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Antitumor Potential of Conjugable Valinomycins Bearing Hydroxyl Sites: In Vitro Studies

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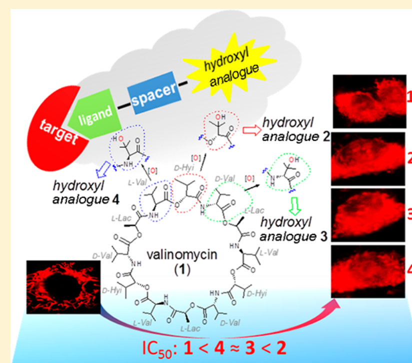
Supporting Information

ABSTRACT: Following our pioneering studies on the direct and efficient introduction of derivatizable hydroxyl handles into the valinomycin (VLM, **1**) structure, a K^+-ionophore with potent antitumor activity, the ensuing conjugable analogues (HyVLMs **2**, **3**, and **4**) have herein been compared to the parent macrocycle for their potential antiproliferative effects on a panel of cancer cell lines, namely, human MCF-7, A2780, and HepG2, as well as rat C6 cells. On the basis of IC_{50} values, we find that hydroxyl analogues **3** and **4** are only moderately less active than **1**, while analogue **2** experiences a heavily diminished activity. Cytofluorimetric analyses of MCF-7 cells treated with HyVLMs suggest that the latter depolarize mitochondria, thus retaining the typical VLM behavior. It is likely that C6 cells, for which the exceptionally potent cytotoxicity of VLM has never reported previously, follow the same fate, as evidenced by alteration of mitochondrial morphology upon incubation with each ionophore.



KEYWORDS: *Valinomycin, hydroxylation, cell cycle disturbances, cell death, targeting, antitumor activity*

Nature is an enormously rich source of potent anticancer drugs, linear and cyclic peptides, depsipeptides, and macrolides being the most renowned leading examples. However, most of such natural products, albeit highly cytotoxic, are poorly selective, with the obvious consequence that their high therapeutic potential cannot be advantageously exploited. In spite of this, ligand-mediated targeting of anticancer therapeutics (LTT) has recently emerged as an elegant and powerful approach to selectively convey drugs or pro-drugs to pathological cells. Excellent reviews covering the many facets of this rapidly advancing area have recently appeared.[1−3]

In view of the recent renewed interest in ionophores for human cancer therapy,[4−6] we have taken up the challenge to build anticancer LTTs based on the potent and broad-spectrum antiproliferative activity of the K^+-ionophore valinomycin (VLM, **1**), a naturally occurring cyclodepsipeptide produced by several Streptomyces.[7] Chemically, it consists of a three-repeating sequence of the tetramer D-α-hydroxyisovaleryl-D-valyl-L-lactyl-L-valyl (D-Hyi-D-Val-L-Lac-L-Val) cyclically arranged to form a 36-membered macro-ring with an internal cavity designed to specifically accommodate K^+, which can hence be safely shuttled across membranes.[7] This causes dissipation of mitochondrial transmembrane potential ($\Delta\Psi_m$) and induction of apoptosis, which has been shown in several mammalian cell types,[8,9] including a number of tumor cell lines.[6,10,11] Because of its unique properties, VLM has captured the attention of many searchers devoted to various fields of science, so that studies detailing its mechanism of action,[12−14] as well as concerning the synthesis of analogues have continued unabated over the past decades.[15]

Apparently, only few studies have put forward the feasibility of VLM-based drug conjugates.[16] Perhaps, the lack of any derivatizable chemical handles (e.g., NH_2, SH, OH, COOH, etc.) in the molecular structure has represented a major obstacle to VLM derivatization. This severe limitation could, in principle, be overcome by adopting synthetic strategies that conjugate efficiency, high selectivity, as well as the possibility of operating under the very mild conditions (e.g., pH close to neutrality, ambient or subambient temperature) that are normally desirable when handling most of natural products.

Along these lines, we have recently developed a convenient route to incorporate hydroxyl handles into the VLM molecular structure, based on the direct reaction of the latter with methyl(trifluoromethyl)dioxirane (TFDO) under extremely

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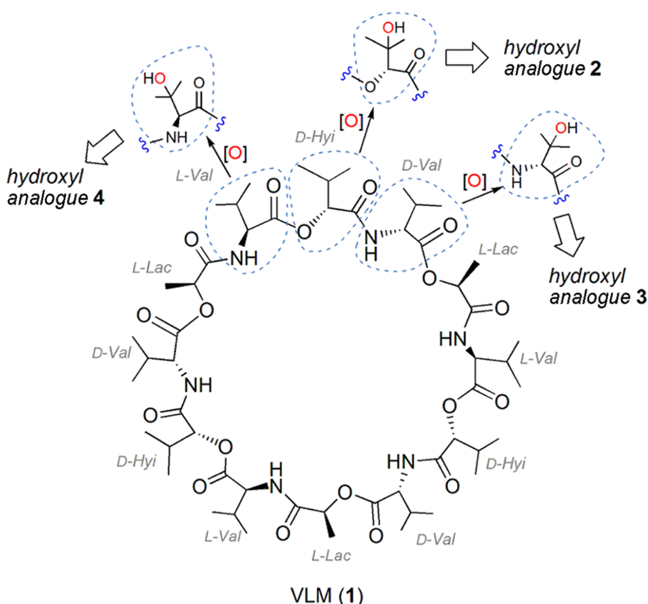
mild conditions.[17] This allowed to readily access a three-entity library of monohydroxyl VLM analogues (HyVLMs), carrying the OH group at the isopropyl side chain of a D-Hyi (**2**), D-Val (**3**), or L-Val (**4**) residue (Chart 1).[17]

Chart 1. Valinomycin (1) and Hydroxyl Analogues Thereof (2−4) Arising from Selective Oxyfunctionalization with TFDO



At this stage, we turn to inspect the levels of bioactivity retained by the modified VLMs **2**−**4**. On the basis of assays for mitochondrial dysfunction induced by analogues **2**−**4** on isolated rat-liver mitochondria, we have recently proved that hydroxyl groups reduce the bioactivity of the various analogues **2**−**4** to an extent that depends on the molecular site involved in the hydroxylation, so that the following order of potency could be established: **1** > **3** ≥ **4** > **2**.[18] However, we found that analogues **2**−**4** strictly retain the cation binding properties of the parent VLM. Nonetheless, on the way to the smart targeting of VLM analogues **2**−**4**, the essential missing step that we provide herein is the in vitro evaluation of their antitumor activity relative to VLM. To this purpose, the potential antiproliferative effects of VLM and HyVLMs were evaluated on four different cancer cell lines, namely, rat C6 glioma cells, MCF-7 human breast carcinoma cells, A2780 human ovarian carcinoma cells, and HepG2 liver hepatocellular carcinoma cells. In all experiments, cancer cell lines were treated with concentrations of compounds **1**−**4**, ranging from 0.0001 to 10 μM, at different incubation times (24, 48, and 72 h), and cell viability was determined quantitatively by the MTT conversion assay.[19] Table 1 collects the IC_{50} values obtained after 72 h of incubation, while the histogram in Figure 1 shows the time-dependent trend of C6 cells survival upon exposure to 0.01 μM **1**−**4** and is representative for the other cases examined.

In agreement with literature,[6] data in Table 1 indicate that VLM (**1**) displays different cytotoxic effects on the panel of selected cancer cell lines, which are dose- and time-dependent (Figure 1); the estimated IC_{50} values cover 3 orders of magnitude, ranging from the low nM for C6 and HepG2 to the low μM for MCF-7 and A2780 cell lines. However, analogues **3** and **4** evidence a similar dose- and time-dependent cytotoxic

Table 1. In Vitro Growth Inhibition of Various Tumor Cell Lines[a]

	IC_{50}[b] (μM)			
compd	A2780	MCF-7	HepG2	C6
1	2.18	1.77	0.0008	0.0004
2	>10	>10	0.1971	0.1752
3	4.53	3.85	0.0043	0.0024
4	2.51	3.91	0.0049	0.0029

[a] Concentration-cell viability profiles for VLM and HyVLMs tested on various tumor cell lines in vitro after 72 h of incubation. The cells were treated with the compounds at different concentrations, and the % of cells viability was calculated. Each point represents a mean value of three independent experiments performed in duplicate. [b] The concentration inducing 50% cell survival inhibition.

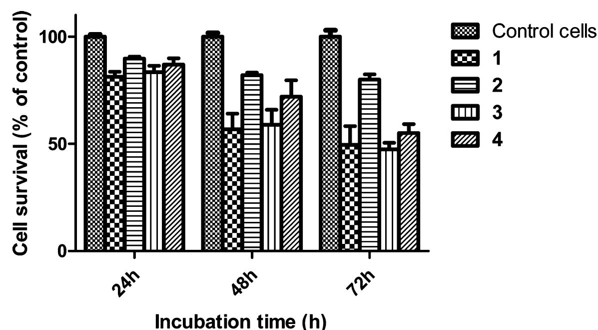


Figure 1. Cell survival of rat C6 glioma cells exposed to compounds **1**−**4**. Cells were seeded in 96 well culture plates at a density of 10,000 cells/well. Compounds were added at 0.01 μM concentration, and plates were incubated at 37 °C in CO_2 incubator for 24 to 72 h. MTT was used to determine viable cells. Same experiment was performed on A2780, MCF-7, and HepG2 cell lines (data not shown).

effect, although anywhere slightly less marked than **1**. By contrast, analogue **2** is remarkably over 200 and 400 times less potent than VLM for HepG2 and C6 cancer cells, respectively, pointing to a major role of the D-Hyi side chains over the D,L-Val counterparts in the VLM bioactivity. In all cases examined, the experimental IC_{50} values follow the order **1** < **4** ≤ **3** < **2**, matching very closely the relative potency of these ionophores based on their effect on mitochondrial bioenergetics parameters.[18]

Because VLM is known to affect the vitality of healthy cells,[8,9,20] we deemed it appropriate to inspect the HyVLM effects on a normal cell line. To this end, the cytotoxicity of compounds **1**−**4** on nontumorigenic breast epithelial cells (MCF-10A) were evaluated in comparison with those obtained for the MCF-7 breast carcinoma cell line. We have chosen this normal cell line in order to evaluate the behavior of normal breast tissue when it is in the presence of such drugs. Accordingly, MCF-10A cells were incubated for 72 h with concentrations of **1**−**4** corresponding to their IC_{50} values for MCF-7 cells (Table 1). Under these conditions, MCF-10A cells were found to be more resistant to VLM and to its analogues, with a cell survival ranging from 50 to 85% (MTT conversion assay).

To highlight the antiproliferative activity of compounds **1**−**4**, we evaluate their ability to interfere with the cells cycle progression of MCF-7 cells by flow cytometry. It is well-known that VLM is a potent apoptotic inducer.[11,12,21] However, in our preliminary experiments, cells treated with compounds **1**−**4** showed only a marked accumulation in S phase after 24 h of

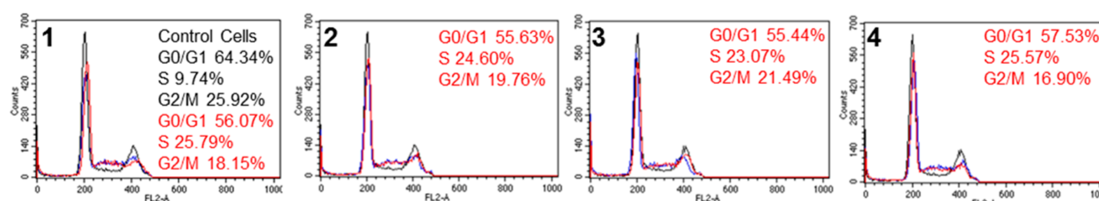


Figure 2. DNA histograms obtained by flow cytometry showing cells in G0/G1, S, and G2/M cell cycle phase, after treatment of MCF-7 cells with **1**−**4** (black trace, control cells; blue trace, cells treated with 2 μM of compounds **1**, **2**, and **4** or with 0.01 μM of **3**; red trace, cells treated with 5 μM of compounds **1**, **2**, and **4** or with 0.05 μM of **3** for 24 h. Percentage values refer to cells treated with 5 μM of compounds **1**, **2**, and **4** or with 0.05 μM of **3**.

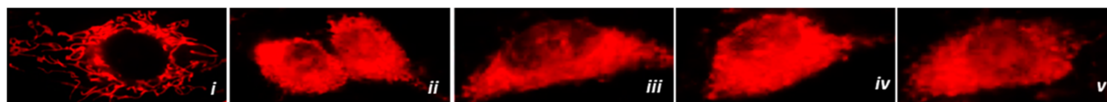


Figure 3. Morphological analysis of mitochondrial network structure in rat C6 glioma cells. Cells seeded at a density of 100,000 cells/well were treated with 50 nM (**1**, **3**, **4**) and 1 μM (**2**) at 37 °C in a 5% CO_2 atmosphere. Mitochondrial structure was evaluated after 72 h after incubation of cells with 25 nM MitoTracker Red CMXRos. As a control, mitochondria of untreated cells are shown in panel *i*. In panels *ii*, *iii*, *iv*, and *v*, cells were treated with **1**, **2**, **3**, and **4**, respectively. Images are representative of 3 independent experiments in which more than 10 cells were examined.

incubation, irrespective to the utilized concentrations (Figure 2; Table S1, Supporting Information). As already suggested by Iguchi et al., an accumulation in S phase could be correlated to apoptosis through the modulation of MAPK and mevalonate pathways.[22,23] After 72 h of incubation, only VLM showed a marked accumulation of cells in the G2/M phase, which might be compatible with an induction of apoptosis. However, analogues **2**−**4** showed quite normal cell cycle histograms, suggesting that the residual cells begin to cycle, and perhaps apoptosis, along with other cell death mechanisms, might be at work.

Although these mechanistic aspects are under investigation, flow cytometry experiments run on MCF-7 cells stained with the mitochondria-specific fluorescent probe JC-1[24] indicate that HyVLMs, similarly to VLM, induce $\Delta\Psi_m$ dissipation, an event that is known to precede the VLM-triggered apoptosis.[11,12,21] In particular, we found that the percentage of JC-1-loaded MCF-7 cells with depolarized mitochondria, upon exposure to compounds **1**−**4** for 72 h, were 16, 8.9, 12.1, and 15.6%, respectively, as opposed to a 1% of MCF-7 control cells. It is noteworthy that these percent values strictly parallel the order of cytotoxicity of compounds **1**−**4** (IC_{50} values of Table 1), as well as their relative order of efficiency (**1** > **3** ≈ **4** > **2**) in depolarizing isolated rat-liver mitochondria.[18]

It is worth mentioning that this work represents, to our knowledge, the first study describing the effect of VLM on C6 glioma cells. The low IC_{50} of VLM and HyVLMs for these cancer cells compare favorably with the higher value (IC_{50} = 0.73 μM) displayed by the most popular *cis*-platin compound;[25] hence, envisaging the potential application of analogues **2**−**4** in the treatment of this aggressive neoplasia. The effect of VLM and HyVLMs on C6 cells was further explored by analyzing the structure of the mitochondrial network in these cells treated with such ionophores (Figure 3). Figure 3*i* shows a representative morphological image of control C6 glioma cells where the typical tubular interconnected mitochondrial network is evident. In contrast, cells treated with 50 nM of **1** (*ii*), **3** (*iii*), and **4** (*iv*) or with 1 μM of **2** (*v*) exhibit, after 72 h of exposure, fragmentation of the mitochondrial network with the CMXRos-fluorescence spread into the cytosol and nucleus. Although the details of the VLM and HyVLM action on C6 require further careful inspection, it seems likely that compounds **1**−**4** induce cell death by targeting mitochondria.

To conclude, this preliminary in vitro investigation on the potential antiproliferative effect on cancer cells of conjugable valinomycin derivatives, evidenced that these compounds (**2**−**4**) are, to a different extent, less cytotoxic than the parent drug (**1**), but still pharmacologically prominent. As these newly VLM analogues possess the extra benefit of a derivatizable chemical handle, the results described herein encourage us in the future development of ligand-targeted compounds **2**−**4** as potential anticancer therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

Detailed information about in vitro biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

VLM, Valinomycin; HyVLM, hydroxyl VLM; D-Hyi, D-α-hydroxyisovaleric acid; L-Lac, L-lactic acid; D-Val, D-valine; L-Val, L-valine; TFDO, methyl(trifluoromethyl)dioxirane; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbo-cyanine iodide

■ REFERENCES

(1) Trapani, G.; Denora, N.; Trapani, A.; Laquintana, V. Recent advances in ligand targeted therapy. *J. Drug Targeting* **2012**, *20*, 1−22.

(2) Heller, A.; Brockhoff, G.; Goepferich, A. Targeting drugs to mitochondria. *Eur. J. Pharm. Biopharm.* **2012**, *82*, 1−18.
(3) Vlahov, I. R.; Leamon, C. P. Engineering folate−drug conjugates to target cancer: from chemistry to clinic. *Bioconjugate Chem.* **2012**, *23*, 1357−1369.
(4) Huczyński, A. Polyether ionophores—promising bioactive molecules for cancer therapy. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 7002−7010.
(5) Smithrud, D. B.; Wang, X.; Tarapore, P.; Ho, S. Crown ether host-rotaxanes as cytotoxic agents. *ACS Med. Chem. Lett.* **2013**, *4*, 27−31.
(6) Marjanović, M.; Kralj, M.; Supek, F.; Frkanec, L.; Piantanida, I.; Smuc, T.; Tusek-Božić, L. Antitumor potential of crown ethers: structure−activity relationships, cell cycle disturbances, and cell death studies of a series of ionophores. *J. Med. Chem.* **2007**, *50*, 1007−1018.
(7) Ovchinnikov, Y. A.; Ivanov, V. T.; Shkrob, A. M. *Membrane Active Complexones*; Elsevier: Amsterdam, The Netherlands, 1974.
(8) Abdalah, R.; Wei, L.; Francis, K.; Yu, S. P. Valinomycin-induced apoptosis in Chinese hamster ovary cells. *Neurosci. Lett.* **2006**, *405*, 68−73.
(9) Paananen, A.; Järvinen, K.; Sareneva, T.; Salkinoja-Salonen, M. S.; Timonen, T.; Hölttä, E. Valinomycin-induced apoptosis of human NK cells is predominantly caspase independent. *Toxicology* **2005**, *212*, 37−45.
(10) Ryoo, I.-J.; Park, H.-R.; Choo, S.-J.; Hwang, J.-H.; Park, Y.-M.; Bae, K.-H.; Shin-Ya, K.; Yoo, I.-D. Selective cytotoxic activity of valinomycin against HT-29 human colon carcinoma cells via down-regulation of GRP78. *Biol. Pharm. Bull.* **2006**, *29*, 817−820.
(11) Morrison, M. L.; Williamson, K.; Price, G. J.; Hamilton, P. W.; Maxwell, P. Phenotypic changes in mitochondrial membrane potential ($\Delta\Psi_m$) during valinomycin-induced depolarisation and apoptosis. *Cell. Oncol.* **2005**, *27*, 231−236.
(12) Lofrumento, D. D.; La Piana, G.; Abbrescia, D. I.; Palmitessa, V.; La Pesa, V.; Marzulli, D.; Lofrumento, N. E. Valinomycin induced energy-dependent mitochondrial swelling, cytochrome c release, cytosolic NADH/cytochrome c oxidation and apoptosis. *Apoptosis* **2011**, *16*, 1004−1013.
(13) Furlong, I. J.; Mediavilla, C. L.; Ascaso, R.; Rivas, A. L.; Collins, M. K. L. Induction of apoptosis by valinomycin−mitochondrial permeability transition causes intracellular acidification. *Cell. Death Differ.* **1998**, *5*, 214−221.
(14) Duax, W. L.; Griffin, J. F.; Langs, D. A.; Smith, G. D.; Grochulski, P.; Pletnev, V.; Ivanov, V. Molecular structure and mechanisms of action of cyclic and linear ion transport antibiotics. *Peptide Sci.* **1996**, *40*, 141−155.
(15) Cheng, Y.-Q. Deciphering the biosynthetic codes for the potent anti-SARS-CoV cyclodepsipeptide valinomycin in *Streptomyces tsusimaensis* ATCC 15141. *ChemBioChem* **2006**, *7*, 471−477.
(16) Vlahov, I. R.; Wang, Y.; Hahn, S. J.; Santhapuram, H. K.; Leamon, C. P. Design and Synthesis of Folate-Valinomycin Conjugate. Abstracts of Papers, 242nd ACS National Meeting, Denver, CO, August 28−September 1, 2011; American Chemical Society: Washington, DC, 2011; MEDI-57.
(17) Annese, C.; Fanizza, I.; Calvano, C. D.; D'Accolti, L.; Fusco, C.; Curci, R.; Williard, P. G. Selective synthesis of hydroxy analogues of valinomycin using dioxiranes. *Org. Lett.* **2011**, *13*, 5096−5099.
(18) Annese, C.; Abbrescia, D. I.; Catucci, L.; D'Accolti, L.; Denora, N.; Fanizza, I.; Fusco, C.; La Piana, G. Site-dependent biological activity of valinomycin analogs bearing derivatizable hydroxyl analogs. *J. Pept. Sci.* **2013**, DOI: 10.1002/psc.2571.
(19) Laquintana, V.; Denora, N.; Musacchio, T.; Lasorsa, M.; Latrofa, A.; Trapani, G. Peripheral benzodiazepine receptor ligand-PLGA polymer conjugates potentially useful as delivery systems of apoptotic agents. *J. Controlled Release* **2009**, *137*, 185−195.
(20) Ndukwe Erlingsson, U. C.; Iacobazzi, F.; Liu, A.; Ardon, O.; Pasquali, M.; Longo, N. The effect of valinomycin in fibroblasts from patients with fatty acid oxidation disorders. *Biochem. Biophys. Res. Commun.* **2013**, *437*, 637−641.
(21) Dallaporta, B.; Marchetti, P.; de Pablo, M. A.; Maisse, C.; Duc, H. T.; Métivier, D.; Zamzami, N.; Geuskens, M.; Kroemer, G. Plasma membrane potential in thymocyte apoptosis. *J. Immunol.* **1999**, *162*, 6534−6542.
(22) Iguchi, T.; Miyakawa, Y.; Yamamoto, K.; Kizaki, M.; Ikeda, Y. Nitrogen-containing bisphosphonates induce S-phase cell cycle arrest and apoptosis of myeloma cells by activating MAPK pathway and inhibiting mevalonate pathway. *Cell. Signalling* **2003**, *15*, 719−727.
(23) Iguchi, T.; Miyakawa, Y.; Saito, K.; Nakabayashi, C.; Nakanishi, M.; Saya, H.; Ikeda, Y.; Kizaki, M. Zoledronate-induced S phase arrest and apoptosis accompanied by DNA damage and activation of the ATM/Chk1/cdc25 pathway in human osteosarcoma cells. *Int. J. Oncol.* **2007**, *31*, 285−291.
(24) Cossarizza, A.; Baccarani-Contri, M.; Kalashnikova, G.; Franceschi, C. A new method for the cytofluorimetic analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazol-carbocyanine iodide (JC-1). *Biochem. Biophys. Res. Commun.* **1993**, *197*, 40−45.
(25) Margiotta, N.; Denora, N.; Ostuni, R.; Laquintana, V.; Anderson, A.; Johnson, S. W.; Trapani, G.; Natile, G. Platinum(II) complexes with bioactive carrier ligands having high affinity for the translocator protein. *J. Med. Chem.* **2010**, *53*, 5144−5154.