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Novel 3-hydroxy vinylboronates influence sphingolipid metabolism, cause apoptosis in Jurkat cells and prevent tumor development in nude mice

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The Letter is dedicated to the memory of Professor Morris Srebnik

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ABSTRACT

A series of novel 3-hydroxy vinylboronates which share structural similarities with sphingolipids were synthesized and tested in vitro and in vivo as anticancer agents. The molecules reduced cancer cell survival in vitro by influencing their sphingolipid metabolism. In a cancer model in nude mice the lead compound **E7** prevented the development of tumor as long as the treatment period continued. Moreover, it delayed tumor growth after the treatment was finished.

Sphingolipids are complex lipids composed of a hydrophilic head group and a lipophilic backbone. Cells use them for the regulation of the fluidity and the sub-domain structure of the lipid bilayers.[1] Besides providing structural integrity in cell membranes, sphingolipids play crucial roles in signal transduction and gene regulation, and are thusly involved in many processes: apoptosis, transport, endocytosis, migration, senescence, proliferation and inflammation.[1–3] In particular, ceramide, its metabolites and the enzymes involved in sphingolipid metabolism participate in tumorigenesis and cancer progression and influence the efficacies of cancer therapies.[1–4] In cells the major sources of ceramide are: The de novo synthesis from serine and palmitoyl CoA; from its metabolite sphingosine (the salvage pathway); or by enzymatic hydrolysis of sphingomyelin and glycosphingolipids. All these pathways and some others minor ones result in ceramide accumulation. Ceramide is metabolized by ceramidase to sphingosine. Sphingomyelin and glycosphingolipids are synthesized from ceramide. Cells control the homeostasis of sphingolipids by regulating the activity of metabolic enzymes. Cancer cells change the sphingolipid metabolism regulation in order to bypass apoptosis, particularly by up-regulating the synthesis of sphingolipids that are generated from ceramide and by down-regulating the metabolism of these sphingolipids.[3–7] This is done in order to decrease the concentration of the pro-apoptotic sphingolipid ceramide in cancer cells.

All these data suggest that many interventions in the metabolic pathways of ceramide should influence cancer development and therapy. Different approaches are being taken in order to influence the metabolism of sphingolipids.[1,8,9]

Vinylboronates are valuable but relatively inert compounds[10] and can be synthesized by various methods.[11] Their utility lies in the ability to transfer the vinyl group from the boron to other elements[10] and metals.[12–17] We synthesized 3-hydroxy-1-alkenylboronates via tributylphosphine stabilized borylzirconacyclopropenes.[18] Boron is appealing as a pharmacore since it can inhibit many of the activities of the carboxyl group, by mimicking it to a point when the biological system recognizes the exchange. Boranes have been at the focus of our laboratory as pharmacologically active chemical agents.[19,20] The use of boron-containing compounds to

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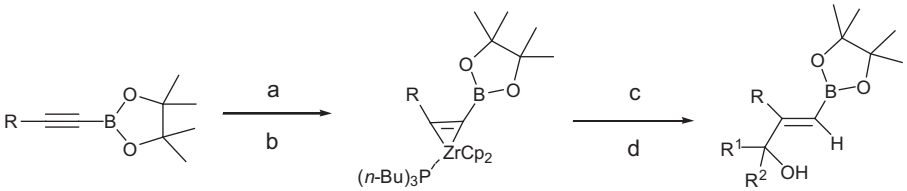


a = Cp_2ZrCl_2/2*n*-BuLi, THF, -78°C, b = $(n\text{-Bu})_3P$, c = $R^1R^2C(O)$, d = HCl/ether.

Scheme 1. Method of 3-hydroxy vinylboronates synthesis.

treat various cancers has until recently been restricted to Boron Neutron Capture Therapy (BNCT).[21] While appealing, BNCT is nonetheless impractical at this stage since it requires a nuclear facility. This may however change in the next decade as a new generation of neutron sources is made available based on various tabletop particle accelerators. In 2003, however, Millenium Pharmaceuticals introduced Velcade® (bortezomib) into the clinic for use against refractory and recurring myelomas.[22] Velcade® is the first entirely synthetic boron-containing compound to be introduced in clinical practice. Its success has prompted intense research into developing other synthetic boranes. To the best of our knowledge, highly substituted vinylboronates have not been tested for anticancer activity of any kind.

We synthesized 3-hydroxy vinylboronates for in vitro studies by the method, presented in Scheme 1, which was developed in our laboratory and published earlier[18]:

3-Hydroxy vinylboronates that were synthesized for in vitro studies are presented in Figure 1.

The anticancer activity of the synthesized 3-hydroxy vinylboronates was evaluated by the MTT assay[23] on several cancer cell lines. The IC_{50} values are presented in Table 1.

The novel 3-hydroxy vinylboronates reduced cancer cells survival in the MTT assays (Table 1). Compounds **E1**, **E2** and **E7** have a similar 3-substitution, but differ in the length of the hydrocarbon chain, with **E7** exhibiting greatest length (8 carbon atoms). Compound **E7** gave lower IC_{50} values, suggesting that a longer hydrocarbon chain, in molecules with cyclopropyl phenyl hydroxyl 3-substitution, results in increased activity. Compounds **E5**, **E6** and **E8** have hydrocarbon chains of different lengths, composed of the chain from an alkyne boronate together with the chain from a ketone. Compound **E8**, with its greatest length (16 carbon atoms) gave higher IC_{50} values. This suggests that a longer hydrocarbon chain, given that the 3-substituted carbon has only hydroxyl and a linear hydrocarbon chain as ligands, results in decreased activity. We have tried to synthesize alkyne boronates using tetra- and octadecynes, in order to combine cyclopropyl phenyl hydroxyl 3-substitution with a longer hydrocarbon chain, but with no success. We assume that the long chains of the alkynes interfere in the course of the reaction.

Compound **E4**, the only molecule that was synthesized using an aldehyde (and not a ketone like others) was not active on myeloma cells and gave IC_{50} value of greater than 100 μM on colon cancer cells. This result led us to conclude that it was not enough for the 3-substituted carbon to have both a hydroxyl and a phenyl groups as ligands (the hydroxyl and the phenyl are present in **E4**), but it should also have another ligand that is not hydrogen.

Comparing all the molecules in Table 1 we conclude that in order to lower the IC_{50} of the molecule it should contain a long hydrocarbon chain and 3-substitution with a cyclopropyl phenyl hydroxyl. The molecule that follows these requirements and reduces cancer cells survival the most is **E7**. So **E7** was selected for further investigations. Compound **E7** was also tested by a MTT assay on Melanoma-624 and leukemia (Jurkat) cells. After 72 h incubation the IC_{50} on Melanoma-624 and on Jurkat cells were 32.31 ± 4.78 and 16.33 ± 0.82 μM, respectively.

Our synthesized 3-hydroxy vinylboronates share several structural similarities with the sphingolipids, particularly sphingosine: an allylic hydroxyl and linear hydrophobic alkyl chains. Figure 2

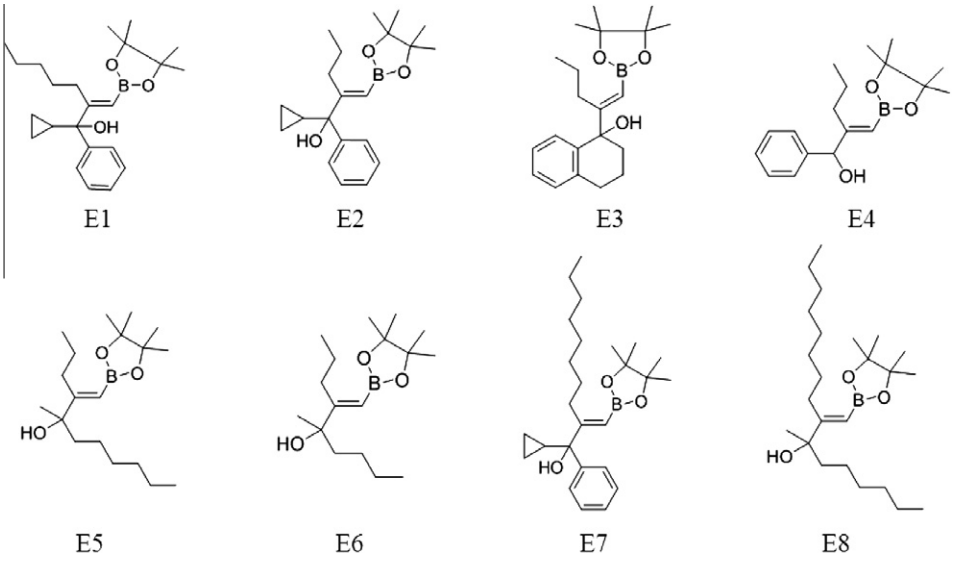


Figure 1. 3-Hydroxy vinylboronates that have been synthesized and examined in this study.

Table 1
IC_{50} values of vinylboronates after 72 h incubation with colon cancer (HT-29) and myeloma (ARH-77) cells

Compound	Cell type	
	HT-29 (μM)	ARH-77 (μM)
E1	Not active	10.38 ± 0.97
E2	83.93 ± 1.33	55.22 ± 2.83
E3	86.83 ± 2.42	64.91 ± 2.92
E4	>100	Not active
E5	57.19 ± 3.33	33.85 ± 0.17
E6	69.62 ± 3.25	54.61 ± 1.64
E7	18.32 ± 0.26	6.36 ± 1.10
E8	>100	79.07 ± 2.27

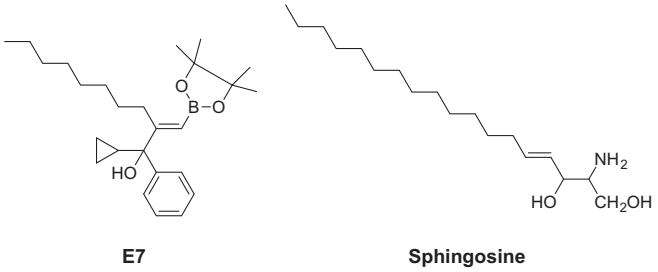


Figure 2. Structures of **E7** and sphingosine.

presents the structure of our lead compound, **E7**, together with the structure of sphingosine.

Compound **E7** gave the best results in MTT assays and thus was chosen for further investigations. Norman Radin published characteristic structural features of many anticancer molecules: an allylic O, N or S; the allylic oxygen is in a carbonyl or an alcohol group; double bonds conjugated to the allylic bond; two or more allylic moieties in each molecule and linear chains.[24] Those structural characteristics are common to many anticancer drugs and sphingolipids and Radin suggests that those structural similarities affect the mechanism of action of the drugs. We tend to agree with him and assume that even if some of the reasons for **E7** activity superiority in MTT assays are maybe not target related (improved cell permeability for example), some are related to better sphingolipid mimicking.

Jurkat cells treated with **E7** for 24 h were examined by FACS. Their distribution in the cell cycle stages is presented in Figure 3. DNA degradation that takes place during apoptosis leads to lower cellular DNA content. This sub G1 DNA content is detected by flow cytometry[25] and apoptotic cells are represented by a sub G1 population.[26,27] FACS examination of Jurkat cells indicate that **E7** at 100 μM causes cells to stop at the sub G1 phase more than twice as much as the controls or lower concentrations of **E7** (Fig. 3). Thus more leukemic cells degrade their DNA and less are involved in cell proliferation in stages G1, S and G2.

In order to track the influence of 3-hydroxy vinylboronates on sphingolipid levels in cancer cells Jurkat cells were incubated with **E7** and a fluorescent ceramide (Bodipy-12-Ceramide)[28] for 72 h. Low concentration (0.25 μM) of fluorescent ceramide (Bodipy-12-Ceramide) is added to the cells at the beginning of the experiment, 30 min after the tested compound. After 72 h of incubation we investigate the fate of this fluorescent ceramide – how much was consumed for sphingomyelin synthesis and how much remained as ceramide. We examine how the tested compound influenced the fate of the fluorescent ceramide. Figure 4 shows the relative percentages of ceramide and sphingomyelin as was detected by HPLC. In another experiment a concentration of 100 μM of **E7** was tested together with naïve cells and vehicle controls of 0.1%

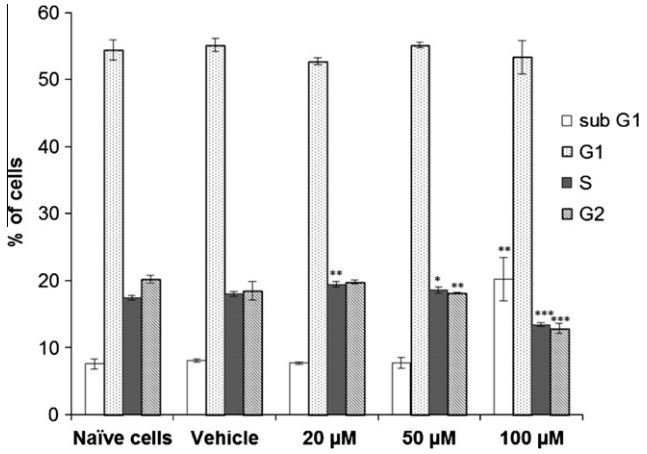


Figure 3. Percentage of Jurkat cells at each stage of cell cycle after incubation with different **E7** concentrations or vehicle (0.2% DMSO) for 24 h. Values are mean, error bars indicate s.d. Asterisks indicate that data are statistically significant compared to a naïve control using a *t* test ($^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001).

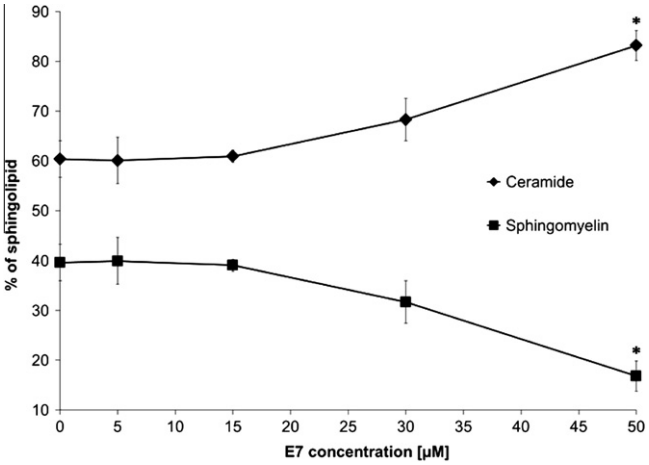
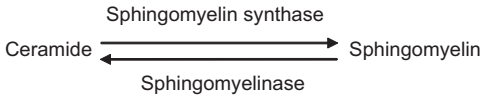


Figure 4. Percentage of ceramide and sphingomyelin in Jurkat cells after 72 h incubation with different concentrations of **E7** or vehicle (0.1% DMSO, signposted as 0 μM **E7**) and a constant concentration of Bodipy-12-Ceramide. Values are mean, error bars indicate s.d. Asterisks indicate that data are statistically significant compared to vehicle control using a *t* test ($^{*}P$ <0.01).

and 0.2% of DMSO. The percentages of the sphingolipids were alike in the control groups, however at the group of 100 μM treatment only ceramide without sphingomyelin was detected by the HPLC. Ceramide is an important suppressor of cancer development. It is a proapoptotic and antiproliferative sphingolipid.[29–31] Ceramide is a metabolite of sphingomyelin and also its building block (Scheme 2).[5]

Compound **E7** increases the percentage of ceramide and decreases the percentage of sphingomyelin in Jurkat cells in a concentration dependent manner (Fig. 4). The effect is seen already at 30 μM and increases at 50 μM. One hundred micromolars of **E7** reduced the concentration of sphingomyelin to a level that was not detected by the HPLC.

The MTT assays that their results are presented in Table 1 were conducted in medium free of FCS. The sphingolipid metabolism study was conducted in medium containing 10% FCS. The IC_{50} of **E7** after 72 h incubation with Jurkat cells in medium containing 10% FCS is 84.01 ± 0.94 μM. The effect of **E7** on percentage of ceramide and sphingomyelin is seen already at 30 μM of **E7** and



Scheme 2. The connection between ceramide and sphingomyelin.

increases at higher concentrations. Compound **E7** increases the percentage of ceramide and decreases the percentage of sphingomyelin while its concentrations are below its IC_{50} in 10% FCS containing medium.

We assume that **E7** is able to inhibit the enzyme sphingomyelin synthase and thus inhibit sphingomyelin synthesis from ceramide. We think this is the reason the concentration of ceramide rises and the concentration of sphingomyelin becomes lower and lower as the concentration of **E7** increases. (From data not shown, **E1** also interfered with sphingolipid metabolism in a similar manner.)

The activity of caspase-3 in several cell lines treated with **E7** was tested with the Z-DEVD–R110 substrate. Caspases are cysteine proteases that attain apoptotic cell death by the cleavage of several substrates.[32] Different incubation times did not change caspase-3 activity in the cells (data not shown). Treatment periods were chosen with the consideration of the fact that there is a time window of caspase activity that ceases with time. Published studies suggest that programmed cell death can be achieved dependently and also independently of caspase activity.[33,34]

Jurkat cells treated with **E7** for 24 h were stained with propidium iodide and Annexin V and checked by FACS to measure the percentage of living non-apoptotic cells (PI and Annexin negative), cells at the early stage of apoptosis (PI negative, Annexin positive), cells at the late apoptotic stage (PI and Annexin positive) and necrotic cells (PI positive, Annexin negative). Representative FACS results are shown in Figure 5. Compound **E7** at 100 μM caused more Jurkat cells to become apoptotic compared to naïve or vehicle treated cells.

The possible synergistic effect that 3-hydroxy vinylboronates may have together with a commercially available anticancer drug was examined by a MTT assay. Jurkat cells were incubated with **E7** together with Doxorubicin for 24 h. Figure 6 shows the results of the assay. Drug combinations are commonly used in cancer therapy. Combinations of **E7** with Doxorubicin led to lower Jurkat cells survival than treatment with each of the compounds by itself (Fig. 6). In anticancer therapy that combines several drugs each of them is given at lower concentration (than if it were given solely) in order to minimize their adverse effects.

The anticancer activity of **E7** was also evaluated in a preliminary study in vivo in female nude mice. During the experiment (50 days) the mice gained weight and except for the tumors were in good health condition, assessed by weighing and clinical examination. The therapeutic effect that **E7** caused in treated mice is presented in Figure 7. Compound **E7** (50 mg/kg) prevented the development of tumors for 3 weeks of the injections. Moreover, it delayed tumor growth after the treatment was finished (last injection was at day 21). Only at day 33 small tumors were measured in two of the **E7**-treated mice. At day 37 an additional mouse from vehicle treated group was measured with a tumor. All mice from 50 mg/kg treatment group survived in good general health condition (except for small tumors in two mice) until the end of the experiment at day 50. From the vehicle treatment group two mice were sacrificed until day 50 because of the animals' well-being requirements of the ethics approval for the experiment.

In conclusion, we synthesized highly substituted vinyl boronates in a sterio and regioselective manner from stable borylzirconacyclopropenes and evaluated their anticancer activity. The molecules reduce the survival of tested cancer cell lines. The selected molecule – **E7** – triggers apoptotic events in Jurkat cells. Compound **E7** interferes with sphingolipid metabolism in Jurkat cells. It increases the concentration of the pro-apoptotic sphingolipid – ceramide, and decreases the concentration of sphingomyelin, that is synthesized from ceramide. Concomitant treatments of Jurkat cells with **E7** and Doxorubicin led to a higher anticancer

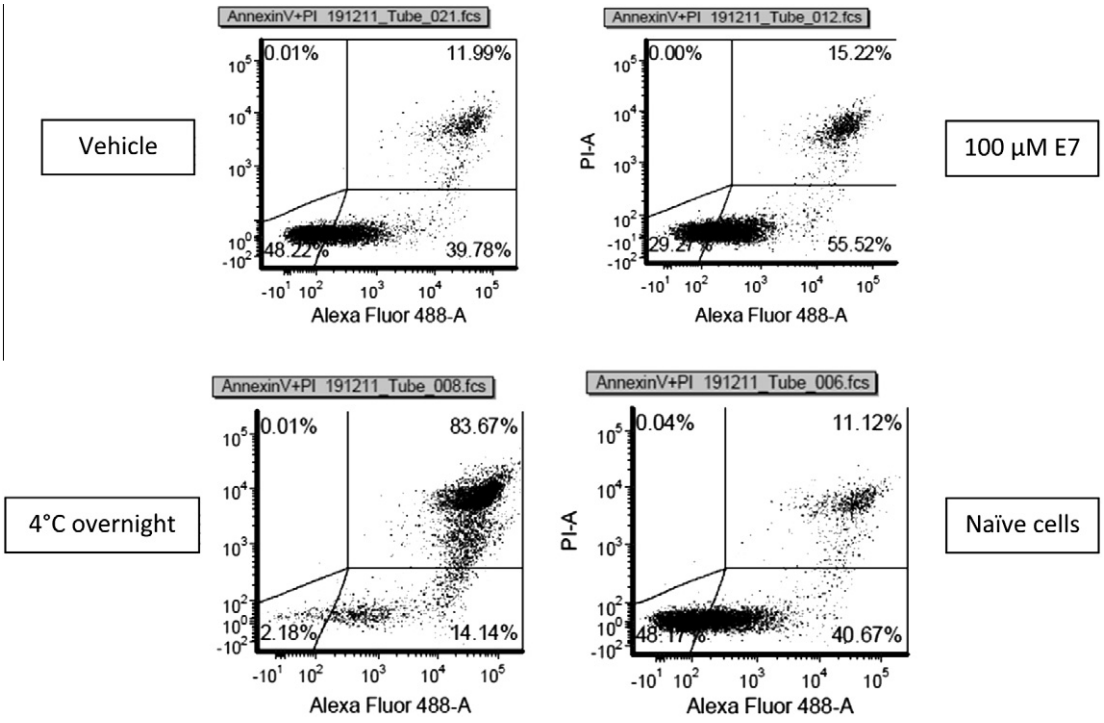


Figure 5. FACS results of Jurkat cells stained with PI and Annexin V after 24 h treatment with the vehicle (0.2% DMSO) or with 100 μM of **E7** or kept at 4 °C overnight or naïve cells.

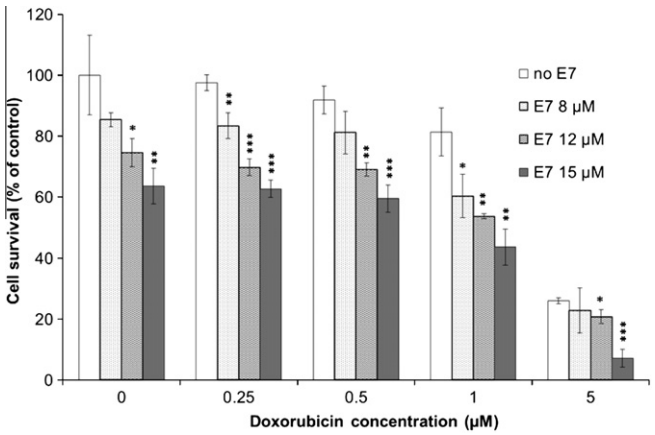


Figure 6. Percentage of survival of Jurkat cells that underwent concomitant treatments with **E7** and Doxorubicin for 24 h. Values are mean, error bars indicate s.d. Asterisks indicate that data are statistically significant compared to same Doxorubicin concentration without **E7** using a *t* test ($^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001).

effect than treatment with each of the compounds alone. In cancer model in nude mice the lead compound **E7** (50 mg/kg) prevented the development of tumors as long as the treatment period continued. Moreover, it delayed tumor growth after the treatment was finished. These findings are supported by the claim, published in several articles, that hydrophobic allyl alcohol derivatives (which are sphingolipid analogues) are efficient as anticancer drugs.[24,35]

Cell culture. HT-29, ARH-77, Melanoma-624 and Jurkat cells were maintained in an incubator at 37 °C in 5% CO_2/95% air atmosphere in RPMI 1640 supplemented medium: supplemented with 10% fetal calf serum and also glucose, glutamine, pyruvate, Pen-Strep and HEPES. Same full medium was used for experiments unless otherwise stated. Media and supplements were purchased from Biological Industries, Kibbutz Beth HaEmek, Israel.

MTT assay. Cells (1×10^6 cells/ml) were plated in 96-wells flat bottom plates (100 μl/well). Medium used was RPMI 1640. HT-29, ARH-77 and Melanoma-624 were left in the incubator overnight for attachment. Then 3-hydroxy vinylboronates were added (5 μl/well). After 24, 48 or 72 h the MTT assay was performed.[23]

Cell cycle analysis. Jurkat cells (1×10^6 cells/ml) were plated in 96-wells round bottom plates (100 μl/well). Compound **E7** solutions or vehicle were added (5 μl/well). After 24 h the cells were harvested and fixed in 70% cold ethanol. The cell cycle analysis was performed in LSR-II flow cytometer after the addition of 50 μg/ml of RNAse and PI to cell suspension in PBS.

Sphingolipid metabolism. Jurkat cells (0.75×10^6 cells/ml) were plated in 24-wells flat bottom plates (750 μl/well). Compound **E7** solutions or vehicle were added (250 μl/well). After 30 min the fluorescent Bodipy-12-Ceramide[28] was added (0.25 μM). After 72 h the cells were harvested. Cells pellets were extracted once with dichloromethane/methanol 1:2 and once with dichloromethane/methanol 1:1. The extractions were dried from solvents, dissolved in ethanol and the concentrations of ceramide and sphingomyelin were determined by the HPLC instrument (Waters®) equipped with Luna 5μ C18(2) 100A column and fluorescence detector (w474) (Ex 505 nm, Em 530 nm).

Caspase-3 activity. Cells (2×10^6 cells/ml) were plated in 24-wells flat bottom plates (500 μl/well). Media used were RPMI 1640 and supplemented RPMI 1640. Compound **E7** solutions or vehicle were added (25 μl/well). After different incubation times cells were harvested. Caspase-3 activity was measured with the EnzChek® Caspase-3 Assay Kit #2 (Molecular Probes®).

Annexin V and PI staining. Jurkat cells (1×10^6 cells/ml) were plated in 96-wells round bottom plates (100 μl/well). Compound **E7** solutions or vehicle were added (5 μl/well). After 24 h the cells were harvested, worked-up with Mebcyto® Apoptosis Kit (Annexin V-FITC Kit) (MBL) and checked in LSR-II flow cytometer.

Synergism studies. Jurkat cells (1×10^6 cells/ml) were plated in 96-wells flat bottom plates (100 μl/well). Medium used was RPMI 1640. Compound **E7** and Doxorubicin were added simultaneously (5 μl/well). After 24 h the MTT assay was performed.[23]

In vivo study in nude mice. The experiment was approved by the Committee for Ethics in Animal Experimentation of The Hebrew University of Jerusalem. Eleven female nude mice 5–6 weeks old were kept under specific pathogen-free conditions in an environment with a controlled temperature (~22 °C) and a 12-h light–dark cycle. The animals had free access to chow and water. Mice were injected sc with 1×10^6 HT-29 cells. At the same day they were randomly divided into two groups according to the intended treatment: 50 mg **E7**/kg (5 mice) or the vehicle: cremophor/ethanol/water for inj. (10:10:80) (6 mice). Mice were injected ip once a

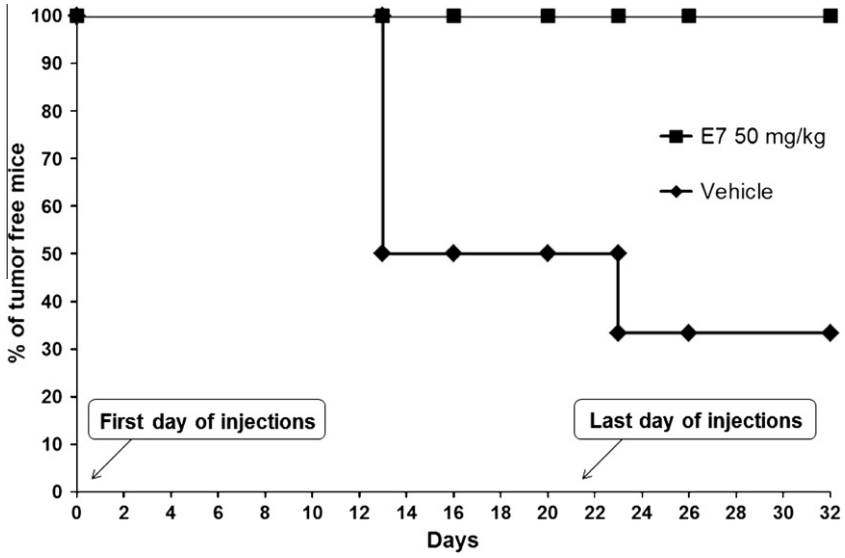


Figure 7. Percentage of tumor-free mice in vehicle ($n = 6$) and **E7** 50 mg/kg ($n = 5$) treated mice.

day with the suitable solution for 3 weeks (15 injections for each mouse). After the treatment period the mice were followed up for another 4 weeks. During the experiment general health condition of the mice was checked daily. Twice a week they were weighed and tumor volume was measured.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.028.

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