Protein Tyrosine Kinase and Protein Kinase C Inhibition by Fungal Anthraquinones Related to Emodin

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(Received for publication April 27, 1995)

The protein kinases are a large family of enzymes that transfer phosphate from adenosine triphosphate (ATP) to proteins as a means of regulating their activity and conformational state. Protein kinase C (PKC) and protein tyrosine kinases (PTKs) belong to two families of enzymes that play an important role in cellular physiology, including cell proliferation and differentiation[1,2]. PTKs catalyze phosphorylation of tyrosine residues of protein substrates whereas PKCs phosphorylate serine or threonine residues.

One of the best understood and most intensely investigated PTKs is the epidermal growth factor receptor (EGF-R) protein tyrosine kinase which upon ligand binding undergoes dimerisation and autophosphorylation followed by the assembly of a signal transduction particle and signal transmission[3]. The deregulation of PTK activity in tumor cells is associated with malignant transformation[4]. PKC has been identified as the receptor for tumor promoting phorbol esters and in addition PKC subtypes have shown transforming potential and deregulation in certain tumor types[5]. Inhibitors of PKC or PTK could have a therapeutic potential as anticancer agents.

In our search for naturally occurring inhibitors of the EGF-R PTK among secondary metabolites of microorganisms, various inhibitors related to emodin were identified in several fungal extracts. The parent compound emodin has recently been described as an inhibitor of $p56^{lck}$ PTK[6]. In the present communication the PTK-inhibitory activities of those compounds are investigated more closely. Furthermore, the compounds were assayed *in vitro* for their ability to inhibit PKC. An HPLC method for a simplified identification of the compounds is presented.

Materials and Methods

Emodin (**1**) and purpurin (**8**) were obtained from Fluka (Buchs, Switzerland), catenarin (**5**) from Apin (Abingdon, U.K.). The fermentation, isolation, and structure elucidation of the paeciloquinones (**9**~**12**) were described earlier[7,8]. Compounds **2**, **3**, **4**, **6** and **7** were isolated from various fungi and identified by their physico-chemical properties, mainly ^{1}H NMR spectroscopy and MS.

HPLC Analysis

The following equipment was used: low pressure gradient delivery system SP8800 (Spectra-Physics), Rheodyne 7125 injection valve, UV-VIS Focus fast-scan detector (Spectra-Physics) with integration software PC-1000 version 2.5 or a diode array detector Gynkotek UVD 340 with software Gynkosoft version 3.5.

Assays for Kinase Inhibition and Antiproliferative Activity

The compounds were tested for inhibition of the protein kinases EGF-R PTK, v-abl PTK, c-src PTK, PKC-α, and PKC-δ as described previously[9~13]. Inhibition of proliferation of EGF-dependent BALB/MK murine keratinocytes and interleukin-3 (IL-3)-dependent FDC-P1 cells was assayed after 3~4 days of drug exposure as described[11].

v-Abl Autophorsphorylation and Western Blot Analysis

The Abelson murine leukemia virus transformed cell line BALB/c AMuLV A.6R.1 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in DULBECCO's modified EAGLE's medium (Life Technologies, Gaitherburg, MD) supplemented with 10% fetal calf serum. Cells were treated with the compounds at a concentration of 1, 10 or 100 μM for 2 hours, and lysates were analyzed by Western blotting using antiphosphotyrosine antibodies as described[14].

Results and Discussion

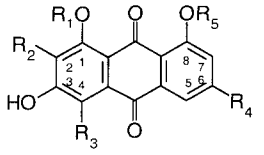
HPLC Analysis

The compounds referred to in the paper are presented in Fig. 1. The HPLC system displayed in Fig. 2 allows the separation of all compounds with the exception of compound **11** which is a minor metabolite[7]. The system is useful for a quick identification of fungal metabolites, as the commercially available emodin can used for calibration of the system. For reference purposes the UV-visible spectra of selected compounds are displayed in Fig. 3.

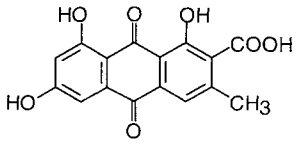
Biological Properties

EGF-R PTK was inhibited by all compounds in a similar range with the exception of endocrocin (**7**) which was inactive (Table 1). This suggested that the binding site of EGF-R PTK could accommodate most structural modifications examined. Since the methylated compounds **3** and **4** were active, not all phenolic groups were necessary for activity. The intracellular inhibitory activity of the anthraquinones against EGF-R PTK was tested in an antiproliferative assay using BALB/MK mouse epidermal keratinocytes, whose proliferation is EGF dependent[15]. The observed antiproliferative potency of

Fig. 1. Structures of emodin (**1**), emodic acid (**2**), 1-methyl-emodin (**3**), 8-methyl-emodin (**4**), catenarin (**5**), 2-chloro-ω-hydroxy-emodin (**6**), endocrocin (**7**), purpurin (**8**) and of paeciloquinones A (**9**), B (**10**), C (**11**) and D (**12**).



	R_1	R_2	R_3	R_4	R_5
1	H	H	H	CH_3	H
2	H	H	H	COOH	H
3	CH_3	H	H	CH_3	H
4	H	H	H	CH_3	CH_3
5	H	H	OH	CH_3	H
6	H	Cl	H	CH_2OH	H



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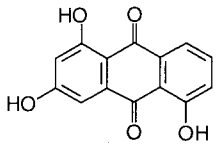

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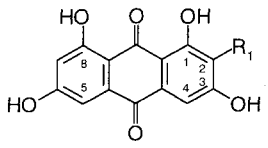
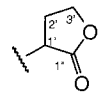
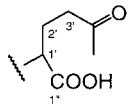


9 R_1 =



10 R_1 =



11 R_1 = CH_2OH

12 R_1 =

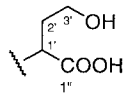
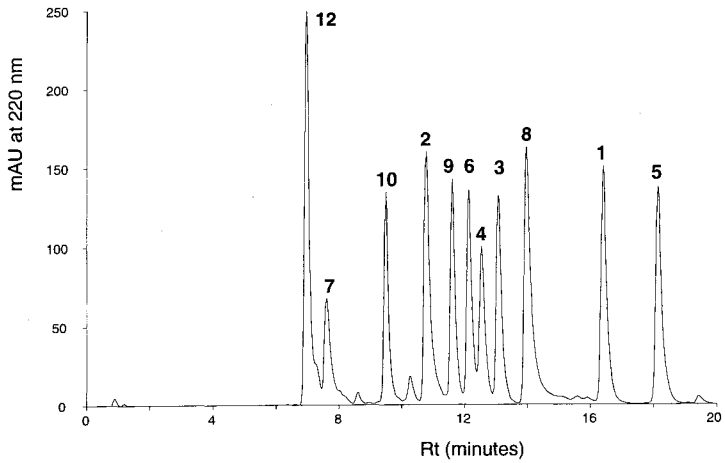


Fig. 2. Reversed phase HPLC chromatogram of a mixture containing 50 μg/ml of compounds **1**~**10** and **12** in methanol.



As purpurin is not stable, the solution has to be freshly prepared. Experimental conditions: analytical reversed-phase cartridge (Nucleosil 100-5 C18, 5 μm; Marcherey-Nagel, Düren, Germany; 4.0 × 100 mm) plus precolumn (4.0 × 30 mm); UV detection at 220 nm; mobile phase A: 2.5 mM aqueous phosphate buffer pH 3.0; mobile phase B: acetonitrile - mobile phase A (8:2); 1.5 ml/minute; gradient elution: from 25% B to 90% B in 20 minutes; 20 μl injected. The numbers indicate the assignment of individual peaks to the respective component.

most compounds was in a similar range as their EGF-R PTK inhibitory activity, suggesting that the compounds with the exception of the acidic compounds **2** and **10** efficiently penetrated into the MK cells.

Autophosphorylation of the intracellular domain of EGF-R PTK is considered to be the first step in signal transduction after ligand stimulation[2]. Inhibition of autophosphorylation of EGF-R PTK in anthraquinone-treated A431-cells was investigated using anti-phosphotyrosine antibodies[14]. Analysis of the cells by ELISA after exposure to serial dilutions of compounds and stimulation with EGF showed that many of the anthraquinones did not inhibit EGF-dependent autophosphorylation of EGF-R in intact cells. Only emodin (**1**) and 2-chloro-ω-hydroxyemodin (**6**) showed some activity in this assay. These two compounds also showed antiproliferative activity against MK cells, but no direct correlation could be demonstrated between inhibition of intracellular autophosphorylation of EGF-R PTK antiproliferative activity against MK cells.

FDC-P1 cells whose growth depends on IL-3[16] and does not involve activation of EGF-R were used as control cells: The antiproliferative potency of the anthraquinones was grossly similar as against EGF-R dependent MK cells. The lack of cell line specificity may indicate that the examined compounds affect another element of the mitogenic signal transduction pathway beyond EGF-R.

Fig. 3. UV/VIS spectra of selected compounds obtained in the HPLC chromatogram of Fig. 2 at pH 3.

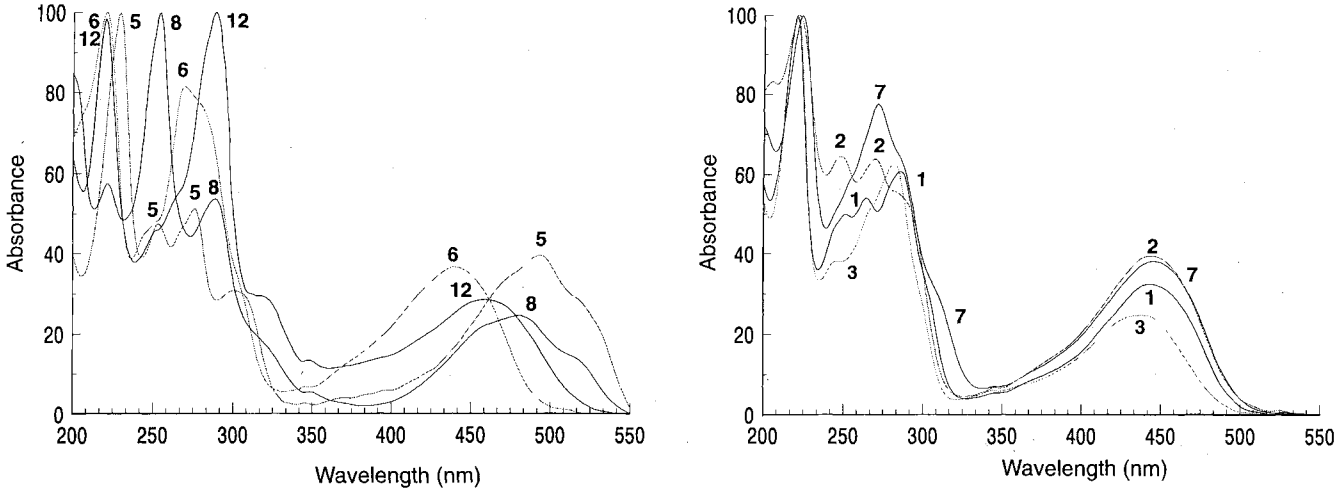


Table 1. Inhibition of EGF-R, v-abl and c-src protein tyrosine kinases, of the PKC subtypes α and δ and of MK- and FDC-P1 cell proliferation by the anthraquinones **1** to **12**.

		IC_{50} (μM)							
		Enzyme assays					Cellular assays: Autophosphorylation		Proliferation
		EGF-R-PTK	v-Abl-PTK	c-Src-PTK	PKC-α	PKC-δ	EGF dependent Tyr-phosphorylation	FDC-P1 (IL-3 dependent)	MK-cells (EGF dependent)
Emodin	(**1**)	12.5	1.6	38	45	>100	25	29	15
Emodic acid	(**2**)	5.6	19	68	92	>100	100	>50	>50
1-Methyl-emodin	(**3**)	16	7.4	230	>100	>100	>100	39	29
8-Methyl-emodin	(**4**)	14	15	380	>100	>100	100	36	37
Catenarin (4-hydroxy-emodin)	(**5**)	12	99	43	90	>100	>100	16	10
2-Chloro-ω-hydroxy-emodin	(**6**)	21	5.4	10	>100	>100	50	12	6.6
Endocrocin	(**7**)	>50	8	13	7.2	21	>100	35	10
Purpurin	(**8**)	20	5.2	64	20	23	n.d.	>50	24
Paeciloquinone A	(**9**)	11	0.59	2	69	>100	>100	49	35
Paeciloquinone B	(**10**)	21	83	>100	>100	>100	>100	>25	>50
Paeciloquinone C	(**11**)	6.7	0.56	9	69	>100	100	>25	10
Paeciloquinone D	(**12**)	8	11	35	5.5	62	>100	18	16

n.d.: Not determined.

This is confirmed by the inhibitory activity of these compounds for other kinases. Most of the compounds showed a low degree of selectivity among the kinases investigated. Emodin shows some selectivity towards v-abl PTK but to a lesser degree than the earlier described paeciloquinones A (**9**) and C (**11**). Although emodin (**1**) and paeciloquinone A (**9**) and C (**11**) showed potent activity against the v-abl PTK *in vitro*, the compounds had no significant effect on v-abl autophosphorylation in intact cells at concentrations up to 100 μM, indicating low penetration or inappropriate intracellular distribution.

Only very few anthraquinones were inhibiting PKC: Paeciloquinone D (**12**) and endocrocin (**7**) were the most potent inhibitors of PKC-α with IC_{50} values around 6 μM. Both compounds contain carboxylic acid groups which are quite close to each other, if the phenolic groups are arranged for the best possible fit as drawn in Fig. 1. The closely related paeciloquinone B (**10**) is, however, not very active suggesting that the enzyme provides no space for large substituents. The selectivity towards inhibition of PKC-α is not sufficient to justify a further *in vitro* evaluation of compounds **7** or **12**.

In conclusion our investigation indicates that most anthraquinones inhibit various protein kinases with a low degree of selectivity. The compounds are not able

to inhibit autophosphorylation of EGF-R PTK and v-abl substrates within the intact cell. This can either be due to low penetration or inappropriate intracellular distribution. Nevertheless, in combination with structural studies on target proteins, emodin and the related anthraquinones could serve as lead structures for kinase inhibitors.

Acknowledgments

We are indebted to JEAN METZGER, BEATRICE ADAM, NICOLE MARTIN, CLAUDIA KOELBING, IRIS OBERKIRCH and ROBERT REUTER for technical assistance.

References

1) NISHIZUKA, Y.: The molecular heterogeneity of protein kinase C and its implications for cellular regulation. Nature 334: 661~665, 1988
2) CHEN, W. S.; C. S. LAZAR, M. POENIE, R. Y. TSIEN, G. N. GILL & M. G. ROSENFELD: Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. Nature 328: 820~823, 1987
3) HONEGGER, A. M.; T. J. DULL, S. FELDER, E. VAN OBBERGHEN, F. BELLOT, D. SZAPARY, A. SCHMIDT, A. ULLRICH & J. SCHLESSINGER: Point mutation at the ATP binding site of EGF receptor abolishes protein-tyrosine kinase activity and alters cellular routing. Cell 51: 199~209, 1987
4) HANAFUSA, H.; B. J. MAYER, C. REICHMAN & M. HAMAGUCHI: Cell transformation by an oncogene that regulates protein kinases. *In* Advances in second messenger and phosphoprotein research. The biology and medicine of signal transduction. *Ed.* Y. NISHIZUKA *et al.* pp. 280~283, Raven Press, New York, 1990
5) BRADSHAW, D.; C. H. HILL, J. S. NIXON & S. E. WILKINSON: Therapeutic potential of protein kinase C inhibitors. Agents Actions 38: 135~147, 1993
6) JAYASURIYA, H.; N. M. KOONCHANOK, R. L. GEAHLEN, J. L. MCLAUGHLIN & C.-J. CHANG: Emodin, a protein tyrosine kinase inhibitor from *Polygonium cuspidatum*. J. Nat. Prod. 55: 696~698, 1992
7) PETERSEN, F.; A. FREDENHAGEN, N. B. LYDON, H. METT, R. DELMENDO, H. B. JENNY & H. H. PETER: Paeciloquinones A, B, C, D, E & F: New potent inhibitors of protein tyrosine kinase produced by *Paecilomyces carneus* I. Taxonomy, fermentation, isolation & biological activity. J. Antibiotics 48: 191~198, 1995
8) FREDENHAGEN, A.; P. HUG, H. SAUTER & H. H. PETER: Paeciloquinones A, B, C, D, E & F: New potent inhibitors of protein tyrosine kinase produced by *Paecilomyces carneus* II. Characterization and structure determination. J. Antibiotics 48: 199~204, 1995
9) MCGLYNN, E.; M. BECKER, H. METT, S. REUTENER, R. COZENS & N. B. LYDON: Large-scale purification and characterization of a recombinant epidermal growth-factor receptor protein-tyrosine kinase. Modulation of activity by multiple factors. Eur. J. Biochem. 207: 265~275, 1992
10) LYDON, N.; B. ADAMS, J. POSCHET, A. GUTZWILLER & A. MATTER: An *E. coli* expression system for the rapid purification and characterization of a v-abl tyrosine protein kinase. Oncogene Res. 5: 161~173, 1990
11) MEYER, T.; U. REGENASS, D. FABBRO, E. ALTERI, J. RÖSEL, M. MÜLLER, G. CARAVATTI & A. MATTER: A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumor activity. Int. J. Cancer 43: 851~856, 1989
12) TRINKS, U.; E. BUCHDUNGER, P. FURET, W. KUMP, H. METT, T. MEYER, M. MÜLLER, U. REGENASS, G. RIHS, N. B. LYDON & P. TRAXLER: Dianilinophtalimides: Potent and selective, ATP-competitive inhibitors of the EGF-receptor protein tyrosine kinase. J. Med. Chem. 37: 1015~1027, 1994
13) MARTE B. M.; T. MEYER, S. STABEL, G. J. R. STANDKE, D. FABBRO & N. E. HYNES: Protein kinase C and mammary cell differentiation: involvement of protein kinase Cα in the induction of β-casein expression. Cell Growth & Differ. 5: 239~247, 1994
14) BUCHDUNGER, E.; U. TRINKS, H. METT, U. REGENASS, M. MÜLLER, T. MEYER, E. MCGLYNN, L. A. PINNA, P. TRAXLER & N. B. LYDON: 4,5-Dianilinophtalimide: A protein-tyrosine kinase inhibitor with selectivity for the epidermal growth factor receptor signal transduction pathway and potent *in vivo* antitumor activity. Proc. Natl. Acad. Sci. U.S.A. 91: 2334~2338, 1994
15) WEISSMAN, B. E. & S. A. AARONSON: BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent balb/c mouse epidermal keratinocyte lines. Cell 32: 599~606, 1983
16) LEE, J. C.; A. J. HAPEL & J. N. IHLE: Constitutive production of a unique lymphokine (IL-3) by the WEHI-3 cell line. J. Immunol. 128: 2393~2398, 1982