Lichen Metabolites. 2. Antiproliferative and Cytotoxic Activity of Gyrophoric, Usnic, and Diffractaic Acid on Human Keratinocyte Growth

Sunil Kumar KC[†] and Klaus Müller*[,‡]

Institut für Pharmazie, Universität Regensburg, D-93040 Regensburg, and Institut für Pharmazeutische Chemie, Westfälische Wilhelms-Universität Münster, Hittorfstrasse 58−62, D-48149 Münster, Germany

Received September 2, 1998

The sensitivity of the human keratinocyte cell line HaCaT to several lichen metabolites isolated from *Parmelia nepalensis* and *Parmelia tinctorum* was evaluated. The tridepside gyrophoric acid (**6**), the dibenzofuran derivative (+)-usnic acid (**1**), and the didepside diffractaic acid (**5**) were potent antiproliferative agents and inhibited cell growth, with IC_{50} values of 1.7, 2.1, and 2.6 μM, respectively. Methyl β-orcinolcarboxylate (**2**), ethyl hematommate (**3**), the didepside atranorin (**4**), and (+)-protolichesterinic acid (**7**) did not influence keratinocyte growth at concentrations of 5 μM. Keratinocytes were further tested for their susceptibility to the action of the potent antiproliferative agents on plasma membrane integrity. The release of lactate dehydrogenase activity into the culture medium was unchanged as compared to controls, documenting that the activity of gyrophoric acid (**6**), (+)-usnic acid (**1**), and diffractaic acid (**5**) was due to cytostatic rather than cytotoxic effects.

In the course of our search for plant-derived antiinflammatory and antiproliferative agents that could be useful for the development into antipsoriatic drugs, we have isolated several compounds from *Parmelia nepalensis* Tayl. and *Parmelia tinctorum* Nyl. (Parmeliaceae):[1,2] (+)-usnic acid (**1**), methyl β-orcinolcarboxylate (**2**), ethyl hematommate (**3**), atranorin (**4**), diffractaic (**5**), gyrophoric (**6**), and (+)-protolichesterinic acid (**7**). Of these structurally dissimilar lichen-derived metabolites, the didepsides **4** and **5** and also the aliphatic α-methylene butyrolactone **7** have been identified as nonredox inhibitors of the biosynthesis of leukotriene B_4 (LTB_4), the product of the 5-lipoxygenase (5-LO) pathway.[1]

Leukotrienes have been considered to be important mediators of inflammation.[3] Among the variety of diseases in which the leukotrienes may play a symptomatic or causative role is the dermatological condition psoriasis.[4,5] Psoriasis is a common, hyperproliferative and inflammatory skin disease, mainly characterized by abnormal keratinocyte proliferation, accumulation of polymorphonuclear leukocytes in the skin, and T-cell activation.[6] LTB_4 stimulates epidermal keratinocyte proliferation,[7,8] and application of LTB_4 to normal human skin induces changes similar to those found in psoriatic skin. Furthermore, the 5-LO pathway has been shown to be involved in T-cell activation.[9] It therefore seems reasonable that inhibitors of the enzyme be targeted for the treatment of psoriasis.

With respect to biological activity studies related to the pathological features of psoriasis, lichen-derived metabolites such as lobaric acid and **7** have been shown to exhibit antiproliferative activity against malignant cell lines,[10] and **7** is also an inhibitor of human DNA ligase I[11] and cellular DNA polymerase β.[12] Probably the most extensively described lichen metabolite is the dibenzofuran derivative **1**, whose antitumor activities were revealed more than two decades ago.[13,14] It was later found to exhibit antimitotic activities.[15,16] However, relatively little information is available about the activity of other small-molecule lichen-derived metabolites on cultured cells.

Table 1. Antiproliferative Activity and Cytotoxicity against HaCaT Cells by Lichen Metabolites

lichen metabolites	AA[a] IC_{50} (μM)	LDH[c] (mU)
(+)-usnic acid (**1**)	2.1 ± 0.7[b]	168
methyl β-orcinolcarboxylate (**2**)	>5	ND
ethyl hematommate (**3**)	>5	ND
atranorin (**4**)	>5	ND
diffractaic acid (**5**)	2.6 ± 0.4[b]	136[c]
gyrophoric acid (**6**)	1.7 ± 0.4[b]	138[c]
(+)-protolichesterinic acid (**7**)	>5	ND
anthralin[d]	0.7 ± 0.3[b]	294

[a] Antiproliferative activity against HaCaT cells, as demonstrated by reduction in cell number over time as compared to control plates. [b] Inhibition of cell growth was significantly different with respect to that of the control, $n = 3$, $p < 0.05$. [c] Activity of LDH (mU) release in HaCaT cells after treatment with 2 μM test compound, $n = 3$, SD < 10%, $p < 0.05$; control: 135 mU. Values are not significantly different with respect to vehicle control. [d] Standard antipsoriatic agent. ND = not determined.

In the present study, we have used HaCaT keratinocytes[17] as a model for highly proliferative epidermis, for example, psoriasis. This nontransformed human cell line is a useful tool in identifying new topical antipsoriatic agents, and it was described as an extremely sensitive target for the antiproliferative action of the antipsoriatic drug anthralin.[18] Accordingly, we evaluated the effects of the lichen metabolites **1**−**7** on the growth of cultured HaCaT cells. Keratinocytes were also tested for their susceptibility to the action of the most potent members of this series on plasma membrane integrity, to confirm that inhibition of cell growth is not a result of membrane damage. This was assessed by the activity of lactate dehydrogenase (LDH) released into the culture medium.

Results and Discussion

The structures of all lichen metabolites (**1**−**7**) assayed are illustrated in the preceding paper. Each compound was tested for antiproliferative effects against HaCaT cells, as demonstrated by reduction in cell number over time as compared to control plates. The concentrations required to inhibit 50% of cell growth are shown in Table 1. The tridepside **6** was the most potent inhibitor, with an IC_{50} value of 1.7 μM. The tricyclic lichen-derived metabolite **1**

* To whom correspondence should be addressed. Phone: +49 251-8333324. Fax: +49 251-8332144. E-mail: kmuller@uni-muenster.de.
[†] Universität Regensburg.
[‡] Universität Münster.

10.1021/np980378z CCC: $18.00
Published on Web 05/06/1999

and the didepside **5** exhibited comparable activity, with IC_{50} values of 2.1 and 2.6 μM, respectively; whereas, the didepside ester **4**, the monomeric esters **2** and **3**, and the butyrolactone **7** were not active at the 5-μM concentration.

In a recent study on lichen-derived metabolites, it was speculated that the observed antiproliferative effects of **7** and lobaric acid may be related to their 5-LO inhibitory activities.[10] However, among the most potent inhibitors of HaCaT cell growth assayed in this study, only **5** was also shown to be a potent inhibitor of the 5-LO enzyme.[1] It is also interesting to note that, although didepside **4** and butyrolactone **7** were not active as inhibitors of cell growth at 5 μM, they were among the most active 5-LO inhibitors of the lichen metabolites.[1] As both assays use intact cell systems, the lack of activity cannot be easily explained by the inability of the compounds to cross the cellular membrane. However, sequestration and dispositional differences may be responsible for the observed absence of the antiproliferative action.

Arachidonate lipoxygenases have been suggested to play an important physiological role in regulating cell survival,[19] and certain inhibitors of LTB_4 biosynthesis were shown to induce antiproliferative effects.[20,21] LTB_4 can effect keratinocyte proliferation,[7,8] and cultured HaCaT keratinocytes express the 5-LO gene.[22] The keratinocyte 5-LO pathway may, therefore, play a primary role in skin biology. According to this study, a role for 5-LO products in modulating proliferation of keratinocytes is only consistent with the data obtained for **5**. However, the weak or negative results obtained with **1** and **6** in the 5-LO assay[1] suggest a different mode of action for the strong antiproliferative activity of these compounds.

To determine whether the compounds may interfere with the functioning of the cell membrane, by causing leakage of cytoplasm through it, cytotoxicity against the cell cultures by the potent cell-growth inhibitors was assessed by the activity of LDH released into the culture medium. The release of LDH is commonly used as an indicator of plasma-membrane damage. In this assay, LDH release by the standard anthralin significantly exceeded that of the vehicle control. On the other hand, the antiproliferative activity of the potent lichen metabolites was due to cytostatic rather than cytotoxic effects, as LDH release was unchanged as compared to controls at 2-μM concentration of the test compounds (Table 1). The only exception was **1**, which gave rise to a slight increase in LDH activity as compared to vehicle controls. This may be related to its ability to generate hydroxyl radicals, whereas the other compounds were devoid of this property.[1] Thus, even though compounds **1**, **5**, and **6** were somewhat weaker inhibitors of cell growth than anthralin, they did not cause damage to the cell membrane as observed with this antipsoriatic agent.

In summary, we found that the lichen-derived metabolites gyrophoric acid (**6**), (+)-usnic acid (**1**), and diffractaic acid (**5**) were potent antiproliferative agents against the growth of human keratinocytes. Whether 5-LO inhibition is a main mechanism for the antiproliferative effect is not clear at present. Aside from the underlying mechanism of action, the combined inhibitory actions, in particular those of diffractaic acid (**5**), against 5-LO and HaCaT cell growth suggest a beneficial effect against inflammatory and hyperproliferative skin diseases such as psoriasis, inasmuch as both pathological features are targeted. The fact that the biological activities of this compound are not mediated by unspecific redox properties or cytotoxic effects on the plasma membrane, as observed for the antipsoriatic agent anthralin,[23] opens new possibilities in the search for antipsoriatic agents.

Experimental Section

General Experimental Procedures. For analytical instruments and methods, see Müller et al.[24]

Isolation and Identification of Lichen Metabolites. For isolation and identification of the lichen metabolites, see Kumar and Müller.[1]

Cell Culture and Determination of Cell Growth. HaCaT cell[17] proliferation assay was performed as previously described.[25,26] In brief, cells (2.5×10^4/1.1 mL suspension per well) were seeded on 24-well multidishes and grown in Dulbecco's modified Eagle's medium (DMEM). After 24 h of growth the medium was replaced, and the test compounds (0.1−5 μM) were added from stock solutions. These were prepared in DMSO and then diluted with DMEM, the final concentration of DMSO was 0.2% in the culture medium. Controls were performed with DMSO or medium alone. Forty eight hours after addition of the test compounds to the culture, the medium was removed and each well was rinsed with phosphate-buffered saline (PBS, 100 μL). The cells were then incubated with sterile 0.5% trypsin, 0.2% EDTA in PBS for 20 min at 37 °C. The detached cells from each well were suspended in DMEM and dispersed into single cells by gentle pipetting through an Eppendorf pipet. Cell growth was determined by enumerating the dispersed cells by phase contrast microscopy. Inhibition was calculated by the comparison of the mean values of the test compound ($n = 3$) with the vehicle control ($n = 3$): (1 − test compound/control) × 100. Inhibition was statistically significant compared to that of the control (Student's t-test; $p < 0.05$). Each IC_{50} value was derived by interpolation of a log inhibitor concentration versus response plot using four or more concentrations of the compound, spanning the 50% inhibition point.

Lactate Dehydrogenase Release.[27] HaCaT cells were incubated with the test compounds (2 μM) for 4 h at 37 °C. Extracellular LDH activity was measured using the UV method with pyruvate and NADH and is expressed in mU/mL. Appropriate controls with the vehicle were performed ($p < 0.05$; $n = 3$, SD < 10%).

Acknowledgment. We thank Mr. K. Ziereis for his excellent technical assistance and Mr. A. H. Banskota for the collection of *P. tinctorum*. S. K. KC thanks the German Academic Exchange Service for a scholarship.

References and Notes

(1) Kumar KC, S.; Müller, K. *J. Nat. Prod.* **1999**, *62*, 817−820 (preceding paper).
(2) Kumar KC, S.; Banskota, A. H.; Manandhar, M. D. *Planta Med.* **1996**, *62*, 93−94.
(3) *Leukotrienes and Lipoxygenases*; Rokach, J., Ed.; Elsevier: New York, 1989.
(4) Grimminger, F.; Mayser, P. *Prostaglandins Leukotrienes Essent. Fatty Acids* **1995**, *52*, 1−15.
(5) Iversen, L.; Kragballe, K.; Ziboh, V. A. *Skin Pharmacol.* **1997**, *10*, 169−177.
(6) Christophers, E.; Mrowietz, U. *Clin. Dermatol.* **1995**, *13*, 131−135.
(7) Kragballe, K.; Desjarlais, L.; Voorhees, J. J. *Br. J. Dermatol.* **1985**, *113*, 43−52.
(8) Chan, C. C.; Duhamel, L.; Ford-Hutchinson, A. *J. Invest. Dermatol.* **1985**, *85*, 333−334.
(9) Los, M.; Schenk, H.; Hexel, K.; Baeuerle, P. A.; Dröge, W.; Schulze-Osthoff, K. *EMBO J.* **1995**, *14*, 3731−3740.
(10) Ögmundsdóttir, H. M.; Zoëga, G. M.; Gissurarson, S. R.; Ingólfsdóttir, K. *J. Pharm. Pharmacol.* **1998**, *50*, 107−115.
(11) Tan, G. T.; Lee, S.; Lee, I.-S.; Chen, J.; Leitner, P.; Besterman, J. M.; Kinghorn, A. D.; Pezzuto, J. M. *Biochem. J.* **1996**, *314*, 993−1000.
(12) Pengsuparp, T.; Cai, L.; Constant, H.; Fong, H. H. S.; Lin, L.-Z.; Kinghorn, A. D.; Pezzuto, J. M.; Cordell, G. A.; Ingólfsdóttir, K.; Wagner, H.; Hughes, S. H. *J. Nat. Prod.* **1995**, *58*, 1024−1031.
(13) Kupchan, S. M.; Kopperman, H. L. *Experientia* **1975**, *31*, 625.
(14) Takai, M.; Uehara, Y.; Beisler, J. A. *J. Med. Chem.* **1979**, *22*, 1380−1384.
(15) Al-Bekairi, A. M.; Qureshi, S.; Chaudhry, M. A.; Krishna, D. R.; Shah, A. H. *J. Ethnopharmacol.* **1991**, *33*, 217−220.
(16) Cardarelli, M.; Serino, G.; Campanella, L.; Ercole, P.; De Cicco Nardone, F.; Alesiani, O.; Rossiello, F. *Cell. Mol. Life Sci.* **1997**, *53*, 667−672.

(17) Boukamp, P.; Petrussevska, R. T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N. E. *J. Cell Biol.* **1988**, *761*, 761−771.
(18) Bonnekoh, B.; Farkas, B.; Geisel, J.; Mahrle, G. *Arch. Dermatol. Res.* **1990**, *282*, 325−329.
(19) Tang, D. G.; Chen, Y. Q.; Honn, K. V. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5241−5246.
(20) Anderson, K. M.; Seed, T.; Plate, J. M. D.; Jajeh, A.; Meng, J.; Harris, J. E. *Leuk. Res.* **1995**, *19*, 789−801.
(21) Dittmann, K. H.; Mayer, C.; Rodemann, H. P.; Petrides, P. E.; Denzlinger, C. *Leuk. Res.* **1998**, *22*, 49−53.
(22) Janssen-Timmen, U.; Vickers, P. J.; Wittig, U.; Lehmann, W. D.; Stark, H.-J.; Fusenig, N. E.; Rosenbach, T.; Rådmark, O.; Samuelsson, B.; Habenicht, A. J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 6966−6970.
(23) Müller, K. *Biochem. Pharmacol.* **1997**, *53*, 1215−1221.
(24) Müller, K.; Gürster, D.; Piwek, S.; Wiegrebe, W. *J. Med. Chem.* **1993**, *36*, 4099−4107.
(25) Müller, K.; Leukel, P.; Ziereis, K.; Gawlik, I. *J. Med. Chem.* **1994**, *37*, 1660−1669.
(26) Müller, K.; Ziereis, K.; Gawlik, I. *Planta Med.* **1995**, *61*, 74−75.
(27) Müller, K.; Huang, H.-S.; Wiegrebe, W. *J. Med. Chem.* **1996**, *39*, 3132−3138.

NP980378Z