Cytoxicity and Mode of Action of Aeroplysinin-1 and a Related Dienone from the Sponge *Aplysina aerophoba*

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Aeroplysinin-1 (**1**) and the structurally related dienone **2** were cytotoxic to Ehrlich ascites tumor (EAT) cells and HeLa tumor cells in the microculture tetrazolium (MTT) and clonogenic assays. Both compounds are bromotyrosine derivatives, isolated from the marine spong *Aplysina aerophoba*. As the effective concentrations in the MTT assay were lower than in the clonogenic assay, **1** and **2** are able to cause growth inhibition as well as actual cell death in these cell lines. With an IC$_{50}$ value of 8.2 μM (MTT assay, 2-h incubation, EAT cells), **1** was the more toxic compound. When the cells were depleted of glutathione by pretreatment with buthionine sulfoximine, they were significantly more sensitive toward **1** and **2** in the MTT assay. A dose-enhancement factor as high as 11.8 was found in EAT cells after 2-h incubation with **2**. Using electron paramagnetic resonance we were able to measure free radical formation of **1** and **2**, yielding the semiquinone structures **3** and **4**, respectively, in a culture medium with tumor cells. It is concluded that free radicals are, at least in part, responsible for the cytotoxicity of **1** and **2**. This conclusion is in line with expectations derived from the chemical structures of both compounds.

The sponge *Aplysina* (formerly *Verongia*) *aerophoba* Schmidt belongs to the family Aplysinidae, which may be found in tropical and subtropical parts of the Atlantic and Pacific Oceans, as well as in the Mediterranean Sea. Most *Aplysina* species are characterized by the presence of secondary metabolites that originate from dibromotyrosine.[1,2]

Aplysina aerophoba occurs in the Mediterranean Sea and in the vicinity of the Canary Islands.[3,4] In this bright yellow sponge isofistularin-3, aerophobin-2, fistularin-1, and other compounds have been found.[4,5] These compounds are precursors for the lower molecular weight constituents, aeroplysinin-1 (**1**) and the dienone **2**, that originate after enzymatic degradation of the former.[5,6] Enzymes and substrates are probably located in different compartments of the sponge or sponge cells, and it is assumed that the degradation reaction occurs as soon as the sponge is damaged, for example, by predators. The enzymatically formed products have been found to be more toxic than the parent compounds,[4] emphasizing the ecological relevance of this system in the sponge's defense.

In several previous reports, cytostatic and antimicrobial effects of these bromotyrosine derivatives have been described.[4,7,8] The aim of the present study was to get insight into the cytotoxicity of compounds **1** and **2** to in vitro cultured tumor cells and into the mode of action underlying this biological activity.

Results and Discussion

The cytotoxicity of aeroplysinin-1 (**1**) and the dienone **2** to Ehrlich ascites (EAT) and HeLa tumor cells was determined using the microculture tetrazolium (MTT) and clonogenic assays. In Table 1 the results of these cytotoxicity tests are listed, expressed as IC$_{50}$ values, as the concentration (in μM) of each test compound causing 50% effect in the respective assays.

Under all experimental conditions aeroplysinin-1 (**1**) was significantly more toxic than the dienone **2**. In the MTT assay, both compounds were significantly more toxic after continuous incubation to HeLa cells, when compared over a 2-h incubation period. In their cytotoxicity toward EAT cells; however, a significant difference between the two incubation periods was found for **2**. Comparing the IC$_{50}$ values of 2-h and continuous incubations, it is clear that **1** and **2** exert the major part of their cytotoxic effect after a relatively short incubation period. In contrast, the cytotoxicity of the reference compound cisplatin increases substantially with increasing incubation time. The IC$_{50}$ values in the MTT assay were always significantly lower than the IC$_{50}$ values in the clonogenic assay after a 2-h incubation. The MTT assay does not distinguish between cytostasis and cytotoxicity, whereas in the clonogenic assay only cell death is measured.[9] This means that both compounds are able to inhibit cell growth (at lower concentrations), next to actual cell death. For comparison, the reference anticancer drug cisplatin was included in the tests (Table 1).

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Table 1. In Vitro Cytotoxicity of Aeroplysinin-1 (**1**), the Dienone **2**, and the Reference Anticancer Drug Cisplatin (IC$_{50}$ values, μM) to Ehrlich Ascites (EAT) and HeLa Tumor Cells in the Microculture Tetrazolium (MTT) and Clonogenic Assays[a]

| | compound | | |
assay	**1**	**2**	cisplatin
MTT assay			
EAT (2-h incubation)	8.2 ± 1.0	46.1 ± 1.8	1.4 ± 0.1
EAT (continuous incubation)	6.2 ± 0.7	26.1 ± 4.1	0.18 ± 0.04
HeLa (2-h incubation)	18.8 ± 2.1	31.9 ± 3.3	4.9 ± 0.1
HeLa (continuous incubation)	5.6 ± 0.7	15.6 ± 0.6	0.7 ± 0.1
clonogenic assay			
EAT (2-h incubation)	37.0 ± 7.0	74.3 ± 3.7	1.1 ± 0.1
HeLa (2-h incubation)	27.5 ± 3.0	58.0 ± 10.9	1.0 ± 0.1

[a] Mean values ($n = 3$) $\pm$ SEM are given.

Table 2. In Vitro Cytotoxicity of Aeroplysinin-1 (**1**) and the Dienone **2** (IC$_{50}$ values, μM) to BSO-Pretreated Ehrlich Ascites (EAT) and HeLa Tumor Cells in the Microculture Tetrazolium (MTT) Assay[a]

| | compound | | | |
treatment	**1**	DEF[b]	**2**	DEF[b]
EAT (2 h)	1.1 ± 0.1	7.5 ± 3.8	3.9 ± 1.0	11.8 ± 3.5
EAT (continuous)	0.7 ± 0.2	8.9 ± 1.3	5.4 ± 1.4	4.8 ± 2.2
HeLa (2 h)	5.4 ± 1.5	3.5 ± 1.5	15.7 ± 0.5	2.0 ± 0.3
HeLa (continuous)	2.8 ± 0.4	2.0 ± 0.6	10.4 ± 2.2	1.5 ± 0.4

[a] Mean values ($n = 3$) $\pm$ SEM are given. [b] Dose enhancement factor (IC$_{50}$ normal cells [Table 1]/IC$_{50}$ BSO-pretreated cells).

The tripeptide glutathione (GSH) is the predominant nonprotein thiol in living systems. As an antioxidant and nucleophile, GSH plays an important role in the cellular defense against pathological changes, as caused by endogenous and foreign agents (xenobiotics), and in the repair of cellular damage. GSH is known furthermore to be an important determinant for the sensitivity of cells to a variety of electrophilic or radical-generating drugs. Reduction of the cellular GSH level may enhance the cytostatic activity of drugs with GSH-dependent detoxification pathways.[10−12] Such reduction can be accomplished by pretreating the cells with buthionine sulfoximine (BSO), a potent and selective inhibitor of GSH synthesis, which selectively interacts with the enzyme γ-glutamylcysteine synthetase.[13] In Table 2 the IC$_{50}$ values of aeroplysinin-1 (**1**) and the dienone **2** for BSO-treated EAT and HeLa cells, after 2-h or continuous incubation in the MTT assay, are listed. In order to compare the cytotoxic effect in GSH-depleted cells to untreated cells, dose-enhancement factors (DEF) are given.

BSO treatment significantly sensitized both cell lines for the cytotoxicity of **1** and **2**. The most pronounced effect, a DEF of 11.8, was found in EAT cells after a 2-h incubation with the dienone **2**. For both compounds the effect was stronger in EAT cells than in HeLa cells. It may be concluded that in both tumor cell lines GSH plays an important role in the defense against the cytotoxic action of **1** and **2**. These results, as well as the types of chemical structures of **1** and **2**, suggested that a free-radical mechanism might be involved in the cytotoxicity of both compounds.

In Figures 1 and 2 the electron paramagnetic resonance (EPR) spectra of **1** and **2**, respectively, at a concentration of 2 mM in culture medium in the presence of EAT cells are given. They prove the ability of both compounds to form radicals under more or less physiological conditions. Figure 1 shows a 1:4:6:4:1 quintet with a splitting constant (a_H) of 1.4 G, derived from four protons. This small constant, in addition to
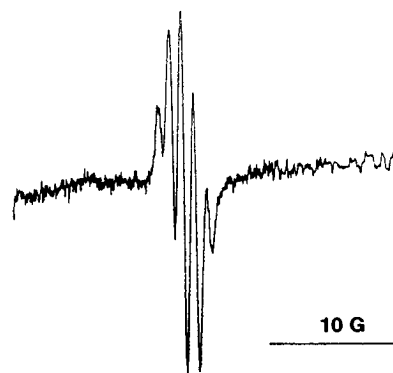
Figure 1. EPR spectrum of the semiquinone radical **3**, originating from aeroplysinin (**1**), 2 mM, in culture medium with 1×10^6 EAT cells/mL.
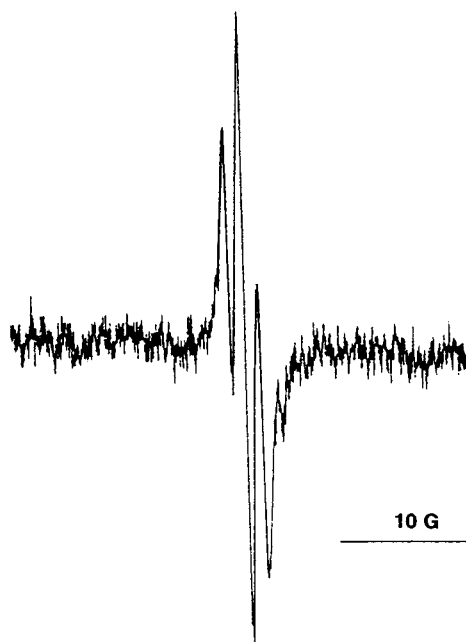
Figure 2. EPR spectrum of the semiquinone radical **4**, originating from the dienone **2**, 2 mM, in culture medium with 1×10^6 EAT cells/mL.

the stability of the radical, points to a semiquinone anion radical as being responsible for the spectrum. Structure **3**, 3,5-dibromo-4-methoxy-1,2-benzosemiquinone, can be derived from aeroplysinin-1 (**1**). We thus observed $a_H = 1.4$ G from three methoxyl protons and from the proton at C-6, making up the spectrum. From the dienone **2** a 1:2:1 triplet was obtained; that is, the responsible radical furnishes two equivalent proton splittings of 1.6 G. The constants observed for structures **3** and **4** are in line with constants observed for similar semiquinone structures.[14]

1   2

3   4
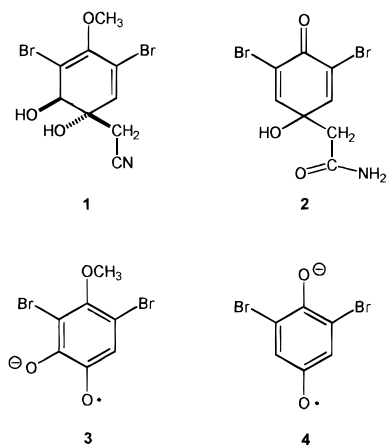
When dissolved in pure H_2O compound **2** also yielded an EPR spectrum, but **1** failed to do so. Thus, in order to obtain free radicals from aeroplysinin-1 (**1**), metabolic activation by living cells may be required. The dienone **2**, in contrast, is highly reactive as such and readily produces the stable free radical. It is remarkable, however, that the more toxic compound requires metabolic activation, while the less toxic compound does not. Usually, if one compound acts directly and another closely related compound requires metabolic activation to act via the same mechanism, it is the directly acting compound that is more potent. Structures **3** and **4** are possibilities for the semiquinone radicals that originate from compounds **1** and **2**, respectively. Semiquinone radicals are known to be very toxic.[15]

It has been shown that aeroplysinin-1 (**1**) and the dienone **2** are powerful inhibitors of the Na^+K^+-ATPase activity of rat brain microsomal fraction.[16,17] Furthermore, **1** displays a strong cytotoxic effect on epidermal growth factor (EGF)-dependent tumor cell lines, due to inhibition of the ligand-dependent protein tyrosine kinase activity of EGF receptors.[8] As electrophilic agents, **1** and **2** may undergo Michael additions with biological nucleophiles.[17] In that case, cytotoxic effects are found, among others, by blocking thiol groups of key enzymes in the cells. The GSH-dependent sensitivity of the EAT and HeLa cells for both compounds is fully consistent with this mechanism. From our results it may be concluded that free radicals are, at least in part, responsible for the cytotoxic effect of **1** and **2** in vitro. Further research will concentrate on DNA strand breakage and interference with the cell cycle distribution in tumor cells after treatment with **1** and **2**. Preliminary results from such experiments are in line with a radiomimetic-type mechanism for these compounds. Although **1** and **2** were less effective than the clinically used anticancer drug cisplatin, their chemical structures may be of interest as leads for future drug development.

The different reactivity of **1** and **2** fits very well in the defensive role of these compounds for *A. aerophoba* when the following hypothesis is used. As soon as a predator attacks this sponge, aeroplysinin-1 (**1**) is formed due to enzymatic degradation of the larger precursors. Compound **1** will generate free radicals after metabolic activation by the predator's cells, which results in an antifeedant effect. Simultaneously, the dienone **2** is liberated, which immediately yields free radicals in the water surrounding the sponge, thereby shielding the sponge from further predation or attack.

Experimental Section

Test Compounds. Aeroplysinin-1 [3,5-dibromo-1,2-dihydroxy-4-methoxy-3,5-cyclohexadiene-1-acetonitrile; (+)-isomer] (**1**) and the dienone **2** (3,5-dibromo-1-hydroxy-4-oxo-2,5-cyclohexadiene-1-acetamide) were isolated from *A. aerophoba* Schmidt, collected near the Canary Islands, as described previously.[3] Shortly thereafter, a MeOH extract of the lyophilized sponge material was evaporated to dryness and subsequently extracted with EtOAc. After evaporation of solvent, the EtOAc fraction was submitted to column chromatography using Sephadex LH-20. Elution with CH_2Cl_2-MeOH (50:50) and CH_2Cl_2-MeOH (90:10) yielded **1** and **2**, respectively. The identity and purity of the isolated compounds (>99%) was checked using various spectroscopic methods, TLC, and HPLC. Physical and spectral data have been published previously for **1**[4,18] and **2**.[4,19]

Cell Lines. Murine EN 19 cells, a cloned Ehrlich ascites tumor (EAT) cell line, were grown in suspension culture in RPMI 1640 (Gibco, Paisley, UK). Human HeLa S3 cervix uteri carcinoma cells were grown in suspension culture in Joklik's modification of MEM (Gibco). Both culture media were supplemented with 10% heat-inactivated fetal calf serum (Gibco) plus 0.2 mg/mL streptomycin and 200 IU/mL penicillin G. The cell lines were cultured routinely at 37 °C in a shaking incubator. The doubling time was $11-13$ h for the EAT and $24-29$ h for the HeLa cells. For all experiments, exponentially growing cells were used, with a viability exceeding 95%, as determined by trypan blue exclusion.

MTT Assay. The MTT assay is a cytotoxicity assay based on the metabolic reduction of a soluble tetrazolium salt (MTT) by mitochondrial enzyme activity of viable tumor cells into an insoluble colored formazan product. This product is measured spectrophotometrically after dissolution in DMSO.[20] Under the experimental conditions used, the enzyme activity and the amount of formazan formed were proportional to the amount of cells. Concentrated stock solutions (200 ×) of the test compounds were made in DMSO (Merck, Darmstadt, FRG) and stored at -20 °C. Cisplatin [*cis*-dichlorodiammineplatinum(II)], used as a reference cytostatic agent, was obtained from Aldrich (Milwaukee, WI). It was dissolved in H_2O immediately before use. After harvesting, the cells were counted and diluted appropriately with culture medium. Of these cell suspension, 50 μL containing 700 and 2400 cells for EAT and HeLa cells, respectively, were pipeted into the wells of a 96-well microtiter plate (Nunc, Roskilde, Denmark). Subsequently, 50 μL of a solution of each test compound, obtained by diluting the stock solution with the appropriate quantity of growth medium, were added to each well. The small amount of DMSO present in the wells (maximum 0.5%) was proven not to affect the experiments. The cells were exposed to the test compounds for either 2 h or continuously (3 days for EAT and 4 days for HeLa cells). In the case of the 2-h exposure, the cells were washed 3 times with 200 μL of culture medium (10 min, 20 °C, 210 × *g*) after the incubation period at 37 °C in a humidified incubator with 5% CO_2. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for a culture period of 3 (EAT) and 4 days (HeLa). After adding a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO), the amount of formazan formed was measured spectrophotometrically

at 520 nm as previously described.[21–23] Cell growth inhibition was calculated using the formula: growth inhibiton (%) = [1 − [(absorbance of treated cells − absorbance of culture medium)/(absorbance of untreated cells − absorbance of culture medium)]] × 100. The IC$_{50}$ value (the concentration of a test compound causing 50% effect) was used as a parameter for cytotoxicity. IC$_{50}$ values were calculated using the curve-fitting program Graphpad.

Clonogenic Assay. The clonogenic assay is a cytotoxicity assay in which the ability of individual cells to form a colony on soft agar is determined, after treatment with a test compound. Cells that have lost this ability are considered to be clonogenically dead.[24,25] Briefly, the test compounds were prepared 10 × concentrated in culture medium, and 0.1 mL was pipeted into 10-mL test tubes. Then, 0.9 mL of the cell suspension was added, yielding a final density of 10^6 cells/mL. The cells were incubated with different concentrations of the test compounds for 2 h in a gently shaking H$_2$O bath at 37 °C, centrifuged (5 min, 20 °C, 90 × g), and provided with fresh medium. The cell suspensions were diluted in order to obtain approximately 100 colonies per plate and mixed with 10^5 feeder cells/mL. The feeder cells were EAT or HeLa cells supralethally irradiated with 200 Gy of X-rays (Philips Müller MG 300 X-ray machine). Subsequently, 0.1 mL of cell suspension was plated onto 60-mm Petri dishes (Greiner, Nürtingen, FRG) containing 0.5% agar (Difco, Detroit) in RPMI 1640 supplemented with 15% newborn calf serum (Gibco) plus 0.2 mg/mL streptomycin and 200 IU/mL penicillin G. After plating, the treated cells that had remained in the test tube were counted to correct for a possible cell loss during the washing procedure. Cells were allowed to grow at 37 °C in a humidified incubator with 95% air and 5% CO$_2$ until colonies (>50 cells) could be counted (1 week for EAT and 2 weeks for HeLa cells). The concentration of a test compound resulting in 50% of the control colonies, calculated using the curve-fitting program Graphpad, was denoted as the IC$_{50}$ value and used as a parameter for cytotoxicity.

GSH Depletion. Depletion of GSH was achieved by culturing the cells for 18 h in medium with 500 μM of BSO (Sigma) prior to treatment with the test compounds. This treatment was proven not to influence cell viability or cell growth. Normal EAT cells contained 7.6 ± 0.7 μg GSH/mg protein and normal HeLa cells 10.2 ± 1.3 μg GSH/mg protein ($n = 3$; mean ± SD). After BSO treatment the GSH contents were reduced by more than 95% of the control values. GSH was measured according to Griffith with GSSG as the standard.[26] Protein was assayed by the method of Lowry *et al.*[27] GSH depletion was not proven to influence the viability of the tumor cells in our experiments.

EPR. Electron paramagnetic resonance (electron spin resonance, ESR) is a spectroscopic technique based upon the absorption of microwave radiation by an unpaired electron (radical) when it is exposed to a strong magnetic field.[28] ESR spectra were run at room temperature on a Varian E4 EPR spectrometer under the following conditions: field set, 3320 G; microwave power, 10 mW; microwave frequency, 9.37 GHz; time constant, 1 s; scan time, 4–12 min; modulation amplitude, 0.63 G; receiver gain, 1.6 × 10^3. Compounds **1** and **2** were tested at 2 mM with 10^6 EAT cells/mL in culture medium and in H$_2$O without cells.

Statistics. For the statistical evaluation of the data, the Student's *t*-test was used. A *p* value <0.05 was considered significant. All results presented are the mean of three independent, separately performed, experiments.

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