Synthesis of a Resveratrol Analogue with High Ceramide-Mediated Proapoptotic Activity on Human Breast Cancer Cells

Filippo Minutolo,[†,||] Giusy Sala,[‡,||] Annalisa Bagnacani,[‡] Simone Bertini,[†] Isabella Carboni,[†] Giorgio Placanica,[†] Giovanni Prota,[†] Simona Rapposelli,[†] Nicoletta Sacchi,[§] Marco Macchia,*[,†] and Riccardo Ghidoni*[,‡]

Department of Pharmaceutical Sciences, University of Pisa, via Bonanno 6, 56126 Pisa, Italy, Laboratory of Biochemistry and Molecular Biology, San Paolo University Hospital, Medical School, University of Milan, 20142 Milan, Italy, and Department of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, New York 14263

Received June 6, 2005

Abstract: Resveratrol, a natural product with a stilbene structure, exerts profound proapoptotic activity in human cancer cells, by triggering the accumulation of ceramide, a bioactive sphingolipid. We studied the biological effects of seven methoxylated and/or naphthalene-based resveratrol analogues and compared these compounds with resveratrol with the objective to identify an analogue with higher ceramide-mediated proapoptotic activity relative to resveratrol. Here we show that the compound with three hydroxyls and a naphthalene ring is the most effective in triggering apoptosis coupled to the induction of endogenous ceramide in human cancer cells.

Resveratrol, a naturally occurring phytoalexin (3,5,4′-*trans*-trihydroxystilbene) present in medicinal plants, grape skin, peanuts, and red wine,[1] has been reported to have chemopreventive activity,[2] by exerting antiproliferative and proapoptotic effects in human cancer cells.[3] We recently showed that resveratrol can affect cancer cell growth and induce apoptosis by triggering the synthesis of endogenous ceramide,[4,5] a bioactive sphingolipid.[6,7] Specifically, we observed that resveratrol leads to the accumulation of ceramide in association with apoptosis both in metastatic breast[4] and prostate[5] cancer cells. Ceramide is a promising pharmacological target when either major apoptotic pathways are disrupted or resistance to DNA damage is elevated. Moreover, drugs that trigger ceramide, while highly effective in malignant cells, are less toxic to normal cells and tissues.[8]

For this reason, objective of the present study was to design and synthesize resveratrol analogues more effective than resveratrol at inducing ceramide-mediated apoptosis in the metastatic breast cancer cell line, MDA-MB-231. In these cells we found that resveratrol induced ceramide by activation of its de novo synthesis.[4]

* Corresponding authors. R.G.: Phone: +39 02 50323250. E-mail: riccardo.ghidoni@unimi.it. M.M.: Dipartimento di Scienze Farmaceutiche, Università di Pisa, Via Bonanno, 6-56126 Pisa, Italy. Phone: +39 050 2219553. Fax: +39 050 2219553. E-mail: mmacchia@farm.unipi.it.

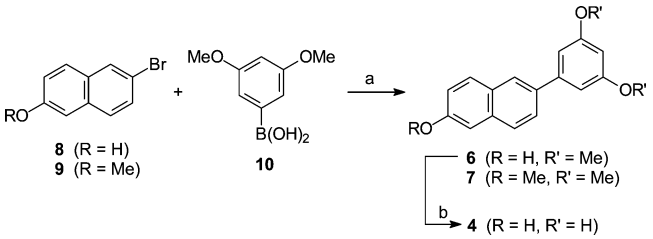
† University of Pisa.

‡ University of Milan.

§ Roswell Park Cancer Institute.

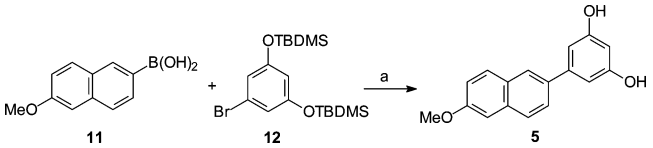
|| These authors equally contributed to the present work.

Scheme 1[a]



[a] Key: (a) $Pd(PPh_3)_4$, K_3PO_4 (aq), dioxane, 80 °C, 16 h. (b) BBr_3, CH_2Cl_2, −78 °C, 3 h.

Scheme 2[a]



[a] Key: (a) (1) $Pd(PPh_3)_4$, K_3PO_4 (aq), dioxane, 80 °C, 16 h. (2) HCl (aq).

Because methoxylated resveratrol analogues have been identified in plants,[9−12] we synthesized three analogues (compounds **1**−**3**), by replacing the hydroxyl groups with methoxyl groups. In addition we tried to overcome both the chemical and metabolic instability of resveratrol by substituting the stilbene double bond present in resveratrol with a naphthalene ring. To this end, we synthesized four naphthalene-based resveratrol analogues that do not contain the unstable double bond within their structure, either with or without methoxyl groups (compounds **4**−**7**). This simple, yet unexplored, change enables preservation of the original geometrical orientation of the pharmacophoric groups of resveratrol, while adding more conformational rigidity to the whole structure.

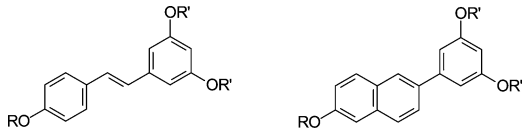
When we tested these seven analogues for their ceramide-mediated proapoptotic activity, we found that the analogue with three hydroxyls and a naphthalene ring was the most effective in triggering apoptosis coupled to the induction of endogenous ceramide in the human breast cancer cell line.

Synthesis of Resveratrol Analogues. Compounds **1**−**3** were synthesized as outlined in the Supporting Information. By using the strategy outlined in Scheme 1, we synthesized compounds **4**, **6**, and **7**. A Suzuki Pd-catalyzed cross-coupling reaction between 3,5-dimethoxyphenylboronic acid (**10**) and 6-bromo-2-naphthol (**8**) or 6-methoxy-2-bromo-naphthalene (**9**) was used to synthesize compounds **6** and **7**, respectively. The completely demethylated compound **4** was obtained by treating **6** with boron tribromide at −78 °C.

The synthesis of monomethylated compound **5** was achieved as shown in Scheme 2. In this case 6-methoxynaphthylboronic acid (**11**)[13] was again submitted to a Suzuki reaction with 3,5-(di-*tert*-butyldimethylsilyloxy)-1-bromobenzene (**12**).[14] Acidic aqueous workup caused complete desilylation of the protected phenolic groups, leading to the synthesis of pure compound **5**.

All Resveratrol Analogues Display Antiproliferative Effect in MDA-MB-231 Cancer Cells. The

10.1021/jm050528k CCC: $30.25
Published on Web 10/04/2005



RES (R = H, R' = H)
1 (R = Me, R' = H)
2 (R = H, R' = Me)
3 (R = Me, R' = Me)

4 (R = H, R' = H)
5 (R = Me, R' = H)
6 (R = H, R' = Me)
7 (R = Me, R' = Me)

Figure 1. Structures of stilbene- and naphthalene-type resveratrol analogues.

seven resveratrol analogues were compared for their growth inhibitory effect on MDA-MB-231 breast cancer cells. Cells were treated with compounds **1**−**7** at different concentrations, ranging from 0.25 μM to 64 μM for 6 days (Figure 2). The sulforhodamine B (SRB) cell proliferation assay was used. The proliferation index at the beginning of the experiment (time 0) was set at 1. The IC_{50} for the seven resveratrol analogues are reported in Table 1, together with the IC_{50} of resveratrol. Compounds **2**, **3**, **4**, and **6** had a stronger antiproliferative effect than resveratrol.

Compound **3** showed the highest antiproliferative effect (IC_{50} = 1.2 μM), followed by compounds **6** (IC_{50} = 7.2 μM), **2** (IC_{50} = 10.0 μM), and **4** (IC_{50} = 12.7 μM). The antiproliferative effect of compounds **1**, **5**, and **7** were by far lower (IC_{50} = >50, 28.3, and 45.1 μM, respectively). Some compounds tested were able to impair cell growth at a concentration as low as 1 μM (Figure 2).

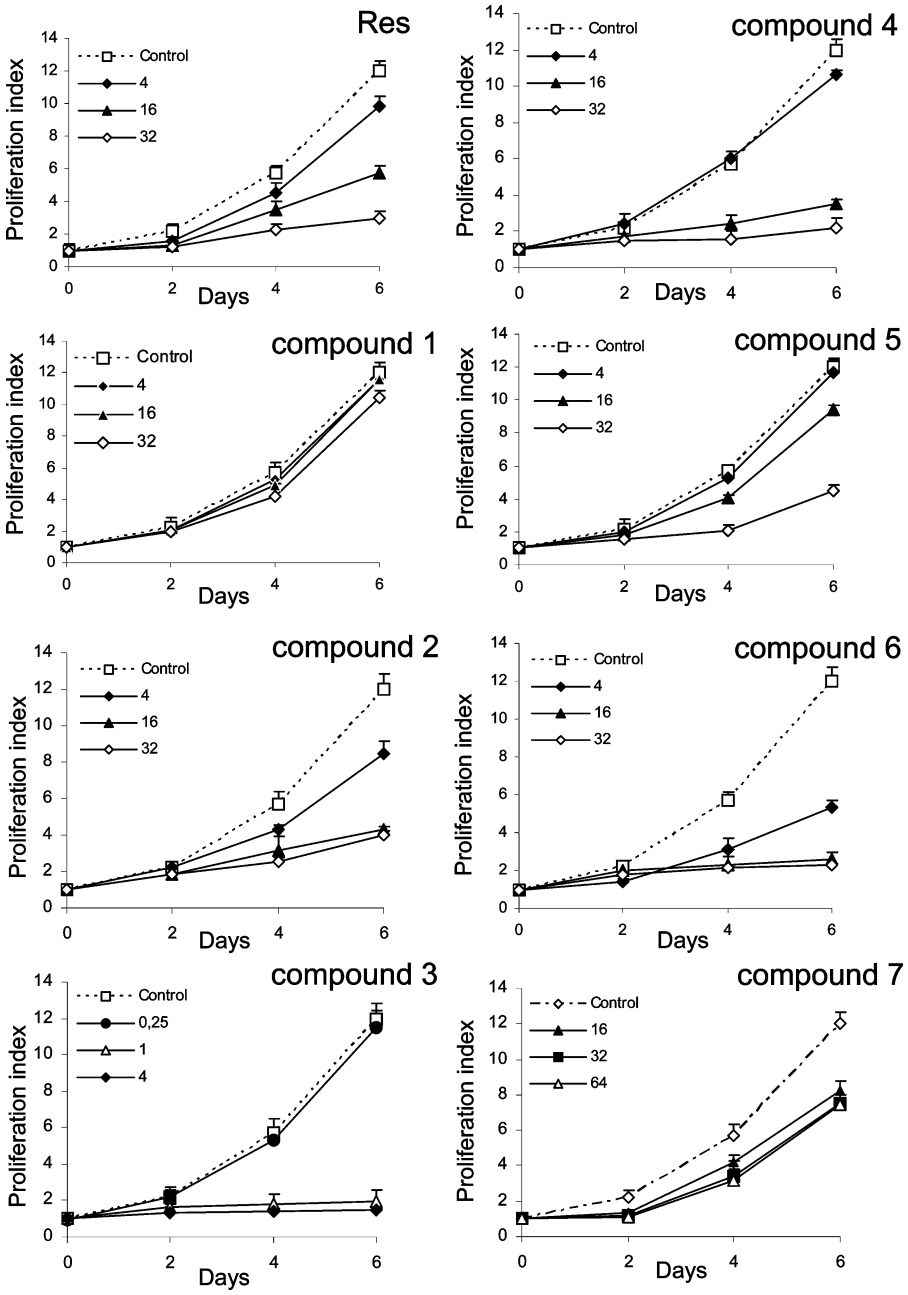


Figure 2. Antiproliferative effect of resveratrol and synthetic resveratrol analogues. Proliferation index was evaluated by the SRB assay in MDA-MB-231 cells treated with 0.25−64 μM resveratrol or synthetic analogues for up to 6 days. The values are the mean ± SD of three independent experiments. The symbols and lines represent different drug concentrations, as shown in the figure insets.

Table 1. IC_{50} Values for Resveratrol and Resveratrol Analogues[a]

compound	IC_{50} (μM)
RES	20.5 ± 2.6
1	>50
2	10.0 ± 1.8
3	1.2 ± 0,2
4	12.7 ± 1.3
5	28.3 ± 3.8
6	7.2 ± 1.0
7	45.1 ± 6.8

[a] IC_{50} (μM) was calculated as the mean ± SD of the proliferation values obtained at 2, 4, and 6 days of treatment with each compound used at different concentrations in three independent experiments.

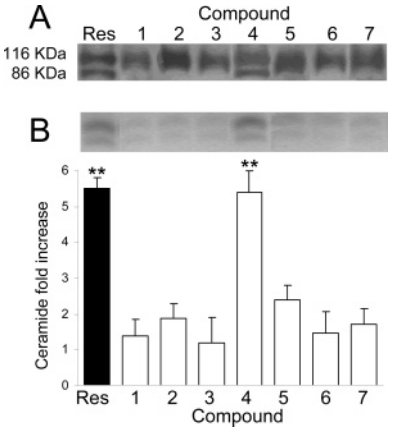


Figure 3. Proapoptotic effect and ceramide accumulation by resveratrol and synthetic analogues. MDA-MB-231 cells were treated for 4 days with resveratrol and resveratrol analogues at the respective IC_{50}. (A) The proapoptotic effect was determined by the PARP cleavage assay, evaluated as appearance of the 86 kDa band. (B) Ceramide accumulation determined by the DGK assay. Ceramide spots of a representative experiment and ceramide quantification, as the mean ± SD of three independent experiments. Ceramide content of treated cells is expressed as fold-increase (** = $p < 0.01$) respect to untreated cells.

Compound 4 Exerts an Antiproliferative Effect through an Apoptotic Mechanism. To assess whether the antiproliferative effect could be traced to an apoptotic mechanism, we tested the induction of poly-(ADP-ribose)-polymerase (PARP) cleavage by the seven analogues after treating MDA-MB-231 cells with the IC_{50} (see Table 1) for 4 days (Figure 3A). The 86 kDa band, which is an evidence of PARP cleavage, was detected only with compound **4**. Thus, only the resveratrol analogue with intact hydroxyls and an additional naphthalene ring exerts an antiproliferative effect via an apoptotic mechanism. Since PARP is a substrate for caspase-3, the proapoptotic effect of compound **4** might be caspase-mediated.

Compound 4 Induces a Level of Endogenous Ceramide Higher Than the Ceramide Level Induced by Resveratrol. Next, we tested whether the antiproliferative effect of the seven analogues involved ceramide accumulation. By using the diacylglycerol-kinase (DGK) assay, we found that MDA-MB-231 cells treated for 4 days with the seven resveratrol analogues at the corresponding IC_{50} (see Table 1) displayed a level of ceramide significantly higher ($p < 0.01$) than the level induced by resveratrol only when treated with compound **4** (Figure 3B). Specifically ceramide was induced more than 5-fold by compound **4** than by resveratrol. A modest, but not significant ceramide accumulation was induced also by compound **5**, a monomethylated, naphthalene ring resveratrol analogue.

Altogether, our data show that the introduction of a naphthalene ring on the intact resveratrol molecule confers an improved ceramide-mediated proapoptotic activity.

In this study we report that there is a relationship between the structure of resveratrol analogues and the ceramide-mediated proapoptotic activity in human cancer cells.

Resveratrol biological activity in cancer cells is limited by its photosensitivity and metabolic instability. Under UV irradiation, resveratrol converts to the (*Z*)-isomer[15a,16a] and this was claimed as one of the reason for the presence of small amounts of (*Z*)-resveratrol in wines. The conversion from an (*E*)- to a (*Z*)-configuration makes resveratrol less stable[16b,c] and consequently diminishes its biological activity. Moreover, stilbene double bonds are readily oxidized by cytochrome P450 monooxygenases into highly reactive epoxides, that may act as carcinogenic metabolites.[15b−d] We then synthesized novel resveratrol analogues with a naphthalene ring instead of a stilbene double bond and tested whether they were able to induce ceramide-mediated apoptosis.

First, we observed that the stabilization of the double bond of resveratrol, accompanied by an increase in molecular rigidity which is operated by the addition of a fused benzene ring as in compound **4**, not only preserves, but considerably improves the biological activity of natural resveratrol (IC_{50} = 12.7 μM of compound **4** versus $\mathbf{IC_{50}}$ = 20.5 μM of resveratrol, and IC_{50} = 28.3 μM of compound **5** versus $\mathbf{IC_{50}}$ > 50 μM of compound **1**). Like resveratrol, yet at higher efficiency, compound **4** exerts an antiproliferative effect via a ceramide-mediated proapoptotic mechanism, as shown by an increase of endogenous ceramide coupled to cleavage of PARP. All the other six compounds tested exerted at the IC_{50} variable antiproliferative effects without significant induction of either ceramide or apoptosis. Thus, both resveratrol and compound **4** with three intact hydroxyl groups seem to affect proliferation with a ceramide-mediated apoptotic mechanism.

Second, we confirmed that ceramide-independent antiproliferative activity of resveratrol can be increased by replacing the 3,5-hydroxy groups with methoxy functions (compounds **2** and **3**), regardless of the presence of two or three aromatic rings. A resveratrol analogue (3,5-di-*O*-methyl-3′-hydroxy-*trans*-stilbene), very similar to compound **2**, has been recently reported to be the most potent antiproliferative analogue of a group of methylated analogues of resveratrol[17] toward the breast cancer cell line MDA-MB-468. The addition of a third methoxy function in the 4′-position of resveratrol (compound **3**) resulted in a dramatic antiproliferative and cytotoxic effect, not associated with ceramide accumulation. In other cell lines[18,19] trimethoxystilbene was found to be highly cytotoxic, and this is due to interference with the mitotic process by microtubule disassembly, impairment of tubulin polymerization, and depletion of polyamine intracellular pool. Interestingly, the presence of an additional aromatic ring to methoxy

derivatives confers a strong antiproliferative potential provided that an hydroxyl group is present (compound **6**). Conversely, the concurrent presence of three methoxy groups and three aromatic rings (compound **7**) abrogated the antiproliferative capacity of the molecule, possibly for its highly lipophilic nature. Interestingly, methoxylation on the 4′-position alone makes the molecule less antiproliferative. This was the case of compound **1** and compound **5** with either two or three aromatic rings, respectively. This argues in favor of an essential role of the 4′-hydroxy group for the antiproliferative activity of resveratrol.[20]

Altogether, our findings show that targeting ceramide signaling by resveratrol analogues might provide novel promising strategies to control cancer cell growth.

Supporting Information Available: Experimental conditions and analytical data of compounds **4**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Aggarwal, B. B.; Bhardwaj, A.; Aggarwal, R. S.; Seeram, N. P.; Shishodia, S.; Takada, Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res.* **2004**, *24*, 2783−2840.

(2) Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218−220.

(3) Signorelli, P.; Ghidoni, R. Resveratrol as an anticancer nutrient: molecular basis, open questions and promises. *J. Nutr. Biochem.* **2005**, *16*, 449−466.

(4) Scarlatti, F.; Sala, G.; Somenzi, G.; Signorelli, P.; Sacchi, N.; Ghidoni, R. Resveratrol induces growth inhibition and apoptosis in metastasis breast cancer cells via de novo ceramide signalling. *FASEB J.* **2003**, *17*, 2339−2341.

(5) Sala, G.; Minutolo, F.; Macchia, M.; Sacchi, N.; Ghidoni, R. Resveratrol structure and ceramide-associated growth inhibition in prostate cancer cells. *Drugs Exp. Clin. Res.* **2003**, *29*, 263−269.

(6) Ogretmen, B.; Hannun, Y.; Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* **2004**, *4*, 604−616.

(7) Patrick Reynolds, C.; Maurer, B.; Kolesnick, R. N. Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Lett.* **2004**, *206*, 169−180.

(8) Selzner, M.; Bielawska, A.; Morse, A. M.; Rudiger, H. A.; Sindram, D.; Hannun, Y. A.; Clavien, P. A. Induction of apoptotic cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer. *Cancer Res.* **2001**, *61*, 1233−40.

(9) Seshadri, T. R. Polyphenols of Pterocarpus and Dalbergia woods. *Phytochemistry* **1972**, *11*, 881−898.

(10) Langkake, P.; Cornford, C. A.; Price, R. J. Identification of pterostilbene as a phytoalexin from Vitis vinifera leaves. *Phytochemistry* **1979**, *18*, 1025−1027.

(11) Paul, B.; Masih, I.; Deopujari, J.; Charpentier, C. Occurrence of resveratrol and pterostilbene in age-old darakchasava, an ayurvedic medicine from India. *J. Ethnopharmacol.* **1999**, *68*, 71−76.

(12) Yao, C. S.; Lin, M.; Liu, X.; Wang, Y. H. Stilbene derivatives from Gnetum cleistostachyum. *Asian Nat. Prod. Res.* **2005**, *7*, 131−137.

(13) Percec, V.; Chu, P.; Kawasumi, M. Toward "Willowlike" Thermotropic Dendrimers. *Macromolecules* **1994**, *27*, 4441−4453.

(14) Bharathi, P.; Zhao, H.; Thayumanavan, S. Toward Globular Macromolecules with Functionalized Interiors: Design and Synthesis of Dendrons with an Interesting Twist. *Org. Lett.* **2001**, *3*, 1961−1964.

(15) (a) Deak, M.; Falk, H. On the chemistry of the resveratrol diastereomers. *Monatsh. Chem.* **2003**, *134*, 883−888. (b) Zbaida, S.; Kariv, R. Biomimetic models for monooxygenases. *Biopharm. Drug Dispos.* **1989**, *10*, 431−442. (c) Metzler, M.; Neumann, H. G. Epoxidation of the stilbene double bond, a major pathway in aminostilbene metabolism. *Xenobiotica* **1977**, *7*, 117−132. (d) Matzler, M. Metabolic activation of diethylstilbestrol. Indirect evidence for the formation of a stilbene oxide intermediate in hamster and rat. *Biochem. Pharmacol.* **1975**, *24*, 1449−1453.

(16) (a) Pervaiz S. Resveratrol: from grapevines to mammalian biology. *FASEB J.* **2003**, *17*, 1975−1985. (b) Trela, B.; Waterhouse, A. Resveratrol: isomeric molar absorptivities and stability. *J. Agric. Food Chem.* **1996**, *44*, 1253−1257. (c) Deak, M.; Falk, H. On the chemistry of resveratrol diastereoisomers. *Monatsh. Chem.* **2003**, *134*, 883−888.

(17) Lion, C. J.; Matthews, C. S.; Stevens, M. F.; Westwell, A. D. Synthesis, antitumor evaluation, and apoptosis-inducing activity of hydroxylated (*E*)-stilbenes. *J. Med. Chem.* **2005**, *48*, 1292−1295.

(18) Schneider, Y.; Chabert, P.; Stutzmann. J.; Coelho, D.; Fougerousse, A.; Gosse, F.; Launay, J. F.; Brouillard, R.; Raul, F. Resveratrol analogue (*Z*)-3,5,4′-trimethoxystilbene is a potent anti-mitotic drug inhibiting tubulin polymerization. *Int. J. Cancer* **2003**, *107*, 189−196.

(19) Belleri, M.; Ribatti, D.; Nicoli, S.; Cotelli, F.; Forti, L.; Tannini, V.; Stivala, L. A.; Presta, M. Antiangiogenic and vascular-targeting activity of the microtubuledestabilizing trans-resveratrol derivative 3,5,4′-trimethoxystilbene. *Mol. Pharmacol.* **2005**, *67*, 1451−1459.

(20) Matsuoka, A.; Takeshita, K.; Furuta, A.; Ozaki, M.; Fukuhara, K.; Miyata, N. The 4′-hydroxy group is responsible for the in vitro cytogenetic activity of resveratrol. *Mutat. Res.* **2002**, *521*, 29−35.

JM050528K