Journal of
Medicinal
Chemistry

© Copyright 2004 by the American Chemical Society

Volume 47, Number 22 October 21, 2004

Letters

Fluorescent Phenylpolyene Analogues of the Ether Phospholipid Edelfosine for the Selective Labeling of Cancer Cells

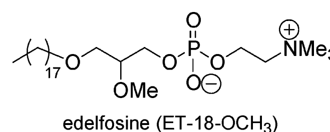
Ernesto Quesada,† Javier Delgado,† Consuelo Gajate,‡ Faustino Mollinedo,‡ A. Ulises Acuña,*,§ and Francisco Amat-Guerri*,†

Instituto de Química Orgánica, C.S.I.C., Juan de la Cierva 3, E-28006 Madrid, Spain, Centro de Investigación del Cáncer, Instituto de Biología Molecular y Celular del Cáncer, C.S.I.C.−Universidad de Salamanca, Campus Miguel de Unamuno, E-37007 Salamanca, Spain, and Instituto de Química Física Rocasolano, C.S.I.C., Serrano 119, E-28006 Madrid, Spain

Received March 8, 2004

Abstract: Edelfosine (ET-18-OCH_3), a synthetic antitumor ether lipid, is taken up by malignant but not by normal cells, triggering apoptosis in a large variety of human tumor cells. The synthesis of the first fluorescent edelfosine analogue (**6**), with apoptotic activity comparable to that of the parent drug, is described. Fluorescence microscopy experiments show that **6** selectively labels human cancer cells, accumulating into specific domains of the plasma membrane.

Ether glycerophospholipids may have one or two hydrocarbon chains linked to the glycerol framework by an ether bond.[1] A number of synthetic ether lipids (EL), collectively known as alkyl ether phospholipids or alkyllysophospholipids, show antitumor activity as well as other useful pharmacological properties,[2] including a potent antiparasitic effect. Interestingly, some EL have been shown to inhibit in a *selective* way the proliferation of cancer cells from a wide variety of solid tumors and leukemias.[3] However, because the therapeutic concentrations of these EL are usually close to their critical micelle concentration (ca. 10^{-6} M), it has been difficult to distinguish specific cytotoxic effects from nonspecific interactions, such as the lytic disruption of cell membranes. This limitation has now been overcome with the finding[3−5] of the selective proapoptotic effect on tumor cells of edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, or ET-18-OCH_3), a prototype for synthetic antitumor ether lipids.



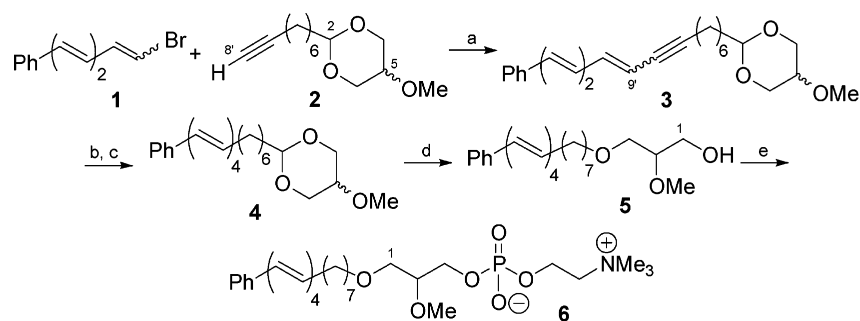
Since apoptosis is a specific event that can be timed and quantitated, the detection of an apoptotic response provides a reliable signal and marker for the putative antitumor activity of different ether lipids. Furthermore, the apoptotic activity of edelfosine seems to account for most of its antitumor activity.[3] This remarkable selectivity has been linked to the unidirectional uptake of the ether lipid by the neoplastic cells, with an efficiency of about an order of magnitude higher than that of noncancer cells.[3,4] However, the molecular mechanisms involved in this process, as well as that of the antineoplastic and antiparasitic activities of edelfosine and related EL, remain to be elucidated.

Fluorescent analogues of drugs are very useful tools for unveiling their mechanism of action and therapeutic targets,[6] as well as for the development of new diagnostic assays. In the case of edelfosine, a fluorescent analogue might also be of utility in the selective labeling of tumor metastasis. Since the available information on structure−apoptotic activity relationship on edelfosine is rather scarce,[3] we reasoned that replacement of the C_{18} aliphatic chain by a lipophilic fluorescent group of similar length might preserve drug activity and selectivity. Previous studies have shown that the *O*-octadecyl chain at C1 of edelfosine can hold some modifications, including the presence of a double bond, preserving the apoptotic activity.[3] On these grounds, a conjugated pentaene group may appear as a convenient candidate, considering that this fluorophore has led to useful

* To whom correspondence should be addressed. For A.U.A.: phone, +34915619400 (+1220); fax, +34915642431; e-mail, roculises@iqfr.csic.es. For F.A.-G.: phone, +34915622900 (+394); fax, +34915644853; e-mail, famat@iqog.csic.es.
† Instituto de Química Orgánica.
‡ Centro de Investigación del Cáncer.
§ Instituto de Química Física Rocasolano.

10.1021/jm049808a CCC: $27.50 © 2004 American Chemical Society
Published on Web 09/21/2004

Scheme 1[a]



[a] Reagents and conditions: (a) **2**/**1** mole ratio = 1.3, $Pd(PPh_3)_2Cl_2$ (7.5 mol %), CuI (25 mol %), Et_2NH, THF, Ar, room temp, 2 h, 90%; (b) Zn(Cu/Ag), $MeOH/H_2O$ 1:1, room temp, 24 h, 95%; (c) I_2 (trace), hexane, Ar, 15 min reflux, 95%; (d) DIBAL-H, MePh, Ar, 0 °C, 93–99%; (e) (i) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Me_3N, acetonitrile, pressure tube, −78 °C; (ii) room temp, 2 h, and 70 °C, 1 h, 23–35%.

fluorescent probes for lipid membranes.[7,8] But this group requires UV excitation at a wavelength (ca. 340 nm) not readily available in a standard fluorescence microscope.

Here, we report the synthesis of the first fluorescent analogue of edelfosine, with a conjugated ω-phenyltetraene group instead of the *n*-octadecyl substituent in position 1 (**6**, Scheme 1). Analogue **6** is characterized by a strong absorption band at ca. 360 nm, a wavelength widely used in fluorescence microscopy, and a bluish fluorescence ($\lambda_{max} \approx 442$ nm) with a modest but still adequate quantum yield (~0.3 in a lipid bilayer). The emitting ether lipid **6** showed both the selectivity for cancer cells and the ensuing apoptotic activity of the parent drug. The relatively simple synthetic method allows for other fluorescent analogues with diverse aromatic groups, chain length, number of conjugated double bonds, or stereochemistry.

Analogue **6** was obtained in five steps (Scheme 1), of which the first three have been previously used in this laboratory[8] and in others[9] for the synthesis of related conjugated polyenes and represent an additional example of the application of the *acetylenic approach*. The first step was a Sonogashira–Hagihara cross-coupling[10,11] between the phenylbromotriene **1** (in the form of 3:7 (1*E*)/(1*Z*) mixture of isomers) and the *cis*- or *trans*-1,3-dioxane **2**, with a terminal acetylenic group at the chain in position 2. *cis*-**2** and *trans*-**2** were simultaneously obtained by acetalization of non-8-ynal with *rac*-2-*O*-methylglycerol. The initial 3:7 (*E*)/(*Z*) ratio of **1** was not changed by the cross-coupling reaction, and *cis*-**3** and *trans*-**3** were respectively isolated as the same (*E*)/(*Z*) mixture. Pure isomers all-(*E*)-*cis*-**3** and all-(*E*)-*trans*-**3** were easily separated from each (*E*)/(*Z*) mixture by precipitation from a dichloromethane solution with *n*-pentane. Partial reduction of the triple bond with activated Zn (Boland method)[12] in any of the isomeric trienyne compounds **3** or mixtures thereof yielded the corresponding (7′*Z*, ...)-tetraene, which after iodine isomerization gave the respective all-(*E*)-*cis*-**4** or all-(*E*)-*trans*-**4** isomers. The subsequent acetal cleavage/reduction of any cis/trans isomer **4** with DIBAL-H[13] produced the all-(*E*) alcohol **5**. All the above reactions had >90% yield. Eventually, one-pot reaction of **5** with 2-chloro-2-oxo-1,3,2-dioxaphospholane and trimethylamine[14] gave the pure fluorescent analogue **6** with 23–35% yield.[15] Compounds **3**–**6** are thermally stable for months if kept frozen in a dark place.

Analogue **6** induced apoptosis in human T-leukemia Jurkat cells, although to a lower extent than the parent drug edelfosine (Figure 1), whereas the control alcohol **5**, lacking the essential phosphocholine headgroup, was inactive. The apoptotic response of **6** was further evidenced by the typical internucleosomal DNA fragmentation in multiples of 180–200 bp (data not shown). In addition, no previous effects were observed in any cell cycle phase in the concentration range assayed here. On the other hand, analogue **6** spared normal peripheral blood lymphocytes, inducing less than 2% apoptosis.

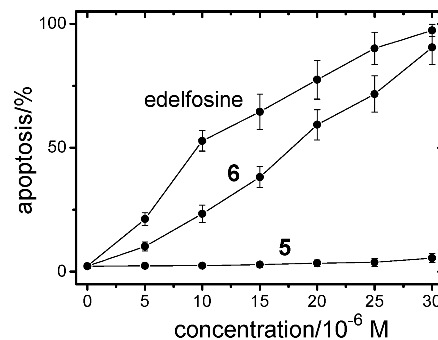


Figure 1. Comparative apoptotic activity of edelfosine, the fluorescent analogue **6**, and the inactive alcohol **5** in human T-leukemic Jurkat cells. Induction of apoptosis was determined as the percentage of cells with a DNA content of less than G_1 (hypodiploidy) in cell cycle analysis. Points were averaged (±SD) from three independent experiments. For further details of the apoptotic assay, see refs 4 and 17.

The selective uptake of the fluorescent analogue **6** was demonstrated by comparing in vitro its incorporation into cancer versus normal nontransformed cells. Human T-leukemic Jurkat cells have been reported to incorporate high levels of edelfosine, whereas normal human peripheral blood lymphocytes failed to take up significant amounts of the lipid.[4,16] Thus, both cell types were incubated with the fluorescent analogue **6**, and after exhaustive washing to remove the loose cell-surface-bonded ether lipid, the cells were analyzed by fluorescence microscopy.[17] Whereas a faint labeling could be hardly observed in normal cells (Figure 2a), **6** was taken up by the plasma membrane of leukemic cells in large quantities (Figure 2b) from which it could be reversibly displaced by adding edelfosine. Interestingly, the fluorescent signal from leukemic cells was not homogeneously distributed over the membrane but accumulated into well-defined domains of the cell plasma membrane. The lateral segregation of the ether lipid, which can easily be detected with fluorescent analogue **6**, may be related to its ability to cluster membrane lipid rafts[18] and suggests its putative enrichment in these lipid

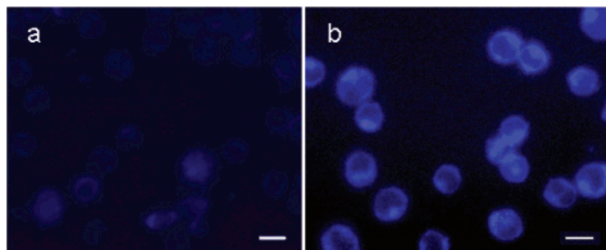


Figure 2. Fluorescence images of (a) normal human peripheral blood lymphocytes and (b) human T-leukemic Jurkat cells, both treated for 7 h with 20 μM fluorescent edelfosine analogue **6**.[17] Practical detection limit was ~5 μM. The bar represents 10 μm.

domains. Further studies on the biochemical basis of this selectivity are in progress.

Acknowledgment. This work was supported by Grants BQU2000-1500, BQU2003-4413, FIS-02/1199, and 1FD97-0622 from the Ministerio de Ciencia y Tecnología, Fondo de Investigación Sanitaria (Spain), and the European Commission. E.Q. and J.D. acknowledge predoctoral grants from the same sources.

Supporting Information Available: Experimental procedures and analytical and spectral data for **1**–**6** and additional fluorescence data of **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Paltauf, F. Ether lipids in biomembranes. *Chem. Phys. Lipids* **1994**, *74*, 101–139.

(2) (a) Munder, P. G.; Westphal, O. Antitumoral and other biomedical activities of synthetic ether lysophospholipids. *Chem. Immunol.* **1990**, *49*, 206–235. (b) Houlihan, W. J.; Lohmeyer, M.; Workman, P.; Cheon, S. H. Phospholipid antitumor agents. *Med. Res. Rev.* **1995**, *15*, 157–223. (c) Arthur, G.; Bittman, R. The inhibition of cell signaling pathways by antitumor ether lipids. *Biochim. Biophys. Acta* **1998**, *1390*, 85–102. (d) Bittman, R.; Perkins, W. R.; Swenson, C. E. TLC ELL-12. *Drugs Future* **2001**, *26*, 1052–1058. (e) Jendrossek, V.; Handrick, R. Membrane targeted anticancer drugs: potent inducers of apoptosis and putative radiosensitisers. *Curr. Med. Chem.* **2003**, *3*, 343–353. (f) Croft, S. L.; Seifert, K.; Duchene, M. Antiprotozoal activities of phospholipid analogues. *Mol. Biochem. Parasitol.* **2003**, *126*, 165–172.

(3) Mollinedo, F.; Fernández-Luna, J. L.; Gajate, C.; Martín-Martín, B.; Benito, A.; Martínez Dalmau, R.; Modolell, M. Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH_3 (edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-XL. *Cancer Res.* **1997**, *57*, 1320–1328.

(4) Gajate, C.; Fonteriz, R. I.; Cabaner, C.; Álvarez-Noves, G.; Álvarez-Rodríguez, Y.; Modolell, M.; Mollinedo, F. Intracellular triggering of Fas, independently of FasL, as a new mechanism of antitumor ether lipid-induced apoptosis. *Int. J. Cancer* **2000**, *85*, 674–682.

(5) Gajate, C.; Mollinedo, F. Biological activities, mechanisms of action and biomedical prospect of the antitumor ether phospholipid ET-18-OCH_3 (edelfosine), a proapoptotic agent in tumor cells. *Curr. Drug Metab.* **2002**, *3*, 491–525.

(6) For the case of the antitumor drug Taxol, see the following. (a) Souto, A. A.; Acuña, A. U.; Andreu, J. M.; Barasoain, I.; Abal, M.; Amat-Guerri, F. New fluorescent water-soluble taxol derivatives. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 2710–2712. (b) Abal, M.; Souto, A. A.; Amat-Guerri, F.; Acuña, A. U.; Andreu, J. M.; Barasoain, I. Centrosome and spindle pole microtubules are main targets of a fluorescent Taxoid inducing cell death. *Cell Motil. Cytoskeleton* **2001**, *49*, 1–15. (c) Lillo, M. P.; Cañadas, O.; Dale, R. E.; Acuña, A. U. Location and properties of the taxol binding center in microtubules: a picosecond laser study with fluorescent taxoids. *Biochemistry* **2002**, *41*, 12436–12449.

(7) (a) Souto, A. A.; Acuña, A. U.; Amat-Guerri, F. A general and practical synthesis of linear conjugated pentaenoic acids. *Tetrahedron Lett.* **1994**, *35*, 5907–5910. (b) Reyes Mateo, C.; Souto, A. A.; Amat-Guerri, F.; Acuña, A. U. New fluorescent octadecapentaenoic acids as probes of lipid membranes and protein–lipid interactions. *Biophys. J.* **1996**, *71*, 2177–2191.

(8) (a) Quesada, E.; Acuña, A. U.; Amat-Guerri, F. New transmembrane polyene bolaamphiphiles as fluorescent probes in lipid bilayers. *Angew. Chem., Int. Ed.* **2001**, *40*, 2095–2097. (b) Quesada, E.; Acuña, A. U.; Amat-Guerri, F. Synthesis of carboxyl-tethered symmetric conjugated polyenes as fluorescent transmembrane probes of lipid bilayers. *Eur. J. Org. Chem.* **2003**, 1308–1318.

(9) Crousse, B.; Alami, M.; Linstrumelle, G. Stereocontrolled synthesis of (*E,E,E*)-chlorotrienes: Efficient intermediates for the construction of all *E* conjugated polyenes. *Tetrahedron Lett.* **1997**, *38*, 5297–5300.

(10) (a) Sonogashira, K.; Tohda, Y.; Hagihara, N. A convenient synthesis of acetylenes: catalytic substitutions of acetylenic hydrogen with bromoalkenes, iodoarenes and bromopyridines. *Tetrahedron Lett.* **1975**, *16*, 4467–4470. (b) Takahashi, S.; Kuroyama, Y.; Sonogashira, K.; Hagihara, N. A convenient synthesis of ethynylarenes and diethynylarenes. *Synthesis* **1980**, 627–630.

(11) Reviews: (a) Campbell, I. B. The Sonogashira Cu–Pd-catalyzed alkyne coupling reaction. In *Organocopper Reagents*; Taylor, R. J. K., Ed.; I.R.L. Press: Oxford, 1994; pp 217–235. (b) Sonogashira, K. Cross-coupling reactions to sp carbon atoms. In *Metal Catalyzed Cross-Coupling Reactions*; Diederich, F., Stang, P. J., Eds.; Wiley-VCH: Weinheim, Germany, 1998; pp 203–229. (c) Sonogashira, K. Development of Pd–Cu catalyzed cross-coupling of terminal acetylenes with sp^2-carbon halides. *J. Organomet. Chem.* **2002**, *653*, 46–49. (d) Tykwinski, R. R. Evolution in the palladium-catalyzed cross-coupling of sp- and sp^2-hybridized carbon atoms. *Angew. Chem., Int. Ed.* **2003**, *42*, 1566–1568.

(12) Boland, W.; Pantke S. (*Z*)-Selective reduction of conjugated alkynes with Zn (Cu/Ag). *J. Prakt. Chem.* **1994**, *336*, 714–715.

(13) Takano, S.; Akiyama, M.; Sato, S.; Ogasawara, K. A facile cleavage of benzylidene acetals with diisobutylaluminum hydride. *Chem. Lett.* **1983**, 1593–1596.

(14) Jia, A.; Haines, H. Diamide analogs of phosphatidyl choline as potential anti-AIDS agents. *J. Chem. Soc., Perkin Trans. 1* **1993**, 2521–2523.

(15) Lower yields were obtained with the two-step phosphorylation frequently used in the synthesis of related phospholipids: (a) Berchtold, R. Preparation of 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine. *Chem. Phys. Lipids* **1982**, *30*, 389–392. (b) Hirth, G.; Barner, R. Synthesis of glyceryletherphosphatides. I. Preparation of 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphorylcholine (*platelet activating factor*) of its enantiomer and of some analogous compounds. *Helv. Chim. Acta* **1982**, *65*, 1059–1084. (c) Stekar, J.; Nössner, G.; Kutscher, B.; Engel, J.; Hilgard, P. Synthesis, antitumor activity, and tolerability of phospholipids containing nitrogen homologs. *Angew, Chem. Int. Ed. Engl.* **1995**, *34*, 238–240.

(16) Cabaner, C.; Gajate, C.; Macho, A.; Muñoz, E.; Modolell, M.; Mollinedo, F. Induction of apoptosis in human mitogen-activated peripheral blood T-lymphocytes by the ether phospholipid ET-18-OCH_3: involvement of the Fas receptor/ligand system. *Br. J. Pharmacol.* **1999**, *127*, 813–825.

(17) Human normal peripheral blood lymphocytes and human T-leukemia Jurkat cells (2.5×10^5 cells/mL) were treated with 20 μM **6** for 7 h in RPMI-1640 culture medium containing 10% fetal calf serum and washed three times with phosphate buffered saline containing 1% bovine serum albumin to eliminate loosely bound **6** to the cells surface. Then 2×10^5 **6**-treated cells were settled onto poly-L-lysine-coated slides, fixed in 4% formaldehyde for 25 min at room temperature, and analyzed using a Zeiss Axioplan 2 fluorescence microscope (Oberkochen, Germany) and a Hamamatsu ORCA-ER-1394 digital camera. The extent of apoptosis was assessed (flow cytometry) as the percentage of cells with a DNA content of less than G1 (hypodiploidy) in cell cycle analysis, using propidium iodide staining in permeabilized cells, after 24 h of incubation with edelfosine or with **5** and **6** in the presence of 10% fetal calf serum. Untreated control cells run in parallel showed 2.3 ± 1.0% apoptosis.

(18) Gajate, C.; Mollinedo, F. The antitumor ether lipid ET-18-OCH_3 induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells. *Blood* **2001**, *98*, 3860–3863.

JM049808A