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CYTOTOXICITY OF ARTEMISININ-RELATED ENDOPEROXIDES TO EHRLICH ASCITES TUMOR CELLS[1]

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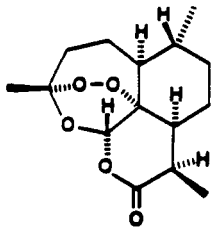
ABSTRACT.—A series of artemisinin-related endoperoxides was tested for cytotoxicity to Ehrlich ascites tumor (EAT) cells using the microculture tetrazolium (MTT) assay. Artemisinin [**1**] had an IC_{50} value of 29.8 μM. Derivatives of dihydroartemisinin [**2**], being developed as antimalarial drugs (artemether [**3**], arteether [**4**], sodium artesunate [**5**], artelinic acid [**6**], and sodium artelinate [**7**]), exhibited a somewhat more potent cytotoxicity. Their IC_{50} values ranged from 12.2 to 19.9 μM. The presence of an exocyclic methylene fused to the lactone ring, as for artemisitene [**9**], led to higher cytotoxicity than **1**. From the two epimeric 11-hydroxyartemisinin derivatives, the *R* form **12** showed a considerably higher cytotoxicity than the *S* form **13**. Opening of the lactone ring of **1** dramatically reduced the cytotoxicity. The ether dimer **8** of **2** was the most potent cytotoxic agent, its IC_{50} being 1.4 μM. The variations in cytotoxicity between the structurally related compounds mostly correlated well with the theoretical capacity of radical formation and stabilization. In some cases lipophilicity or the presence of an electrophilic moiety seemed to have a determinant influence on cytotoxicity. The artemisinin-related endoperoxides showed cytotoxicity to EAT cells at higher concentrations than those needed for in vitro antimalarial activity, as reported in the literature.

Sesquiterpene lactones are natural products, displaying a variety of biological activities, including cytotoxicity (1,2). Artemisinin or qinghaosu [**1**], a sesquiterpene lactone endoperoxide from *Artemisia annua* L. (Asteraceae), and derivatives prepared from dihydroartemisinin [**2**], such as artemether [**3**], arteether [**4**], sodium artesunate [**5**], artelinic acid [**6**], and sodium artelinate [**7**], are promising antimalarial agents, because of their activity against chloroquine-resistant *Plasmodium falciparum* strains and their effectiveness in the treatment of life-threatening cerebral malaria (3,4). Efforts are being made to make these potent and rapidly acting antimalarial drugs available world-wide, and clinical studies are on-going (5). In addition, studies are in progress directed to the design of dihydroartemisinin derivatives with improved antimalarial potency and better physico-chemical properties than artemisinin itself (6–10) and in order to search for analogues with simpler structures (11–13).

These drugs are known to be toxic to malaria parasites, but no information is available on the cytotoxicity of artemisinin derivatives to mammalian cells. It is, however, important to know to what extent representatives of this class of compounds affect mammalian cells. First, possible side effects when treating malaria patients with these drugs may be explained with this knowledge and avoided if derivatives are used that are potent against the malaria parasite without exhibiting such cytotoxicity. Second, the chemical structure of artemisinin may be used as a lead for the possible development of new cytostatic agents that specifically act on rapidly proliferating tissue.

[1]Dedicated to the memory of Dr. Theo M. Malingré.



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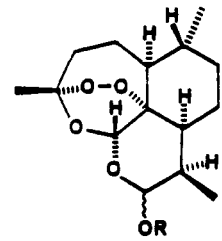
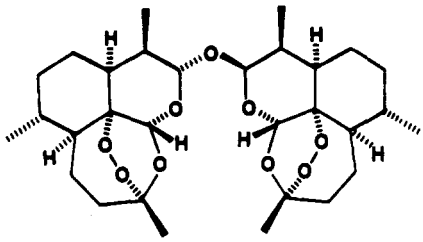


2 R=H (α+β)
3 R=CH_3 (β)
4 R=CH_2CH_3 (β)
5 R=$COCH_2CH_2COONa$ (α)
6 R=$CH_2C_6H_4COOH$ (β)
7 R=$CH_2CC_6H_4COONa$ (β)



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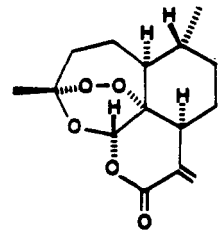

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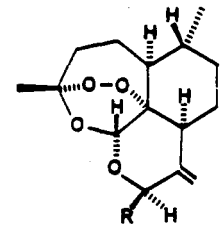


10 R=OOH
11 R=$OOCH_2CH_3$

Finally, by testing a range of endoperoxides with variations in their chemical structure, insight into structure-activity relationships can be obtained.

In the present study, we tested **1** and related compounds, including the above-mentioned antimalarial drugs **2–7**, as well as a series of related semi-synthetic derivatives **8–16**, for their cytotoxicity to Ehrlich ascites tumor (EAT) cells, using the microculture tetrazolium (MTT) assay (14). In this case, tumor cells were chosen as a model for normal tissue damage. In addition, artemisinic (arteannuic) acid [**17**] and arteannuin B [**18**], two precursors of the artemisinin biosynthesis (4), were tested. For comparison, the monoterpene endoperoxide ascaridole [**19**], the sesquiterpene lactone eupatoriopicrin [**20**], benzoyl peroxide, and H_2O_2 were included. The discussion on structure-activity relationships is focussed on the theoretical radical reactivity of these compounds. As reference anticancer drugs, doxorubicin and cisplatin were used.

RESULTS AND DISCUSSION

The MTT assay, used to test the artemisinin-related endoperoxides for cytotoxicity, is based on the metabolic reduction of soluble tetrazolium salts into insoluble colored formazan products by mitochondrial dehydrogenase activity of the tumor cells. Under the conditions used in this study, the enzyme activity and the amount of formazan formed were proportional to the amount of living cells (14–16).

As parameters for cytotoxicity, the IC_{50} and IC_{80} values, the drug concentrations causing respectively 50 and 80% growth inhibition of the tumor cells, were used. In Table 1 the IC_{50} and IC_{80} values for the tested compounds in the MTT assay against EAT cells are listed.

All endoperoxides are able to form oxygen radicals via homolytic oxygen peroxide cleavage because the oxygen-oxygen bond is relatively weak (17). It is suggested that the

TABLE 1. In vitro Cytotoxicity of Artemisinin-Related Endoperoxides to Ehrlich Ascites Tumor Cells.[a]

Compound	IC_{50} (μM)	IC_{80} (μM)
Artemisinin [**1**]	29.8±4.5	346±4
Dihydroartemisinin [**2**]	83.4±9.3	233±9
Artemether [**3**]	14.3±0.7	>125[b]
Arteether [**4**]	13.1±1.4	212±8
Sodium artesunate [**5**]	19.9±2.6	53.6±4.1
Artelinic acid [**6**]	16.1±0.8	103±3
Sodium artelinate [**7**]	12.2±2.2	47.9±0.9
Ether dimer of **2** [**8**]	1.4±0.01	2.4±0.03
Artemisitene [**9**]	6.8±1.1	16.0±1.1
Hydroperoxyartemisitene [**10**]	37.8±3.4	59.2±3.2
Ethylperoxyartemisitene [**11**]	11.5±1.1	38.3±4.7
11-Hydroxyartemisinin [**12**]	48.7±2.7	125±11
11-Hydroxy-11-*epi*-artemisinin [**13**]	>125[c]	
Anhydrodihydroartemisinin [**14**]	29.2±1.2	207±6
Formate ester **15**	91.0±4.7	156±6
Ketone **16**	>175[d]	
Artemisinic acid [**17**]	>175[e]	
Arteannuin B [**18**]	47.0±2.4	>50[f]
Ascaridole [**19**]	>500[g]	
Eupatoriopicrin [**20**]	5.7±0.1	13.4±0.2
Benzoyl peroxide	337±43	415±9
Hydrogen peroxide	13.1±0.5	17.3±0.3
Doxorubicin	0.35±0.1	1.0±0.1
Cisplatin	3.3±0.1	5.8±0.2

[a]Mean values (n=3)±SD are given.
[b]Highest concentration used; survival level 26.7±6.8%.
[c]Highest concentration used; survival level 98.8±0.9%.
[d]Highest concentration used; survival level 98.1±0.3%.
[e]Highest concentration used; survival level 100%.
[f]Highest concentration used; survival level 44.8±1.9%.
[g]Highest concentration used; survival level 74.8±5.1%.

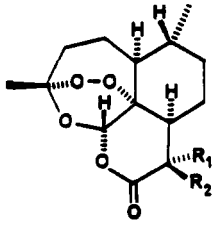
differences in cytotoxicity of the various endoperoxides may at least partly be due to differences in free radical generating capacity of these compounds. The reactivity of the drugs is determined by the degree of delocalization of the unpaired electron of the free radicals. Delocalization occurs in an electron-rich environment, as a result of resonance. The molecule gains stability and will become less reactive in that case (18).

When we compare artemisinin [**1**] and dihydroartemisinin [**2**] with the ethers and the ester of **2**, compounds **3**–**7**, it can be seen that **1** and **2** were significantly less cytotoxic than artemether [**3**], arteether [**4**], sodium artesunate [**5**], artelinic acid [**6**], and sodium artelinate [**7**]. Compound **1** possesses an electron-rich carbonyl group, and the hydroxy group of **2** is able to dissociate, yielding a negatively charged oxygen. In both compounds a source of free electrons is available, leading to delocalization of the free radicals, thereby stabilizing the molecule and mitigating the cytotoxicity. In **3** and **4**, electron-rich substitutents are lacking and no direct stabilization of the radicals can occur, which may result in an increased reactivity and cytotoxicity. In **6** and **7**, ethers with a carboxybenzyl fragment, delocalization of the radicals probably occurs to some extent via charge transfer interactions with the aryl moiety. Because of the longer distance to the radical center, however, the stabilization will be less effective than in **1** and **2**. This is reflected in the enhanced cytotoxicity of **6** and **7**, compared to **1** and **2**. The ester group of **5** possesses radical stabilizing properties but less than the carbonyl or hydroxy functions of **1** and **2**.

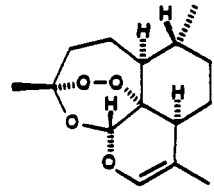
The 3-fold difference in cytotoxicity between **1** and **2** (IC_{50} values) might be explained by the poor stability of **2** in aqueous environment (17) and thus in the culture medium of the tumor cells. This argument, however, does not explain why **2** is more potent than **1** as an antimalarial agent (19). In addition, the hydroxy group of **2** makes the molecule more polar than **1**. Due to a higher lipophilicity, better penetration through cell membranes may result in higher intracellular concentrations of the drug. Increasing cytotoxicity with increasing lipophilicity has been reported for several other sesquiterpene lactones (20,21).

Of all endoperoxides tested, compound **8**, the ether dimer of **2**, was the most cytotoxic agent. Compound **8** was 22 times more cytotoxic than **1** and 60 times more than **2**, the parent compound. Although **8** possesses two reactive peroxide centers, it is unlikely that this can fully explain the large difference in cytotoxicity. Lipophilicity may play an important role here. In addition, the dimer can be a good radical generator because of homolytic cleavage of the ether bridge linking the two monomeric units.

Artemisitene [**9**] differs from **1** because of the exocyclic methylene group fused to the lactone ring. This compound, which can be prepared chemically from **1** (22), has also been found in *A. annua* plants grown in the United States (23) and in Saudi Arabia (24). It could not, however, be detected in plants originating from the Netherlands (25). Compound **9** was significantly more cytotoxic than **1**. As an electrophilic moiety, the exocyclic methylene functionality is apt to undergo a Michael-type addition with



12 R_1=Me, R_2=OH
13 R_1=OH, R_2=Me



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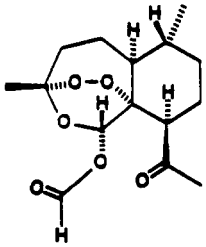

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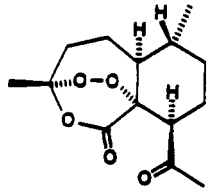

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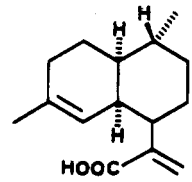

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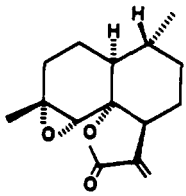

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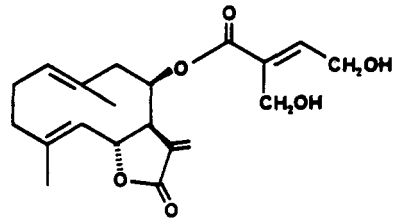

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biological nucleophiles, such as sulfhydryl groups of enzymes, glutathione, and proteins as well as parts of DNA. This has been described for several sesquiterpene lactones, including eupatoriopicrin [**20**]. As a result, cell death may be caused by this mechanism (26) and thereby explain the higher toxicity of **9** compared to **1**. On the other hand, very short-lived and highly reactive primary carbon radicals may be formed due to the methylene group in **9** and in ethylperoxyartemisitene [**11**]. In hydroperoxyartemisitene [**10**], a rearrangement may occur, yielding a five-membered cyclic peroxide, that generates oxygen radicals with a lower reactivity than the carbon radicals. This might explain the lower cytotoxicity of **10** as compared to **9** and **11**. The presence of an electron-rich peroxy group in **11** may also underlie the lower reactivity as compared to **9**. In addition, the accessibility to the reactive site, the exocyclic methylene, can become impaired due to steric hindrance of the rather bulky ethylperoxy group, reducing the cytotoxicity (27).

Two epimeric hydroxyartemisinin derivatives, namely 11-hydroxyartemisinin [**12**] and 11-hydroxy-11-*epi*-artemisinin [**13**], were tested. The *R* form showed a considerably higher cytotoxicity than the *S* form, probably due to steric effects influencing the penetration of the cell membrane.

From the moderate to low in vitro cytotoxicity of compounds **2**, **12**, and **13**, it may be concluded that the presence of a tertiary alcohol is responsible for this fact, probably because it reduces the lipophilicity of the molecule.

The cytotoxicities of 9,10-dehydrodeoxyartemisinin [**14**] and **1** were equal in terms of their IC_{50} values. The π-electrons of the double bond in the ring of **14** stabilize the free radicals, reducing the cytotoxicity.

Opening of the lactone ring of **1** led to a dramatic loss of the in vitro cytotoxicity (compounds **15** and **16**). Disruption of the lactone and lactol rings of, **1** and **2**, respectively, however, has yielded a series of structurally simplified tricyclic trioxanes with considerable antimalarial potency (13). For maximal cytotoxicity, in contrast, a tetracyclic ring system seems a prerequisite.

The sesquiterpene artemisinic acid [**17**] and the sesquiterpene lactone arteannuin B [**18**] are precursors of **1** (4). In Chinese traditional medicine, extracts of *A. annua* have been employed since ancient times as a remedy against fever and malaria (28). In the aerial parts of the plant, **17** and **18** are more abundant than **1** (25). It is therefore likely that the extracts have contained higher concentrations of **17** and **18**. For **17** no cytotoxic effect could be detected in our study, and **18** was considerably less cytotoxic than **1**. These observations are of importance for the judgment of the safety or the evaluation of the possible general toxicity of the popular *Artemisia* preparations.

Eupatoriopicrin [**20**], the principal sesquiterpene lactone from *Eupatorium cannabinum* L. (29) was about nine times more cytotoxic than **18**. Both compounds are sesquiterpenes with an exocyclic methylene group fused to the lactone ring, and **18** bears a potentially reactive epoxy group. Important for the observed difference in cytotoxicity is the role of the dihydroxytiglic acid side chain of **20**, esterified with the hydroxy group. In a previous study (21) it has been shown that eupatolide, the corresponding germacranolide alcohol obtained after hydrolysis of **20**, was about ten times less active than the parent compound.

For several compounds, including **1**, **3**, **4**, **6**, and **7**, a large difference existed between the IC_{50} and IC_{80} values. The survival curves of these endoperoxides revealed two phases (not shown). First, there was a rapid decrease in cell survival with increasing drug concentrations. This was followed by less cell kill at the higher concentrations. The break-point was found at a survival level of about 40%. Extrapolation of the second phase of the survival curve to the Y-axis revealed that the tested populations of tumor cells contained 40–60% of the less sensitive cells. This may indicate a selectivity, such as a

cell-cycle-dependent effect of the compound mentioned. For example, cells in the *S* phase may be more resistant. Interestingly, FACS analysis showed that 63% of a population of EAT cells is in that particular cell cycle phase.

Ascaridole [**19**], a monoterpene endoperoxide present in *Chenopodium* oil that was formerly used because of its anthelmintic properties but appeared to be too toxic for the host, had a very low toxicity to EAT cells. The radical reactivity of **19** seems very low because of the strong stabilizing effect of the π-electrons of the double bond. Benzoyl peroxide and H_2O_2 were used for comparison, as an organic and inorganic peroxide, respectively. Benzoyl peroxide, used in the treatment of acne, had a low cytotoxicity. H_2O_2 was remarkably toxic. The anthracycline derivative doxorubicin and cisplatin, two established anticancer agents, were included as reference drugs. Compound **8** possessed an in vitro cytotoxicity comparable to these two anti-tumor compounds.

Compound **1**, with an IC_{50} value of 29.8 μM against EAT cells, exhibits toxic effects against *P. falciparum* in vitro at the much lower concentration of 0.1 μM (3). Such differences are also seen when the cytotoxicity of **2–7** is compared to their antimalarial activity in vitro (7). This emphasizes the selective toxicity of these compounds against malaria parasites. The antimalarial actvity of **1** and related drugs is also ascribed to the generation of reactive oxygen species, including free radicals, initiating radical reactions (30,31). This cascade of events has been suggested to be mediated by a reaction with intraparasitic hemin and may explain the selective toxicity (30,32).

Comparing the cytotoxicity with the antimalarial activity of compounds **1–7**, it appeared that all derivatives of **1** are stronger antimalarial drugs (7,33). This was also found for the cytotoxicity to EAT cells, except for **2**, that was almost three times less toxic to EAT cells than **1**.

In conclusion, the artemisinin-related endoperoxides showed cytotoxicity to EAT cells at considerably higher concentrations than those needed for in vitro antimalarial activity. For safe antimalarial drugs, a high ratio between these activities is desired as for dihydroartemisinin [**2**]. In vivo, the liver is the main site of metabolism of **3–7**, yielding **2** as a major metabolite that is held responsible for the antimalarial activity (17,34,35). The variations in cytotoxicity between the structurally related compounds mostly correlated well with the theoretical capacity to radical formation and stabilization. In some cases lipophilicity or the presence of an electrophilic moiety could be an explanation for the cytotoxicity. As free radicals can cause damage to vital cellular structures, further investigations into the mechanism of action of these compounds are in progress, including their possible effect on cellular membranes and DNA. The dimer of dihydroartemisinin, compound **8**, may be an interesting candidate for further studies as an anticancer drug because of its strong cytotoxic activity.

EXPERIMENTAL

TEST COMPOUNDS.—Artemisinin [**1**], isolated from *A. annua*, was manufactured by ACF Beheer BV (Maarssen, The Netherlands). Dihydroartemisinin [**2**] was prepared at the Department of Pharmacognosy, Groningen, through $NaBH_4$ reduction of **1** (6). For the preparation of the ethers and ester **3–7**, compound **2** was used. Artemether [**3**] was manufactured in Kunming Pharmaceutical Factory (Kunming, China). Arteether [**4**] was manufactured by Sapec S.A. (Lugano, Switzerland). Sodium artesunate [**5**] (Roteria® injection) was manufactured by Guilin No. 2 Pharmaceutical Factory (Guilin, China) and marketed by Canada Rotam Ent Ltd. (Chaiwan, Hong-Kong). Artelinic acid [**6**] and sodium artelinate [**7**] were manufactured by ACF Beheer BV. Compounds **8–16** were prepared at the Department of Pharmacognosy, Riyadh. The ether dimer **8** (33) of dihydroartemisinin [**2**] was obtained in quantitative yields by treating **2** with BF_3 as previously described (7) for obtaining **14**, except that hplc grade Et_2O, not anhydrous Et_2O, was used as a solvent. Artemisitene [**9**], hydroperoxyartemisitene [**10**], 9,10-dehydrodeoxyartemisinin [**14**], and formate ester **15** were prepared from **1** as described (22). Ethylperoxyartemisitene [**11**] was prepared from **10** by alkylation with ethyl iodide in the presence of Ag_2O (36). 11-Hydroxyartemisinin [**12**], 11-hydroxy-11-*epi*-artemisinin [**13**], and the ketone **16** were obtained from **14** as previously

described (37). Artemisinic acid [**17**] and arteannuin B [**18**] were isolated from *A. annua* and were a gift from Prof. Dr. D.L. Klayman, Washington. Compounds **3**, **4**, **5**, **17**, and **18** were obtained through ACF Beheer BV. Ascaridole [**19**] was isolated from *Chenopodium* oil and eupatoriopicrin [**20**] from *E. cannabinum* at the Department of Pharmacognosy, Groningen (29). The identity and purity of the test compounds were checked using spectroscopical and chromatographical methods. Benzoyl peroxide was purchased from OPG (Utrecht, The Netherlands). H_2O_2 was from Merck (Darmstadt, FRG), doxorubicin (Adriamycin®) was from Farmitalia (Brussels, Belgium), and cisplatin was from Aldrich (Milwaukee, WI).

CELL CULTURING.—Ehrlich ascites tumor (EAT) cells were grown in suspension culture in RPMI 1640 medium (Gibco, Paisly, Scotland), supplemented with 10% heat-inactivated fetal calf serum (Gibco) plus 50 $\mu g \cdot ml^{-1}$ streptomycin and 50 $IU \cdot ml^{-1}$ penicillin G. The cell line was cultured routinely at 37° in a humidified incubator with 5% CO_2 at the Department of Radiobiology. The doubling time of the cells was ca. 12 h. Exponentially growing cells were used in all experiments. The viability of the cells used in the experiments exceeded 95% as determined with trypan blue.

MTT ASSAY.—Cytotoxicity after treatment of EAT cells with the test compounds was determined using the microculture tetrazolium (MTT) assay and compared to untreated controls (14). Concentrated stock solutions of the test compounds were made in DMSO (Merck) and stored at −20°. Exponentially growing cells were harvested, counted, and diluted appropriately. The cell suspensions (50 ml containing 400 cells) were pipetted into 96-well microtiter plates (Nunc, Roskilde, Denmark). Subsequently, 50 µl of a solution of the test compounds, obtained by diluting the stock solution 100× with culture medium, was added to each well. The final DMSO concentration of 0.25% in the wells was proven not to affect the experiments. After a 2 h incubation period with the agents at 37° in a humidified incubator with 5% CO_2, the cells were washed 3× with 150 µl medium (10 min, 20°, 210×*g*). The test plates were then incubated at 37° in a humidified incubator with 5% CO_2 for 3 days. A solution of 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) was prepared at 5 $mg \cdot ml^{-1}$ in phosphate buffered saline (PBS; 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl; pH 7.4). Of this solution 20 µl was added to each well. After an incubation period of 3 h 45 min at 37° in a humidified incubator with 5% CO_2, the medium was removed after centrifugation (15 min, 20°, 210×*g*). The formed formazan product was dissolved in 200 µl DMSO. After thorough mixing, the absorbance was measured at 520 nm using a scanning microtiterwell spectrophotometer (Titertek Multiscan, Flow Laboratories, Irvine, Scotland). Cell survival was calculated using the formula: Survival (%)=[(absorbance of treated cells−absorbance of culture medium)/(absorbance of untreated cells−absorbance of culture medium)]×100. IC_{50} and IC_{80} values (the drug concentrations causing respectively 50 and 80% growth inhibition of the tumor cells, indicated as 50 and 20% survival, respectively) were determined from the survival data.

STATISTICS.—For the statistical evaluation of the data, the paired Student's *t*-test was used. A P value <0.05 was considered significant. All survival experiments were carried out in triplicate (separate experiments).

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