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# PHOTOSENSITIZED INHIBITION OF GROWTH FACTOR-REGULATED PROTEIN KINASES BY HYPERICIN

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**Abstract**—The naphthodianthrone hypericin causes a photosensitized inhibition of protein kinases involved in growth factor signalling pathways. Nanomolar concentrations of hypericin inhibit the protein tyrosine kinase activities (PTK) of the epidermal growth factor receptor and the insulin receptor, while being ineffective towards the cytosolic protein tyrosine kinases Lyn, Fgr, TPK-IIB and CSK. Photosensitized inhibition by hypericin is not restricted to receptor-PTKs since the Ser/Thr protein kinases (protein kinase CK-2, protein kinase C and mitogen-activated kinase) are also extremely sensitive to inhibition ($IC_{50}$ value for protein kinase CK-2 = 6 nM). A comparison of the hypericin-mediated inhibition of the epidermal growth factor-receptor PTK and protein kinase CK-2 revealed that the inhibition is irreversible, strictly dependent upon irradiation of the enzyme–inhibitor complex with fluorescent light and likely mediated by the formation of radical intermediates (type I mechanism). Although the exact molecular basis for the selectivity of enzyme inhibition by hypericin remains unknown, our results suggest that distantly related protein kinases could still share common reactive domains for the interaction with hypericin.

*Key words:* hypericin; tyrosine kinase; MAP kinase; casein kinase 2; inhibition; signal transduction

Recent developments in cellular biology have implicated the unbalanced action of growth stimulatory proto-oncogenes and growth-inhibitory tumour suppressor genes in the aetiology of cancer. When a proto-oncogene is either overexpressed or mutated or when a tumour-suppressor gene is inactivated, the cell receives an unrestrained signal to grow. It is also universally recognized that protein phosphorylation plays a crucial role in cellular processes such as growth, differentiation and transformation. All proto-oncogenes known so far are elements of cellular signalling pathways with many being protein kinases, including receptor tyrosine kinases (ErbB, Fms, Kit, Neu, Trk), non-receptor protein tyrosine kinases (Abl, Lck, Src, Yes, Fgr, Fps) and protein serine/threonine kinases (Mos, Pim-1, Raf) [1]. Protein kinases are therefore potential targets for chemotherapy of proliferative diseases. In accordance with this rationale, much effort has recently been put into the design of selective PTK‖ blockers and the evaluation of their anti-proliferative activity.

Several naturally occurring compounds which inhibit the growth of cultured cells by virtue of their specific interference with PTK activities [2–6] have been isolated (e.g. erbstatin, herbimycin A, staurosporine, flavonoids) and many have been chemically synthesized (e.g. hydroxycinnamamides, tyrphostins, thiazolidine-diones and sulphonyl-benzoyl-nitrostyrenes). A generalization as to the basic structure of a PTK-inhibitor is difficult to put forward since good inhibitors have been shown to belong to several different chemical classes of compounds. However, the majority of synthetic strategies have been based on the idea of mimicking tyrosine in an attempt to find nonphosphorylatable analogues that compete with selective PTK substrates. Consequently, many chemically synthesized inhibitors are substituted hydroxylated aromatic molecules.

One important aspect in designing new PTK blockers is to evaluate their specificity towards the different members of protein kinase families. An inhibitor which selectively blocks one enzyme involved in cell transformation could possibly serve as an anticancer agent as well as a probe for the identification of the physiological role of the kinase involved. However, since cancer is a concerted multistep process, it might be advantageous to simultaneously block several oncogenic alterations of signal transduction pathways; a drug which inhibits several protein kinases of related structure–function could also be a useful therapeutic tool.

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‖ Abbreviations: EGF-R, epidermal growth factor-receptor; Ins-R, insulin-receptor; PTK, protein tyrosine kinase; CSK, c-Src kinase; CK-2 casein kinase 2; CK-1, casein kinase 1; PK-C, protein kinase; PK-A, cAMP-dependent protein kinase; MAP-K, mitogen-activated kinase; MBP, myelin basic protein; GS-1 peptide, glycogen synthase-1 peptide; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; DABCO, 1,4-diazabicyclo[2,2,2]-octane; 2,5-DMF, 2,5-dimethylfuran; HPLC, high-performance liquid chromatography.

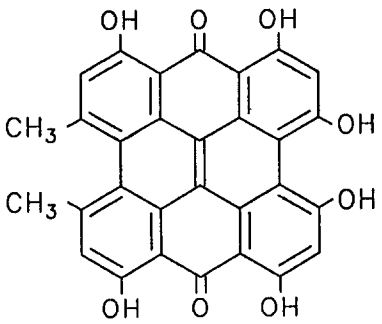

Fig. 1. Structure of hypericin.

We have recently shown that hypericin, the main constituent of plants of the genus *Hypericum*, inhibits the PTK activity of the EGF receptor at submicromolar concentrations [7]. This compound has a naphthodianthrone structure containing the hydroxylated styrene-skeleton of erbstatin (Fig. 1). It exhibits photodynamic properties responsible for inducing hypericism in cattle which ingest large amounts of *Hypericum* in pastures [8]. In recent years, interest in hypericin has greatly increased because the drug was reported to have antiviral and antineoplastic properties [9–17]. Intrigued by the photodynamic activity of this drug, we examined its effect on different Tyr and Ser/Thr kinases in detail, giving particular emphasis to enzymes involved in signal transduction and the regulation of cell growth.

## MATERIALS AND METHODS

*Materials.* Hypericin (TLC grade, purity > 99% from Carl Roth GmbH + Co, Karlsruhe, Germany) was dissolved in DMSO (Janssen Chimica) and stored at −20° in dark conditions (stability > 2 months). One unit of protein kinase activity is defined as the amount of enzyme which incorporates 1 μmol Pi into the substrate per minute at 30°.

The Lyn (sp. act. 0.15 U/mg using angiotensin II as substrate), TPK-IIB (sp. act. 2 U/mg) and Fgr (sp. act. 0.1 U/mg) were purified from the particulate fraction of rat spleen as previously described in Refs 18, 19 and 20, respectively. CSK (sp. act. 0.005 U/mg using angiotensin II as substrate) expressed in *Escherichia coli* was a generous gift from Dr S. Fischer and R. Benarous (INSERM, Unité 332, Paris, France). Protein kinase CK-2 (sp. act. 1 U/mg using casein as the substrate), casein kinase 1 (CK-1, sp. act. 0.4 U/mg) were prepared from porcine spleen as described in Ref. 7. Recombinant *Zea mays* protein kinase CK-2 α-subunit was kindly donated by Dr O.-G. Issinger (Institut für Humangentik, Universität des Saarlandes, Hamburg, Germany). PK-A (bovine, catalytic subunit, sp. act. 0.41 U/mg using histone type II-AS as the substrate) and PK-C (sp. act. 1 U/mg using histone type III-S as the substrate) were purchased from Sigma and Boehringer, respectively. The MAP-K (sp. act. 1 U/mg using MBP as the substrate) and the p13-Sepharose 4B bound cdc2 kinase (sp. act. 0.14 mU/10 μL of p13-Sepharose 4B beads, using histone type III-S as the substrate) were purified from *Xenopus* oocytes as described in Refs 21 and 22, respectively. Inhibitor-2 of the inactive type-1 protein phosphatase was prepared according to Ref. 23. Histones type III-S and type IIA-S, MBP, polylysine (average molecular mass of 46 kDa), angiotensin II (DRVYIHPF), poly(Glu,Ala,Tyr) 6:3:1, Poly-($Glu_4$,$Tyr_1$), 1,2-dioleoyl-*sn*-glycerol (c18:1, [*cis*]-9), L-α-phosphatidyl-L-serine, DABCO and 2,5-DMF were from Sigma Chemical Co (St. Louis, MO, U.S.A.). Whole casein was prepared as described in Ref. 24. The peptides RRREEESEEE, YRRAAVPPSPSPSLSRHSSPHQSEDEE (GS-1) and RRLSSLR (S6) were synthesized with a Milligen 9050, using the 9-fluorenylmethoxycarbonyl mode and purified using reverse-phase HPLC on a Delta-Pack C18 column from Waters. [γ-$^{32}$P]ATP was purchased from Amersham International.

*Cell culture and preparation of crude membrane fractions.* Human epidermoid carcinoma A431 cells were obtained from ATCC (U.S.A.). The 3T3 HIR mouse fibroblast cell line expressing more than $10^6$ human insulin receptors/cell was kindly provided by Dr J. Whittaker (Division of Endocrinology, Dept. of Medicine, Suny at Stony Brook, New York, U.S.A.). A431 cells were grown at 37° in a humidified 5% $CO_2$ atmosphere in DMEM containing 4.5 g/L glucose (Gibco), 2 mM L-glutamine (Gibco), penicillin (100 IU/mL), streptomycin (100 IU/mL), nystatin (50 U/mL) and 10% foetal calf serum. HIR cells were cultivated in DMEM containing 4.5 g/L glucose, 2 mM L-glutamine, 5% foetal calf serum with the addition of non-essential amino acids, pyruvate (1 mM) and gentamycin (50 μg/mL). The membrane-bound EGF-R and Ins-R were obtained from A431 and HIR cells, respectively, according to the method of Thom *et al.* [25] as detailed in Ref. 7.

*Phosphorylations by membrane-bound receptors.* EGF-R and Ins-R autophosphorylations were carried out by incubating 2.5 μg of either A431 or HIR membrane fractions in a final volume of 25 μL essentially as previously described for the EGF-R in [7]. In the case of the Ins-R no dithiothreitol was added to the phosphorylation buffer [7]. Activation of the Ins-R was achieved by preincubating the membranes with insulin (125 nM) for 1 hr at 25° followed by the addition of the appropriate amounts of hypericin or DMSO (control) and irradiation for 15 min at 30°. The autophosphorylation reaction was then started by adding 10 μM [γ-$^{32}$P]ATP (specific activity 45,000 cpm/pmol) to the mixture and allowed to continue for 1 hr at 25°. All autophosphorylation reactions were stopped by boiling the samples for 4 min in 1% SDS denaturation buffer and the resulting proteins were subjected to SDS-PAGE as described in Ref. 7. The $^{32}$P-labelled protein bands corresponding to the EGF-R (170 kDa), the β-chain of the Ins-R (93 kDa), were detected by autoradiography, cut out from the gels and quantified by liquid scintillation counting.

Phosphorylation of poly(Glu,Ala,Tyr) 6:3:1

(0.25 mg/mL) by the EGF-R was essentially carried out as described in Ref. 7 with the exception that after photoactivation the subsequent phosphorylation of the exogenous substrate was performed on ice for 20 min. In the case of the Ins-R, after activation of the receptor and irradiation in the presence of different amounts of inhibitor, 0.50 mg/mL Poly($Glu_4$,$Tyr_1$) was added to the phosphorylation mixture and the reaction allowed to proceed for 1 hr at 25°. Quantification of the $^{32}Pi$ incorporated into the substrate was determined by TCA precipitation as detailed in Ref. 7.

*Phosphoamino acid analysis.* The phosphoamino acid content of the *in vitro* autophosphorylated EGF-R and Ins-R, after SDS-PAGE, was determined after partial acid hydrolysis according to Cooper *et al.* [26].

*Protein kinase assays.* Protein kinases were preincubated in the presence of 0.3% DMSO (control) or with different concentrations of hypericin in 0.3% DMSO either in subdued light or under irradiation with a standard fluorescent lamp (Osram Dulux S, 9 W) as indicated. The light dose over 15 min was 3.6 J/cm$^2$ as measured with a IL 1400 radiometer (International Light). Preincubation of the enzymes with hypericin was performed in a final volume of 30 μL for 15 min at 30°. Subsequently, the phosphorylation reaction was carried out in a final volume of 50 μL containing 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 40 μM [$\gamma$-$^{32}$P]ATP (specific activity 1000–2000 cpm/pmol) and the respective substrates and cofactors indicated below.

MAP kinase (40 μU) and cdc2 kinase (0.14 mU) were assayed with MBP (1 mg/mL) and histone type III-S (0.15 mg/mL), respectively.

CK-1 (12 μU) and protein kinase CK-2 (10 μU) were measured with casein (2.2 mg/mL) in the presence of 150 mM NaCl. The peptides RRREEESEEE (2 mg/mL), YRRAAVPPSPSPSLSRHSSPHQSEDEE (GS-1) (1.7 mg/mL) and inhibitor-2 (0.13 mg/mL) were also used as alternative protein kinase CK-2 substrates. Protein kinase CK-2 autophosphorylation was measured after preincubating CK-2 (166 ng) with hypericin or DMSO (control) in a 15 μL mix. The autophosphorylation reaction was started by the addition of 12 μM [$\gamma$-$^{32}$P]ATP (specific activity 40,000 cpm/pmol) and measured over a period of 15 min at 30°.

PK-C (2 μU) activity was measured with histone III-S (0.25 mg/mL) as substrate in the presence of 50 μM $CaCl_2$, 125 μg/mL L-α-phosphatidyl-L-serine, 12.5 μg/mL 1,2-dioleoyl-*sn*-glycerol (c18:1, [*cis*]-9) and only 5 mM $MgCl_2$.

PK-A was measured with histone II A-S as the substrate as in Ref. 7.

The tyrosine kinases Lyn, Fgr, PTK-IIB and CSK were measured with 2 mM angiotensin II as substrate in the presence of 10 μU [18] of the enzyme, while the ATP concentration of the reaction was lowered to 15 μM. The Lyn, PTK-IIB and CSK enzymes were assayed in the presence of 5 mM $MnCl_2$ instead of 20 mM $MgCl_2$, while in the assay mixture of Fgr 10 μM $Na_3VO_4$ was included to stop possible tyrosine dephosphorylation during the assay. In the case of Lyn, 1 μM polylysine was included.

Substrate phosphorylation was allowed to proceed for 10–20 min at 30° and was stopped by spotting aliquots of the reaction mixture onto paper squares of either P-81 paper (MBP, histones, inhibitor-2, RRREEESEEE and GS-1 peptides) or Whatman paper (casein). The unbound [$\gamma$-$^{32}$P]ATP was removed by successive washes in 0.5% $H_3PO_4$ (P-81 paper) or in ice-cold 10% TCA (Whatman paper). Determination of the $^{32}$P-phosphate bound to angiotensin II was performed as detailed in Ref. 27.

*Statistical analysis.* Each experiment was repeated three times and the mean and the standard deviation were calculated as described in Ref. 7.

Table 1. Selectivity of hypericin-induced protein kinase inhibition

| | $IC_{50}$ (nM) |
|---|---|
| *PTKs* | |
| *Membrane-bound receptor PTK* | |
| EGF-R | 102*/35** |
| Ins-R | 20*/29** |
| *Cytosolic PTKs* | |
| src-related PTK | |
| Lyn | 4000 |
| Fgr | > 20,000 |
| src-unrelated PTK | |
| PTK-IIB | > 20,000 |
| CSK | > 20,000 |
| *Protein Ser/Thr kinases* | $IC_{50}$ (nM) |
| PK-A | 10,000 |
| cdc2 kinase | > 20,000 |
| CK-1 | 3000 |
| PK-C | 27 |
| Protein kinase CK-2 | 6 |
| MAP-K | 4 |

* Autophosphorylation assay.
** Phosphorylation of an exogenous substrate.

## RESULTS

### *Specificity of hypericin inhibition*

In order to investigate the specificity of hypericin as a protein kinase inhibitor, we tested a number of very different protein kinases such as: the EGF-R and Ins-R tyrosine kinases, non-receptor tyrosine kinases (Lyn, Fgr, PTK-IIB, CSK) as well as different Ser/Thr kinases (PK-A, PK-C, CK-1, protein kinase CK-2, MAP-K, cdc2 kinase). Because of the documented photodynamic property of the hypericin-induced inhibition of the EGF-R PTK [7], all protein kinases were preincubated with or without hypericin while irradiated with fluorescent light. The $IC_{50}$ values (50% of photosensitized inhibition by hypericin in comparison to controls without hypericin) for the inhibition of the different kinases are listed in Table 1, and an analysis of the data allows us to draw the following conclusions:

(1) the membrane-associated Ins-R PTK shows a similar sensitivity to hypericin as the membrane-bound EGF-R PTK;
(2) the cytosolic tyrosine kinases Lyn, Fgr, PTK-IIB and CSK are all quite resistant to

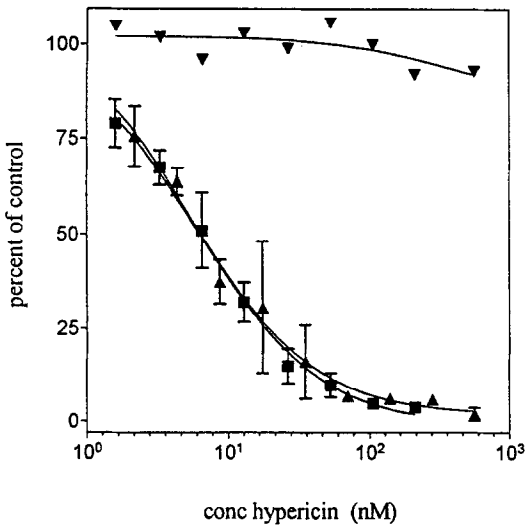


Fig. 2. Light-dependence of protein kinase CK-2 inhibition by hypericin. Protein kinase CK-2 (10 μU) was preincubated in the presence of the indicated amounts of hypericin either under photoactivating (■) or subdued light (▼) conditions. Protein kinase CK-2 activity was evaluated using casein (2.2 mg/mL) as described in the Materials and Methods section, unless otherwise mentioned. Each experiment was repeated three times and the mean and the standard deviation are shown. Results are expressed as percentage of the casein kinase activity in control samples without hypericin. (▲) Represents the recombinant α-subunit (250 ng) of protein kinase CK-2 from *Zea mays*.

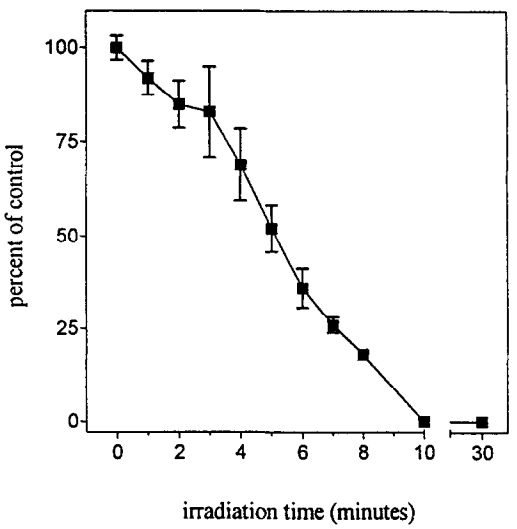


Fig. 3. Irradiation dose-dependence of protein kinase CK-2 inhibition. Protein kinase CK-2 (40 μU) was incubated with or without 140 nM hypericin under photoactivating conditions for different periods of time. At each time point, aliquots were withdrawn and protein kinase CK-2 activity measured using casein (2.2 mg/mL). Results are expressed as a percentage of casein kinase activity in control samples without hypericin.

photosensitized inhibition by hypericin, their calculated values of $IC_{50}$ being two to three orders of magnitude higher than the values found for receptor-associated PTKs;

(3) hypericin-mediated photosensitized inhibition is not restricted to membrane-bound PTK activities, since the strongest inhibition is exhibited by two cytosolic Ser/Thr protein kinases, namely MAP kinase ($IC_{50}$ = 4 nM) and protein kinase CK-2 ($IC_{50}$ = 6 nM).

This last observation is rather intriguing since it implies that some structural domain(s) of rather distantly-related protein kinase families such as receptor tyrosine kinases and Ser/Thr kinases must be quite similar, since both classes of enzymes are extremely susceptible to photosensitized inhibition by hypericin. We therefore studied the hypericin-induced inhibition of protein kinase CK-2 in more detail and compared it to the results previously obtained for the EGF-R PTK [7].

*Light requirement and irradiation dose-dependence of protein kinase CK-2 inhibition by hypericin*

As we previously observed with the EGF-R PTK [7], inhibition of protein kinase CK-2 by hypericin was dramatically dependent upon irradiation with fluorescent light. Figure 2 clearly shows that concentrations of hypericin that totally abolished protein kinase CK-2 activity were completely ineffective when preincubation was performed in subdued light; the calculated $IC_{50}$ value increased more than 1000-fold (not shown).

It is becoming more and more evident that the β-subunit of protein kinase CK-2 is not absolutely required for kinase activity, but that it modulates the activity of the enzyme as well as its responses towards effector molecules [28]. We therefore examined whether the recombinant free catalytic α-subunit of protein kinase CK-2 from *Zea mays*, which shows a high degree of homology with its mammalian counterpart [29], was also inhibited by nanomolar concentrations of hypericin as in the pig spleen purified protein kinase CK-2 tetrameric enzyme.

As is shown in Fig. 2, the free recombinant α-catalytic subunit exhibited the same high susceptibility to photosensitized inhibition ($IC_{50}$ = 5 nM) as the mammalian tetrameric enzyme. This rules out the participation of the regulatory β-subunit in protein kinase CK-2 inhibition by hypericin.

In order to test whether the inhibition of protein kinase CK-2 by hypericin was light dose-dependent, the inhibition process was monitored over a period of 30 min under photoactivating conditions. As is shown in Fig. 3, a 10 min irradiation is required for complete inhibition of protein kinase CK-2 activity by 140 nM hypericin. Since irradiation of a similar concentration of hypericin in the absence of protein kinase CK-2 in the same medium and for the same time period did not cause a subsequent measurable inhibition of protein kinase CK-2, it can be concluded that the inhibition depends upon the time of contact between hypericin and the kinase under irradiating conditions. The progressive increase in enzyme

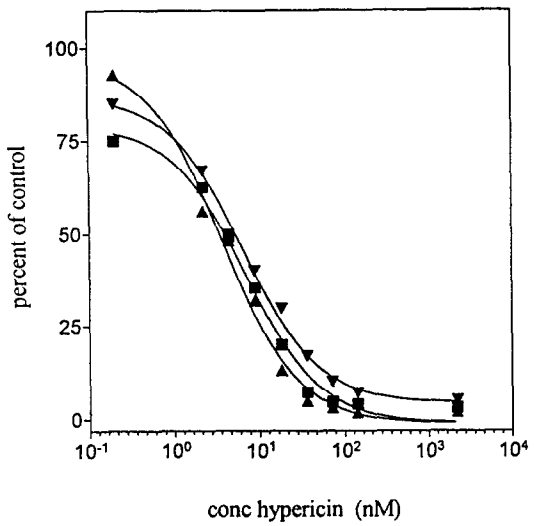


Fig. 4. Substrate dependence of hypericin-mediated photosensitized protein kinase CK-2 inhibition. Protein kinase CK-2 activity was measured after preincubation of the enzyme (10 $\mu$U) with different concentrations of hypericin under photoactivating conditions, using (▼) 2 mg/mL RRREEESEEE, (■) 1.7 mg/mL YRRAAVPPSPSPSLSRHSSPHQSEDEE (GS-1) peptide, or (▲) 0.13 mg/mL inhibitor-2 as substrates as described in the Materials and Methods section. Results are expressed as a percentage of kinase activity in control samples.

inhibition with irradiation time suggests an irreversible process ultimately resulting in complete enzyme inhibition.

*Effects of substrates and cofactors*

Figure 4 shows the hypericin photosensitized-induced inhibition of protein kinase CK-2 measured with inhibitor-2 or the synthetic peptides RRREEESEEE and YRRAAVPPSPSPSLSRHSSPHQSEDEE (GS-1) as substrates. An identical pattern of inhibition was observed as with casein as substrate, suggesting that photosensitized inhibition by hypericin is not dependent on the exogenous substrate used (see Table 1). As expected, hypericin also blocked the intramolecular autophosphorylation of the protein kinase CK-2 $\beta$-subunit (not shown). The higher concentration of hypericin required for reaching half maximal inhibition ($IC_{50}$ = 140 nM) of the latter process reflects the higher concentration of the enzyme needed in the autophosphorylation experiment and suggests a stoichiometric reaction between enzyme and inhibitor.

The inclusion of stimulatory polybasic compounds such as spermine or histones in the preincubation mixture did not alter the pattern of hypericin inhibition either (not shown).

It is worthwhile mentioning that the inhibition of protein kinase CK-2 by heparin, the most powerful protein kinase CK-2 inhibitor known thus far ($IC_{50}$ = 1.4 nM), is very much substrate-dependent [30].

*Irreversibility of hypericin-induced inhibition*

Since EGF-R PTK activity was found to be irreversibly affected by hypericin [7], we examined whether the same was true for protein kinase CK-2 photosensitized inhibition. A protein kinase CK-2-hypericin mixture was thus subjected to treatments that would dissociate a reversible inhibitor–enzyme complex. The photosensitized hypericin-inhibited protein kinase CK-2 was either subjected to serial dilutions or chromatographed on a small G-25 Sephadex column. The Sephadex particle retains the free hypericin molecule which can be visualized by the addition of organic solvent (acetone) and subsequent exposure to 365 nm ultraviolet light; under these conditions hypericin exhibits a red fluorescence. The photosensitized inhibition by hypericin was however not reversed by dilution nor by gel filtration (Fig. 5), which supports the idea that protein kinase CK-2 is irreversibly inhibited.

Although calculation of a true $K_i$ value is not possible in the case of irreversible enzyme inhibitions, some information concerning the nature of the inhibition may still be gained from kinetic experiments which examine substrate-protection of the enzyme. In general, if an irreversible inhibitor binds at the active site, it may be expected that the substrate will protect against inhibition and consequently decrease the velocity at which inhibition occurs.

Complex mixed kinetics were obtained for increasing concentrations of hypericin with both peptide and protein substrates, with a major effect on the $K_m$ values for the peptide RRREEESEEE at lower (4.4 nM) inhibitor concentration (Fig. 6). Similar kinetics were observed when a protein (casein) was used as the phosphorylatable substrate in Fig. 6 (not shown). This would suggest that hypericin inhibitory action is indeed obstructed by the presence of protein kinase CK-2 substrates at the active site and that both substrate and hypericin share common or overlapping binding sites on the enzyme. This idea was reinforced by the observation that peptides or proteins which are not substrates for protein kinase CK-2, such as the S6 peptide or MBP, do not substantially interfere with photoinhibition by hypericin (not shown). Alternatively, the binding of hypericin to an allosteric site on the enzyme could cause a conformational change which prevents access of the substrate to the binding site. By the same token, the presence of the substrates at the active site of the kinase could allosterically hinder the binding of hypericin at a separate high affinity inhibitor site. A third explanation for the observed results would be that hypericin, through a photodynamic process, causes irreversible oxidative damage to specific amino acid residues at the active centre resulting in the irreversible inhibition of the protein kinase. This mechanism would not necessarily implicate a covalent binding of the hypericin molecular to the enzyme.

*Effect of singlet oxygen quenchers and Triton X-100*

Upon irradiation with visible light, ground state hypericin (HYP) is converted to the lowest excited single state ($^1$HYP*) which, after intersystem crossing

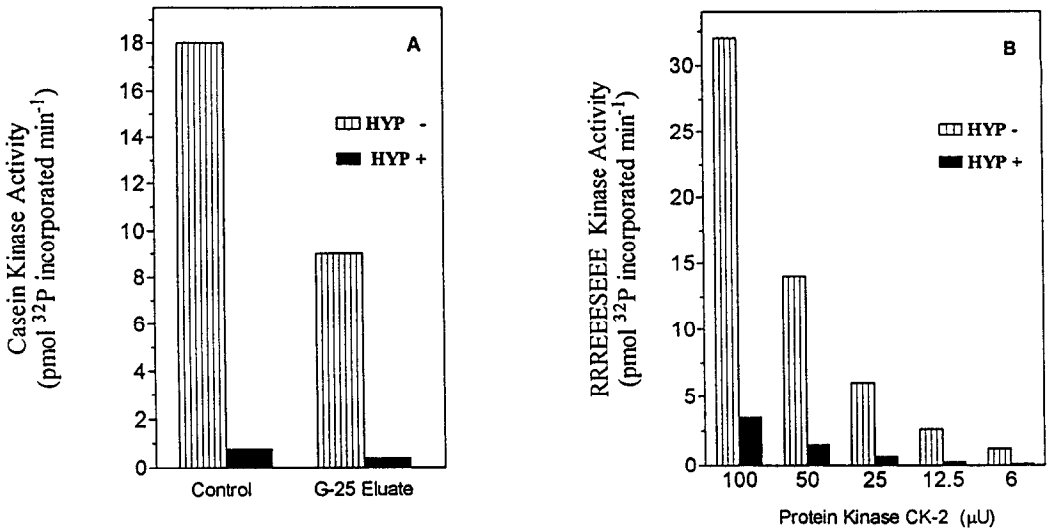


Fig. 5. Irreversibility of hypericin protein kinase CK-2 photosensitized inhibition. Panel A: Protein kinase CK-2 (1 mU) was incubated with or without 8 $\mu$M hypericin and irradiated for 15 min. The mixtures were then diluted 4-fold in buffer A (50 mM Tris–HCl pH 7.5, 150 mM NaCl) and centrifuged through a 1 mL G-25 Sephadex column equilibrated in the same buffer. Protein kinase CK-2 activity was determined in an aliquot (30 $\mu$L) of the eluate (G-25 Eluate) using casein (2.2 mg/mL) as described in the Materials and Methods section. Identically diluted samples (control) were assayed directly without the gel filtration step. Panel B: Protein kinase CK-2 (100 $\mu$U) was incubated with 2 $\mu$M hypericin under photoactivating conditions. Then the enzyme–inhibitor complex was subjected to serial dilutions in the preincubation medium and protein kinase CK-2 activity assayed using the RRREEESEEE peptide (2 mg/mL) as described in the Materials and Methods section.

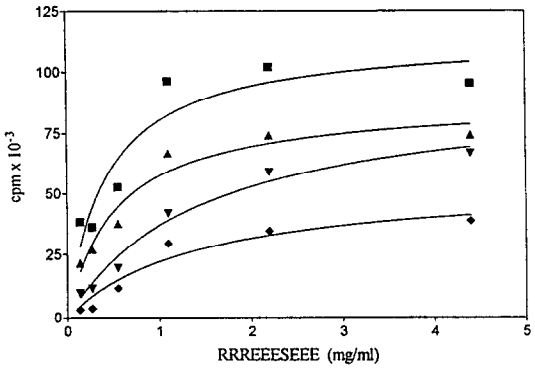


Fig. 6. Michaelis–Menten plots for the photosensitized inhibition of protein kinase CK-2 by different concentrations of hypericin. Protein kinase CK-2 (10 $\mu$U) was preincubated in the presence of various amounts of the RRREEESEEE peptide without (■) or with (▲) 4.4 nM, (▼) 18 nM and (◆) 72 nM hypericin. After preincubation residual protein kinase CK-2 activity was measured by the addition of 25 $\mu$M [$\gamma$-$^{32}$P]ATP (specific activity 2000 cpm/pmol).

Table 2. Effect of singlet-oxygen quenchers and detergent on protein kinase CK-2 inhibition (%) by hypericin

| | Hypericin (nM) | |
|---|---|---|
| | 800 | 35 |
| Controls | 100 | 65 |
| DABCO 5 mM | 100 | 73 |
| 2,5-DMF 5 mM | 96 | 61 |
| Triton X-100 0.25% | 10 | 0 |

with spin conversion, gives the less energetic but longer-lived triplet state ($^3$HYP*). The latter may mediate its biological effects by returning to the ground state involving free radical intermediates (e.g. hypericin semiquinone radical (HYP$^{\cdot-}$)) and ground state oxygen (type I mechanism). However, singlet oxygen ($^1O_2$) can also be generated when the excitation energy of triplet hypericin is transferred to ground triplet state oxygen. Singlet oxygen is the prime oxidant in type II photosensitization processes [31].

Therefore, in order to gain some insight into the mechanism of hypericin-mediated protein kinase CK-2 inhibition we performed the photosensitization reaction in the presence of effective concentrations of singlet oxygen quenchers (DABCO, 2, 5-DMF). It should be noted that azide, another efficient singlet-oxygen quencher, as well as 1,4-benzoquinone, a quencher of the superoxide anion radical, could not be used in our experiments since they exhibited a direct effect on protein kinase CK-2 activity (not shown). As is summarized in Table 2, none of the singlet oxygen quenchers used interfered with the photosensitized inhibition. This would suggest that, as in the case of the EGF-R PTK [7], the

photosensitized inhibition of protein kinase CK-2 by hypericin is triggered by the formation of active radical intermediates (type I reaction).

Table 2 also shows that inclusion of the non-ionic detergent Triton X-100 in the preincubation mixture abolishes the inhibitory effect of hypericin on protein kinase CK-2, as observed for the EGF-R PTK [7].

## DISCUSSION

Recently, increasing attention has been given to photodynamic therapy because of the discovery that many photosensitizer compounds, either naturally occurring or synthesized, exhibit remarkable anti-cancer and antiviral activities (reviewed in Ref. 32). Some of these drugs have been known for many years and although their application as photodynamic agents has been exploited for clinical studies, very little is known about their mechanism of action at the biochemical or molecular level.

This is the first study on hypericin dealing with the target selectivity of the photosensitizer and implicating protein kinases involved in signalling pathways of cell proliferation and transformation. Surprisingly, among the kinases tested, hypericin showed a very high reactivity towards two families of distantly related protein kinases: receptor-tyrosine kinases (EGF-R and Ins-R) and Ser/Thr protein kinases (PK-C, protein kinase CK-2 and MAP-kinase). In all instances, hypericin affected the kinase activities at nanomolar concentrations by a mechanism which involves a light dose-dependent irreversible inhibition.

Although in some instances the photosensitizing effects of hypericin on biomolecules have been associated with the formation of reactive singlet-oxygen species (type II mechanism), hypericin semiquinone radicals and other active intermediates have also been shown to play an important role in the photosensitized action of this drug (reviewed in Ref. 32). The photosensitized inhibition of protein kinases by hypericin (Ref. 7 and this work) seems to be mediated by the formation of reactive radical intermediates (type I mechanism) rather than by singlet oxygen species (type II mechanism). However, we cannot exclude the possibility that steric factors imposed by the structure of the enzyme render singlet oxygen quenchers ineffective. More detailed mechanistic studies involving laser flash spectroscopy and/or steady-state irradiation followed by amino acid analyses would be necessary to completely reject type II processes.

The abrogation of hypericin-mediated protein kinase photosensitized inhibition by the nonionic detergent Triton X-100 could be caused by the sequestration of the lipophilic molecules of hypericin into the micelles created by the detergent in an aqueous environment. This could explain why many studies where the effect of hypericin as inhibitor of viral reverse transcriptase activity was evaluated in the presence of various amounts of detergent have failed to produce consistent results [9, 11, 12, 33].

The exact structural basis of the high sensitivity for photosensitized inhibition of these kinases is unknown. A crystallographic analysis of the structure of the catalytic subunit of these protein kinases would unequivocally shed more light on the critical residues involved in irreversible hypericin inhibition and possibly reveal a common interacting domain. Moreover, the development of structural analogues of hypericin could be important for the further investigation of the interactions between the photosensitizer and the protein kinases and could eventually lead to the selection of specific protein kinase blockers. Finally, the use of hypericin and/or its (more specific) analogues in *in vivo* studies may be exploited to evaluate the role of its target protein kinase(s) in malignant cellular processes such as tumour formation.

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