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Targeting Anthracycline-Resistant Tumor Cells with Synthetic Aloe-Emodin Glycosides

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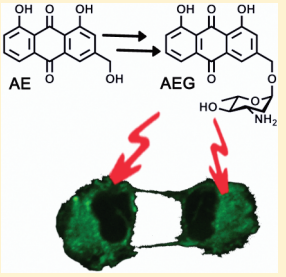
Supporting Information

ABSTRACT: The cytotoxic activity of aloe-emodin (AE), a natural anthranoid that readily permeates anthracycline-resistant tumor cells, was improved by the attachment of an amino-sugar unit to its anthraquinone core. The new class of AE glycosides (AEGs) showed a significant improvement in cytotoxicity—up to more than 2 orders of magnitude greater than those of AE and the clinically used anthracycline doxorubicin (DOX)—against several cancer cell lines with different levels of DOX resistance. Incubation with the synthetic AEGs induced cell death in less than one cell cycle, indicating that these compounds do not directly target the cell division mechanism. Confocal microscopy provided evidence that unlike DOX, AEGs accumulated in anthracycline-resistant tumor cells in which resistance is conferred by P-glycoprotein efflux pumps. The results of this study demonstrate that AEGs may serve as a promising scaffold for the development of cytotoxic agents capable of overcoming anthracycline resistance in tumor cells.



KEYWORDS: Anthracycline analogues, aloe-emodin, anthranoids, antitumor agents, P-glycoproteins

Anthracyclines such as doxorubicin (DOX, Figure 1) are highly efficient antineoplastic agents commonly used in the treatment of hematopoietic and solid tumors. These chemotherapeutic agents act by stabilizing the ternary complex between double-stranded DNA and topoisomerase II and lead to the inhibition of DNA transcription and replication.[1−3]

One of the major limitations on the clinical use of anthracylines results from the emergence of tumor cells with resistance to these chemothrapeutic agents.[4,5] To date, well over 2000 analogues of anthracyclines have been synthesized in search of compounds with improved clinical performance; yet, very few have demonstrated superior anticancer activity and become clinically useful.[6] Synthetic efforts have generally focused on altering the anthraquinone and/or sugar scaffolds of the parent anthracylines.[7−10]

In search of novel directions that would provide anthracycline-like compounds with activity against resistant tumors, our attention was drawn to the unique properties of a family of natural anthranoids that includes danthron, rhein, and aloe-emodin (AE, Figure 1).[11−13] These anthranoids have demonstrated antitumor activity in cell lines derived from lung carcinomas and ovarian cancers.[13] Interestingly, although its activity was modest, AE demonstrated similar levels of permeability and cytotoxicity against several tumor cell lines and their corresponding DOX-resistant lines. On the basis of these observations, we reasoned that it should be possible to design AE analogues that maintain the structural properties that render this anthranoid scaffold permeable into anthracycline-resistant tumor cells while enhancing the rather modest antitumor activity of AE.

Like the C-7 benzylic position in the structure of DOX (Figure 1), the C-15 benzylic alcohol of AE is suitable for the preparation of AE glycosides (AEGs). In these AE analogues, the carbohydrate is positioned similarly to that in DOX, and we expected that this modification should improve the affinity for DNA. Previous studies showed that 2,3,6-trideoxy-3-amino-L-sugars, such as the daunosamine moiety of DOX (Figure 1), contribute around 40% of the binding energy to the target DNA.[14] Thus, we chose to modify AE with this carbohydrate family.

We therefore prepared two 3-azido-4-*O*-acetyl-protected glycosyl acetate donors, acosamine derivative **D-1** and ristosamine derivative **D-2**, differing in the absolute configuration of their C-3 azides, in three synthetic steps from commercially available 3,4-di-*O*-acetyl-L-rhamnal (Scheme 1) as previously described.[15,16] Lewis acid-catalyzed activation of the glycosyl acetates and AE in THF gave anomeric mixtures of AEGs **1a**−**4a** (Scheme 1), which were readily separated by reverse-phase HPLC.[9] Removal of the acetyl groups under mild basic conditions gave AEGs **1b**−**4b**, which were purified by size exclusion column chromatography on Sephadex LH-20. The azido groups were transformed to the corresponding free amines by catalytic hydrogenation, and reverse-phase HPLC purification gave the final AEGs **1**−**4** in good yields. AEGs **1**−**4** represent all four combinations of two structural descriptors: the configurations of the glycosidic linkage (α or β) and the carbohydrate C-3 amine (axial or equatorial). All compounds were fully characterized by ^{1}H, ^{13}C NMR, and HRMS.

Cytotoxicities of the four compounds were tested by determining the IC_{50} values after incubation with leukemia, ovarian,

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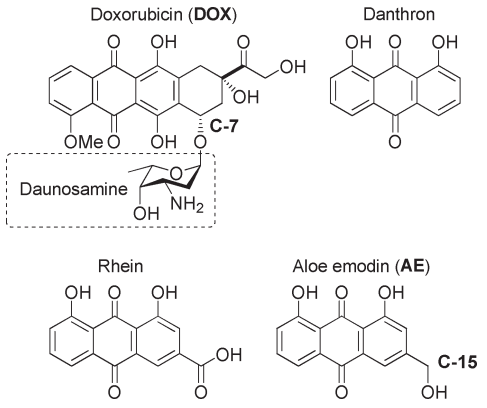
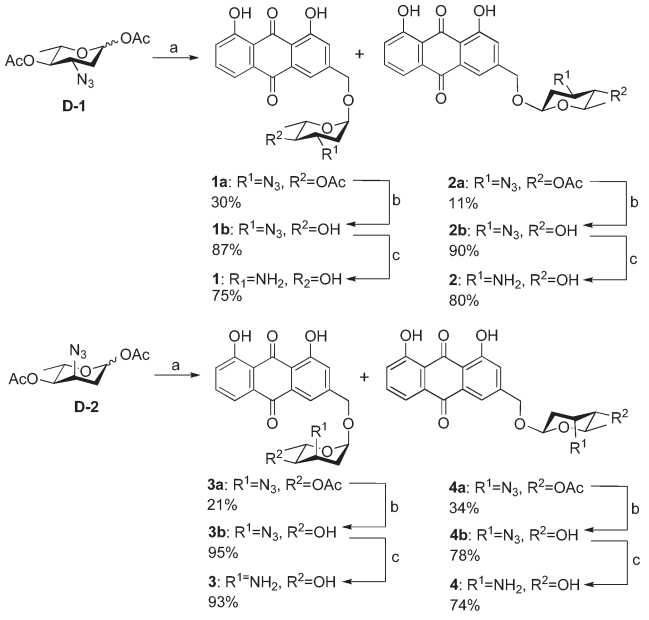


Figure 1. Structures of DOX and common anthranoids.

Scheme 1. General Synthetic Scheme for the Preparation of AEGs 1−4[a]



[a] Reagents and conditions: (a) AE, TMSOTf (cat.), THF, M.S. 4Å, 0 °C, 18−40 h. (b) K_2CO_3, MeOH:DCM/9:1. (c) MeOH:DCM/5:1, H_2/Pd, TFA (cat.).

and breast cancer lines (Table 1) using procedures previously described.[17] The MOLT-4 leukemia cells exhibited high sensitivity toward DOX (IC_{50} = 0.20 ± 0.07 μM); however, AE had no effect on MOLT-4 cells even at 20 μM (cells were 100% viable). AEGs **1**−**4** had improved activity in MOLT-4 cells relative to AE with IC_{50} values ranging from 5.8 ± 1.3 μM for AEG **1** to 12.8 ± 0.7 μM for AEG **4**. The ovarian cancer line OVCAR-3 was less sensitive to DOX with 61% cell viability at 20 μM. In OVCAR-3 cells, acosamine AEGs **1** and **2** demonstrated potent cytotoxic activity (IC_{50} = 5.2 ± 0.1 and 6.4 ± 0.2 μM, respectively), whereas AEG **3** was less active (IC_{50} = 15.7 ± 0.8 μM), and 60% cell viability was detected for cells that were exposed to a 20 μM concentration of AEG **4**.

The DOX-resistant breast cancer line MCF-7 was resistant to AE (83 and 77% viability at 20 μM DOX and AE, respectively). AEG **1** was the most active AEG in MCF-7 cells (IC_{50} = 7.1 ± 0.3 μM). AEGs **2** and **3** were less active (IC_{50} = 11.9 ± 0.6 and 12.7 ± 1.0 μM, respectively), and AEG **4** was the least active with 83% MCF-7 cell viability at a concentration of 20 μM. SKOV-3 cells, derived from an ovarian cancer, were relatively insensitive to DOX, AE, and AEGs **2** and **4** (63, 98, 92, and 84% cell viability at 20 μM, respectively). In SKOV-3, the α-acosamine AEG **1** was again the most cytotoxic of the AEGs (IC_{50} = 6.9 ± 0.5), and α-ristosamine AEG **3** was more modestly active (13.5 ± 1.3 μM).

Table 1. IC_{50} Values after 24 h of Incubation with AEGs 1−4[a]

	MOLT-4	OVCAR-3	MCF-7	SKOV-3	NAR
DOX	0.20 ± 0.07	>20	>20	>20	>100
AE	>20	>20	>20	>20	>100
AEG 1	5.8 ± 1.3	5.2 ± 0.1	7.1 ± 0.3	6.9 ± 0.5	8.6 ± 0.6
AEG 2	7.6 ± 1.6	6.4 ± 0.2	11.9 ± 0.6	>20	>100
AEG 3	5.4 ± 0.4	15.7 ± 0.8	12.7 ± 1.0	13.5 ± 1.3	18.0 ± 1.3
AEG 4	12.8 ± 0.7	>20	>20	>20	28.3 ± 2.3

[a] Values are given in μM.

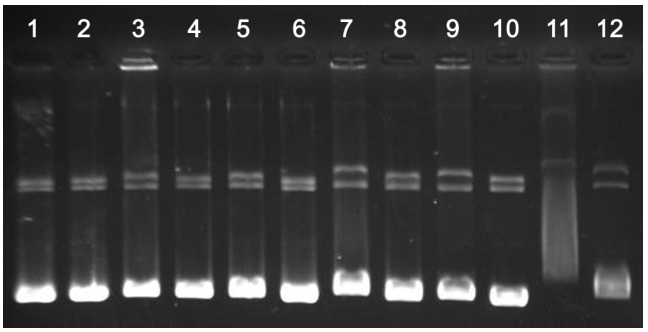


Figure 2. Supercoiled plasmid DNA unwinding detected by gel electrophoresis. Plasmid DNA incubated with (1) no drug, (2) 200 μM AE, (3) 200 μM AEG **1**, (4) 20 μM AEG **1**, (5) 200 μM AEG **2**, (6) 20 μM AEG **2**, (7) 200 μM AEG **3**, (8) 20 μM AEG **3**, (9) 200 μM AEG **4**, (10) 20 μM AEG **4**, (11) 200 μM DOX, and (12) 20 μM DOX.

Finally, AEGs **1**−**4** were evaluated against the highly DOX-resistant NAR cells, ovarian cancer cells in which resistance is conferred by overexpression of P-glycoprotein (P-gp) efflux pumps.[18] Viability of NAR cells was not at all affected by 100 μM DOX, and these cells exhibited 78% viability in the presence of 100 μM AE. In NAR cells, AEG **1** was once again the most potent AE analogue with an IC_{50} value of 8.6 ± 0.6 μM, at least 2 orders of magnitude higher than either DOX or AE. The single structural difference between the α-acosamine of AEG **1** and the β-acosamine of AEG **2** resulted in a dramatic drop in the activity of the later (65% cell viability at 100 μM). α-Ristosamine AEG **3** was more active then the β-ristosamine AEG **4** (IC_{50} = 18.0 ± 1.3 and 28.3 ± 2.3 μM, respectively) in NAR cells.

In all of the tested cell lines, the combination of an α-glycosidic linkage and an equatorial carbohydrate C-3 amine in AEG **1** resulted in the most potent cytotoxic activity. The significant differences between the cytotoxic activities of AEGs **1**−**4**, which differ solely in their carbohydrate stereochemistry, rule out the possibility that the amino-sugars only contribute to improve the solubility of these compounds. The clear differences in the IC_{50} values of AEGs **1**−**4** in each of the tested cell lines suggest that the carbohydrate segments are involved directly in the mechanism that leads to their improved cytotoxicity.

To determine whether the addition of the carbohydrate to AE resulted in an improvement in the DNA interactions of the

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synthetic AEGs, DNA intercalation was qualitatively studied by testing the supercoiled plasmid DNA unwinding by AEGs **1**−**4** using a gel electrophoresis protocol.[13,19,20]

Briefly, samples containing one of the AEGs, DOX, or AE were preincubated with PBR322 DNA plasmid, and samples were loaded on a 1% agarose gel, run for 4 h at 70 V, and then stained with ethidium bromide. As was previously reported, at 200 μM, AE had no observable effect on DNA migration (Figure 2, lane 2), indicating that AE has low affinity for DNA.[13] However, concentrations of 200 and 20 μM DOX significantly retarded DNA migration (Figure 2, lanes 11 and 12, respectively). As compared to DOX, AEGs **1**−**4** had a weak effect on DNA migration at a concentration of 200 μM, and no effect was detected at 20 μM.

These results indicate that the attachment of the carbohydrate to AE resulted with a very modest effect on the DNA intercalation properties of AEGs **1**−**4**. Furthermore, the measured IC_{50} values of potent AEGs range between 5 and 10 μM. At these values, no supercoiled plasmid DNA unwinding was detected at all. Although the attachment of an amino-sugar to AE was aimed at improving the DNA intercalation properties of the synthetic AEGs, the results of the supercoiled plasmid DNA unwinding gel assay suggest that AEGs are unlikely to derive their cytotoxic activity by targeting DNA, and that a different mechanism of action may account for their improved cytotoxicity.

The cellular effect of the most potent analogue, AEG **1**, was further investigated by using the trypan blue dye exclusion test to determine the number of viable cells present in a cell suspension.[21] We arbitrarily chose OVCAR-3 and NAR cell lines for this experiment. The most significant effect was observed when cells from either of the chosen lines were incubated with 10 μM DOX for 24 h. In the case of DOX, there was a significant decrease in the total number of cells as compared to the untreated control; however, no cells stained with the dye, indicating dead cells, were detected. In contrast to DOX, all of the cells in samples from both lines that were incubated with 10 μM AEG **1** for 24 h had trypan blue-stained cytoplasms, indicating that all of the cells in the sample were dead. The results of the viability tests indicate that, unlike DOX, which slows cell proliferation, AEGs caused cell death in less than one cell cycle. Hence, a 24 h incubation period was sufficient to study the IC_{50} values of the synthetic AEGs.

From the observations that AEGs induced cell death in less than one cell cycle, we propose that, unlike anthracyclines, these compounds act in a cell-cycle independent manner. To test this hypothesis, we studied the cytotoxicity of AEG **1** on normal human lymphocyte samples obtained from two individuals. Human lymphocytes were insensitive to 24 h of incubation with 20 μM DOX (100% viability as compared with the control containing untreated cells). At 20 μM, AE had limited effect (77−100% viability as compared with the control containing untreated cells) on these cells. Unlike DOX and AE, AEG **1** demonstrated significant cytotoxicity to human lymphocytes with IC_{50} values around 3 μM for both of the tested blood samples. Furthermore, AEG **1** demonstrated identical IC_{50} values even after a short 3 h incubation period with human lymphocytes.

Because the cytotoxic effect of AEG **1** could be detected after a very short incubation time, we tested the hemolytic effect of this compound on red blood cells obtained from rat to determine if these compounds may act as detergents and rupture cell membranes. After 1 h of incubation with 50 μM AEG **1**, a very modest hemolytic effect was observed for both DOX and AEG **1**, and no effect was observed for AE (2, 3, and 0% hemolysis, respectively). Hence, AEGs are unlikely to act by inducing cell lysis as do detergents such as Triton X100 (100% lysis after 1 h of incubation).

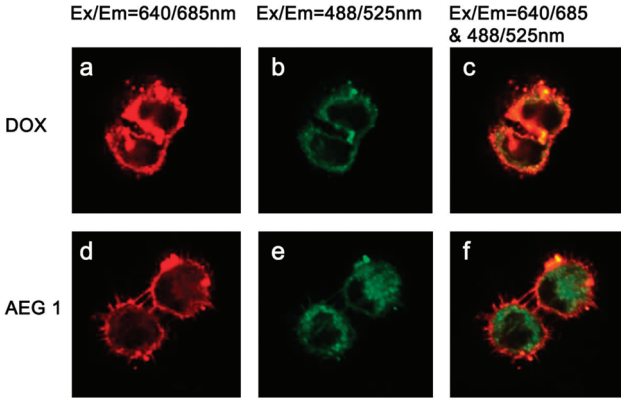

Figure 3. Confocal microscopy images of NAR cells preincubated with DOX or AEG **1** for 2 h. Cells were incubated with (a−c) 5 μM DOX or (d−f) 5 μM AEG **1**. The plasma membrane was stained with carbocyanine tracer DiD (red), whereas DOX and AEG **1** appear green.

A possible explanation for the improved potency of the synthetic AEGs as compared to DOX in DOX-resistant cells was provided by data from confocal microscopy (Figure 3). Cell permeability of the drugs was evaluated in DOX-resistant NAR cells. Cells were preincubated with either DOX or AEG **1**, which was the most potent AE analog against this cell line. A 2 h incubation time and a concentration of 5 μM DOX and AEG **1** were chosen to avoid significant cell damage during the experiment. After incubation, cells were fixed in paraformaldehyde, and the plasma membranes were stained with a carbocyanine tracer DiIC18 (5)-DS (DiD). Confocal microscopy (Ex/Em = 488/525 nm for DOX and AEG **1** and Ex/Em = 640/685 nm for DiD) provided evidence that DOX accumulated mainly in the plasma membrane (Figure 3a−c). In contrast, AEG **1** accumulated inside cells (Figure 3d−f). Hence, confocal microscopy showed that AEG **1** had significantly higher permeability than DOX into DOX-resistant tumor cells in which resistance is conferred by P-gp-mediated drug efflux.

In conclusion, a novel class of AEGs was designed and synthesized. Certain AEGs exhibited improved cytotoxic activity relative to DOX and AE in several tumor cell lines with different levels of anthracycline resistance. A combination of an α-glycosidic linkage and an equatorial C-3 amine resulted in the most potent cytotoxic activity of the analogues evaluated. AEG **1**, with this preferred structural combination, exhibited high levels of cytotoxicity against all of the tested cell lines and was at least 2 orders of magnitude more potent then DOX and AE against NAR cells, a DOX-resistant ovarian cancer line that overexpresses P-gp efflux pumps. In contrast to the expected effect of the amino-sugar, AEGs did not effectively interact with plasmid DNA; DOX had significantly higher affinity for plasmid DNA, suggesting that AEGs may exert their activity by targeting other cellular targets. Indeed, trypan blue staining of cell samples that had been incubated with AEG **1** indicated that no viable cells were present in the sample in less than one cell cycle. When cells were incubated with DOX, the rate of cell proliferation was reduced, but no dead cells were detected. AEG **1** did not demonstrate selective activity against tumor cells; it exhibited similar levels of cytotoxicity against normal human lymphocytes and tumor cells. Even though AEG **1** treatment

resulted in rapid cell death, it had no significant hemolytic activity and is therefore unlikely to act by causing cell lysis. Confocal microscopy provided evidence that, unlike DOX, AEG **1** permeated anthracycline-resistant tumor cells. The results of this study demonstrate that AEGs may serve as a promising scaffold for the development of antineoplastic agents that should overcome anthracycline resistance in tumors in which resistance is conferred by P-gp efflux pumps. Additional and more detailed mode of action studies are currently underway.

■ ASSOCIATED CONTENT

Supporting Information. Synthetic procedures; characterization by ^{1}H, ^{13}C NMR, and HRMS; supercoiled plasmid DNA unwinding gel electrophoresis protocol ; IC_{50} protocol; trypan blue cell viability test protocol; protocol for the preparation of peripheral blood cell populations; protocol for the preparation of red blood cells (RBC) populations; RBC lysis assay protocol; confocal microscopy protocol; and ^{1}H, ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
£We dedicate this paper to the memory of our dear colleague, Professor Eliezer Flescher.

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