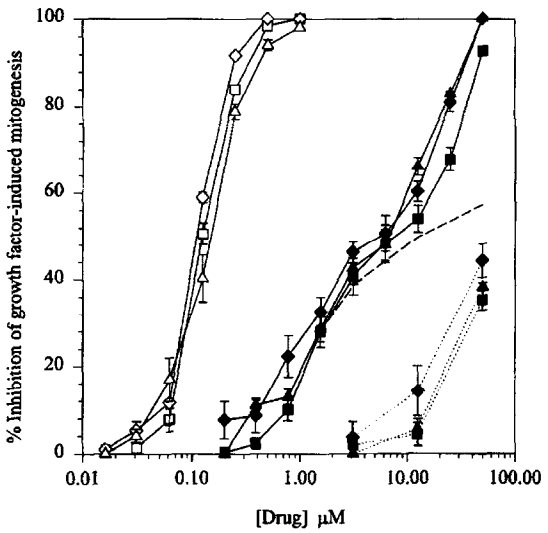


Fig. 4. Effect of anthralin (white symbols) or hypericin (black symbols) on mitogenesis induced by 1 ng/mL EGF (squares), 2.5 IU/mL PDGF (diamonds) or 0.4 ng/mL aFGF (triangles). DNA synthesis was measured by incorporation of [^{125}I]UdR into NR6/HER fibroblasts during S-phase as described in Materials and Methods. The drugs were added with the growth factor (solid lines) or 20 hr later during S-phase (dotted lines). Data points are the mean ± SEM of three individual experiments. The dashed line shows the effect of hypericin on EGF-induced DNA synthesis after correction for its effect when added at 20 hr.

Effect of light exposure on response to hypericin

Hypericin is a photodynamic compound that produces ROS following exposure to visible light. To determine if production of ROS contributed to the inhibitory effect of hypericin, experiments were performed under high and low light conditions. Unless otherwise stated, experiments were set up in low light (<50 lux) and incubations were performed in the dark. Light treatment in the presence of hypericin consisted of irradiation with fluorescent light at 500 lux or 4500 lux for 30 min at room temperature.

Figure 5 shows that light had a marked effect on hypericin-induced inhibition of [^{125}I]EGF binding and EGF-induced mitogenesis in NR6/HER cells. Under low light conditions, hypericin had little effect but at the highest light intensity it almost completely blocked [^{125}I]EGF binding and DNA synthesis. In the previous experiments, shown in Figs. 2 and 4, hypericin-treated cells had been exposed to 500 lux for 40 and 15 min, respectively during assay preparation. Therefore, the effect of hypericin appeared to be due to light activation. We also determined the effect of light on ligand-induced EGF-R phosphorylation. Under limited light conditions (Fig. 3, where hypericin-treated cells had been exposed to 500 lux for a maximum of 5 minutes, and Fig. 6B)

Table 2. Inhibition of cellular proliferation by anthralin or hypericin

	IC_{50} (μM)	
Cell line	Anthralin	Hypericin
NR6	0.5	1.9
NR6/HER	0.4	1.1
A431	0.4	1.0
HN5	0.7	0.8

Cells were cultured for 4 days in serum-free medium in the presence of anthralin or hypericin as described in the Materials and Methods. IC_{50} values are the mean of three individual experiments.

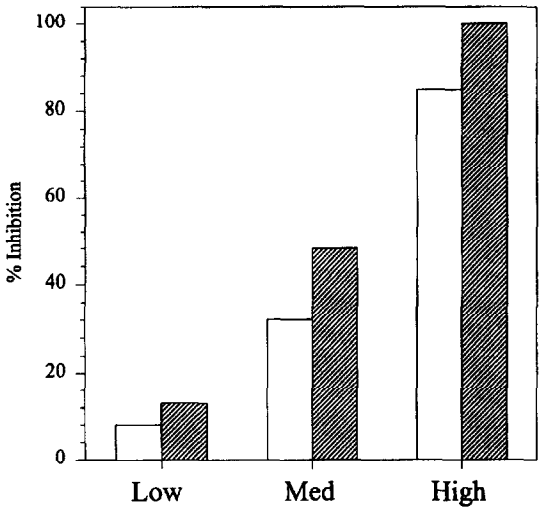


Fig. 5. Effect of light on hypericin-induced inhibition of [^{125}I]EGF binding (white bars) or EGF-induced mitogenesis (hatched bars). [^{125}I]EGF binding was measured as described in Materials and Methods. Cells were incubated with 6 μM hypericin for 2 hr then exposed to low (<50 lux), medium (500 lux), or high (4500 lux) light conditions for 30 min prior to incubation with [^{125}I]EGF. Mitogenesis induced by 1 ng/mL EGF was assayed as described in Materials and Methods. 6 μM hypericin was added to the cells with the growth factor and then irradiated for 30 min as described above. Data points are the mean of two determinations.

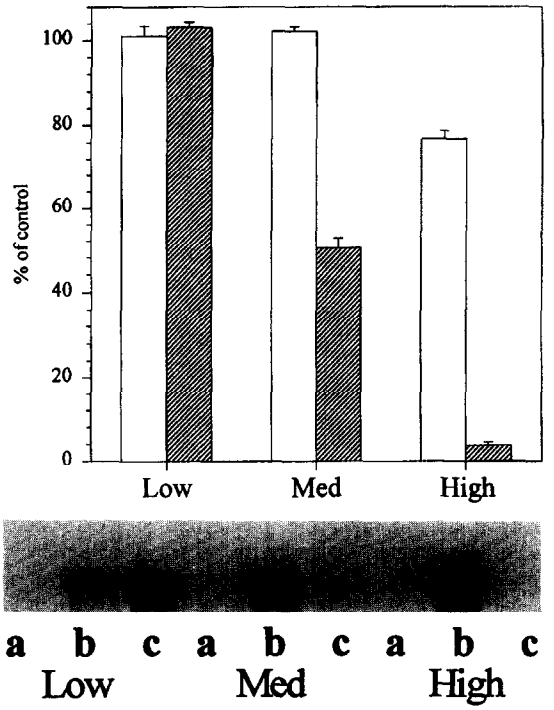


Fig. 6. Effect of light on [^{125}I]EGF binding and EGF-R phosphorylation in hypericin-treated cells. HN5 cells were incubated with 50 μM hypericin for 30 min at room temp. under low (<50 lux), medium (500 lux), or high (4500 lux) light conditions. All subsequent procedures were performed at <50 lux or in the dark. Cell number, [^{125}I]EGF binding and EGF-R phosphorylation induced by 100 ng/mL EGF were determined as described in Materials and Methods. (A) Graph shows cell number (white bars) or [^{125}I]EGF binding/cell (hatched bars) expressed as a % of control cells that were irradiated but not treated with hypericin. Data points are the mean ± SE of four determinations. (B) Western blot shows the effect of different light intensities on EGF-R phosphorylation in (a) unstimulated control cells; (b) control cells stimulated with EGF; (c) cells treated with hypericin then stimulated with EGF.

hypericin did not inhibit receptor phosphorylation. However, following light exposure [^{125}I]EGF binding/cell and EGF-R phosphorylation were inhibited in parallel (Fig. 6).

DISCUSSION

In this study, we have shown that the structurally related compounds, anthralin and hypericin, both inhibit DNA synthesis and cellular proliferation but that neither compound appeared to directly affect EGF-R tyrosine kinase activity. Further, only hypericin causes a time- and dose-dependent reduction in EGF-R number and affinity on HN5 squamous carcinoma cells.

The finding that anthralin had little effect on EGF binding to HN5 cells contrasts with a previous report [6] where anthralin reduced binding of [^{125}I]EGF to keratinocytes. However, these authors demonstrated that 100 times more anthralin was required to cause a 50% reduction in receptor binding than was required to inhibit keratinocyte growth in culture by 98% (approx. 5 μM versus 50 nM). In the present study, we found a similar discrepancy between the effects of anthralin on EGF-R number and its growth inhibitory properties. Together these observations suggest that growth inhibition by anthralin is not a direct result of modulation of EGF-R number. Indeed, the equivalent effects of anthralin on aFGF- or PDGF-induced mitogenesis, and the lack of any differential effect of anthralin on proliferation of cells expressing different numbers of EGF-R, indicates that anthralin shows no specificity towards the EGF-R.

EGF, PDGF, or aFGF are three distinct mitogens that have receptors with intrinsic, ligand-activated tyrosine kinase activity. The signal transduction pathways of these growth factors have a number of features in common, namely formation of receptor/protein complexes *via* Src-homology 2 (SH2) domains, stimulation of phosphatidyl inositol turnover and activation of Ras and the MAP kinase pathway [19]. Anthralin may, therefore, affect the mitogenic signal at one or more points downstream of receptor signalling. In support of this, anthralin has been reported to inhibit protein kinase C (PKC) [20]. Activation of PKC is thought to play a key role in signal transduction for a variety of cellular processes, including mitogenesis [21]. Alternatively, growth inhibition by anthralin (a relatively unstable compound that undergoes auto-oxidation under physiological conditions [22]), may be due to inhibition of mitochondrial function by formation of ROS or secondary free radicals [23, 24].

Comparatively, hypericin is a more stable compound than anthralin but it can be induced to form ROS by photosensitization [25]. By varying the light conditions during hypericin treatment of cells we have shown that the observed inhibitory effects of hypericin were largely, if not exclusively, due to production of ROS.

Unlike anthralin, hypericin induced a time- and dose-dependent reduction in EGF-R number and affinity. At

concentrations of hypericin less than 10 μM, the observed inhibition of EGF binding to cells may account for its inhibition of EGF-induced DNA synthesis as both effects showed similar dose-responses. Hypericin is a hydrophobic compound that associates with plasma membranes; upon illumination, production of ROS can cause membrane disruption and protein cross-linking [26]. In view of its equivalent potency in inhibiting DNA synthesis in response to EGF, aFGF, and PDGF, we suggest that it may irreversibly modify the extracellular and transmembrane domains of membrane proteins, including growth factor receptors with a consequent inhibition of ligand binding and cellular signalling.

The potency of hypericin was enhanced in the proliferation assay when compared to the mitogenesis assay. Two factors appear to contribute to this effect. First, in the proliferation assay, the treated cells were repeatedly exposed to light (approximately 500 lux for 15 min/day over four days) as the assay medium was changed on a daily basis and, therefore, production of ROS would have been greater. Secondly, the effect of hypericin is time-dependent and the proliferation assay was of a longer duration. In this case, the action of hypericin is consistent with accumulation of an irreversible modification.

In the mitogenesis assays, use of high concentrations (10–50 μM) of hypericin caused a second effect of the compound to become evident as it began to have a marked effect on DNA synthesis when added 20 hours after mitogen, when the cells were in S-phase. As the mitogenesis assay depends on uptake and utilization of [^{125}I]UdR by thymidine kinase, hypericin may have been affecting these processes. Alternatively, as hypericin has been shown to inhibit PKC with an IC_{50} of 3.4 μM [27], effects of hypericin on intracellular Ser/Thr protein kinases such as PKC cannot be excluded.

In view of the fact that hypericin inhibited [^{125}I]EGF binding, it was difficult to discriminate between inhibition of ligand activation and direct inhibition of kinase activity. Inhibition of EGF binding paralleled inhibition of ligand-induced EGF-R phosphorylation and both effects were light-dependent. This contrasts with the findings of De Witte *et al.* [9] who reported that hypericin inhibited EGF-R kinase activity in A431 cell membranes under light and dark conditions. These conflicting results may reflect differences in the assay systems used. In the present study, intact cells were exposed to hypericin and this may have limited access of hypericin to the activate site of the kinase which is located intracellularly, whereas De Witte *et al.* used cell membranes where the kinase domain was immediately accessible to the drug.

In conclusion, we have shown that both anthralin and hypericin inhibit mitogen-induced DNA synthesis. Hypericin appears to act by a light-sensitive mechanism leading to nonspecific modification of cell surface receptors which subsequently reduces their ability to bind ligand and, hence, activate cellular signalling. Anthralin has no effect on ligand binding over the concentration range where it affects DNA synthesis and cellular proliferation. It may, therefore, act intracellularly on a target which is common to several growth factor signalling pathways or by inhibition of mitochondrial function.

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