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Action of a novel pyrrolo[1,2-*c*][1.3]benzodiazepine on the viability of Jurkat and neuronal/glial cells

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Abstract—We have designed and synthesized several structural isomers of anthramycin (heterocycles **2**, **3**, **5**, **6**, and **8**) and found that, in particular, pyrrolobenzodiazepine **8** induces DNA cleavage and formation of small fragments of DNA. The cytotoxic effects of **8** were manifested with both non-transformed primary neuronal/glial cells and transformed Jurkat cells. The other compounds did not change the viability either of transformed or of non-transformed cells, and induced DNA cleavage to a lesser extent.

Interactions between the drug molecules and the guanine N^2 of DNA are becoming important aspects in the design of new antitumor compounds. Many natural antibiotics, including anthramycin,[1] daunorobicin and doxorubicin,[2] and ecteinascidins,[3] are shown to bind to the N^2 of guanine in the minor groove of DNA. Anthramycin, an antitumor alkylating antibiotic produced by Streptomyces refuineus, covalently binds to the N^2 amine of the guanine 9 residues through its C11 position.[4] The cytostatic and antitumor effects of anthramycin and related benzodiazepines are believed to be due to their selective interaction with DNA,[5] which causes inhibition of nucleic acid synthesis,[6] and production of excision-dependent single- and double-strand breaks in cellular DNA.[7] The DNA adduct induced by anthramycin causes only minimal distortion of the DNA helix and is poorly recognized and, hence, poorly removed by the repair proteins.

The action of anthramycin on the apoptosis of cancer cells is not fully understood, but it may be assumed that like other DNA-alkylating antibiotics it induces apoptosis by a mitochondrial pathway.[8]

Another aspect of pyrrolobenzodiazepine activity is the inhibition of molecular interactions between DNA and transcription factor Sp1.[9] Inhibition of DNA binding activity of Sp1 to the cognate 'G–C' box causes neuroprotection against oxidative stress induced by various excytotoxic stimuli.[10] Because of the many side effects of anthramycin antibiotics (e.g., nausea, vomiting, diarrhea, myelosuppression, and a dose-limiting cardiotoxicity), there have been numerous attempts to obtain improved derivatives with enhanced activity or reduced side effects. Taking into account the potential neuroprotective and anti-carcinogenic effects of anthramycin related benzodiazepines, we investigated the change in survival of transformed human leukemic Jurkat and non-transformed neuronal/glial cells, induced by novel pyrrole, pyrrolobenzazepine, and pyrrolobenzodiazepine derivatives.

The tested heterocycles **2**, **3**, **5**, **6**, and **8** were prepared from a common starting material, namely, (2-nitrophenyl)(1-tosyl-1*H*-pyrrol-2-yl)methanone **1**[11] (Scheme 1). Compound **1** was reduced by catalytic hydrogenation over 5% palladium-on-carbon to give the corresponding amine, which was then detosylated to compound **2** in aqueous 2 N NaOH. Compound **2** was either further reduced to 2-(1*H*-pyrrol-2-yl)methyl)benzenamine **3** with sodium borohydride in refluxing propan-2-ol, or

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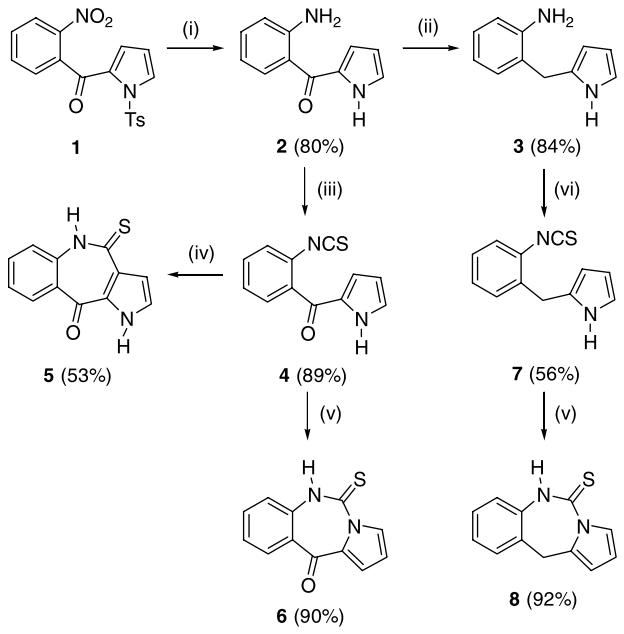

Scheme 1. Reagents and conditions: (i) (a) H_2, 5% Pd–C, MeOH, rt, 8 h, (b) aq 2 N NaOH, MeOH, reflux, 5 h; (ii) $NaBH_4$, IPA, reflux, 24 h; (iii) $CSCl_2$, toluene, Et_3N, rt, 1 h; (iv) $AlCl_3$, $MeNO_2$, rt, 6.5 h; (v) K_2CO_3, DMF, rt, 3 h; (vi) $CSCl_2$, toluene, Et_3N, −45 °C, 5 min.

transformed into the isothiocyanate **4** by reaction with thiophosgene. Amine **3** was converted into isothiocyanate **7** similarly. When **4** was subjected to $AlCl_3$ in nitromethane, ring closure via intramolecular electrophilic substitution between C-3 of the pyrrole ring and isothiocyanate group afforded pyrrolo[3,2-*c*][1]benzazepine-10(1*H*)-one-4(5*H*)-thione **5**. On the other hand, treatment of **4** and **7** with potassium carbonate in DMF afforded pyrrolo[1,2-*c*][1.3]benzodiazepines **6** and **8**, by intramolecular nucleophilic addition of the generated pyrrolyl anion to the isothiocyanate group.[12]

N-Methyl-D-aspartate (NMDA) receptor-mediated cell death is complex, probably involving elements of apoptosis in neuronal cells. The cytotoxic effects are mediated by increased Ca^{2+} influx through activated NMDA receptor. Associated with Ca^{2+} influx is an increase in reactive oxygen species (ROS) that appears to originate in the mitochondria. Ca^{2+} overloading reduces the membrane potential and disrupts electron transport, resulting in the increased production of ROS.[13] Some types of DNA-binding drugs through transcription factors Sp1 inhibit apoptosis and DNA damage in rat cortical neurons caused by excitotoxic oxidative stress.[10] Hence, the effects of heterocycles **2**, **3**, **5**, **6**, and **8** on NMDA-receptor mediated cell death were examined.[14] It was found that none of the examined compounds have any protective properties against NMDA-induced excitotoxicity. In the presence of these compounds, the reduction rate of MTT did not change (Table 1). Moreover, compound **8** increased cell damage after treatment of cells by NMDA, suggesting that this compound intensifies NMDA-dependent excitotoxicity.

Jurkat cells are a human leukemic cell line, which expresses CD4 clusters and has the ability to produce several cytokines in response to stimuli. It is a well-established model for the study of apoptotic death pathways of cancer cells.[15] Therefore, in the next series of experiments, Jurkat cells[14] were incubated in the presence of heterocycles **2**, **3**, **5**, **6**, and **8** (Table 2). It was found that MTT uptake was significantly decreased only in the presence of compound **8**, suggesting that this derivative has anti-proliferative and pro-apoptotic activity.

Table 1. Effects of 1 μM heterocycles **2**, **3**, **5**, **6** and **8** on the reduction of MTT in glial/neuronal cells

Additions	MTT reduction (O.D. 570 nm)[a]
Control	0.56 ± 0.08
2	0.65 ± 0.07
3	0.38 ± 0.08
5	0.55 ± 0.09
6	0.62 ± 0.06
8	0.58 ± 0.01
+ NMDA	0.29 ± 0.05
+ NMDA + **2**	0.30 ± 0.07
+ NMDA + **3**	0.22 ± 0.04
+ NMDA + **5**	0.25 ± 0.07
+ NMDA + **6**	0.28 ± 0.06
+ NMDA + **8**	0.17 ± 0.03

[a] Cell viability after exposure to 100 μM NMDA. The data are presented as means ± SEM for triplicate determination.

We have also analyzed DNA strand breaks in cells treated with 1 μM each of heterocycles **2**, **3**, **5**, **6**, and **8** using pulsed field gel electrophoresis[16] (Fig. 1). The additional DNA fragments of 3–4 Mb appeared in Jurkat cells following a 20 h treatment only with tricycle **8**, suggesting strongly that this compound induced DNA cleavage. Apparently, in this case DNA cleavage is initiated by heterocycle **8** mediated inhibition of topoisomerases that results in provoking poly(ADP)-ribose polymerase activation and apoptosis. Other compounds cause the formation of DNA fragments to a lesser extent.

The data presented point out that compound **8** causes cell death in both transformed Jurkat and non-transformed primary neuronal/glial cells, apparently by inducing damage to DNA. However, the mechanism of action of this pyrrolobenzodiazepine derivative differs from anthramycin and other alkylating minor groove binders, since compound **8** does not contain the imine or carbinolamine groups that would react with amines to form covalent bonds. It is suggested that compound

Table 2. Effects of 1 μM heterocycles **2**, **3**, **5**, **6**, and **8** on the reduction of MTT in Jurkat cells

Additions	MTT reduction (O.D. 570 nm)[a]
Control	0.47 ± 0.05
2	0.39 ± 0.07
3	0.34 ± 0.03
5	0.45 ± 0.07
6	0.41 ± 0.08
8	0.22 ± 0.05

[a] Cell viability after exposure for 24 h assessed by the MTT tests. The data are presented as means ± SEM for triplicate determination.

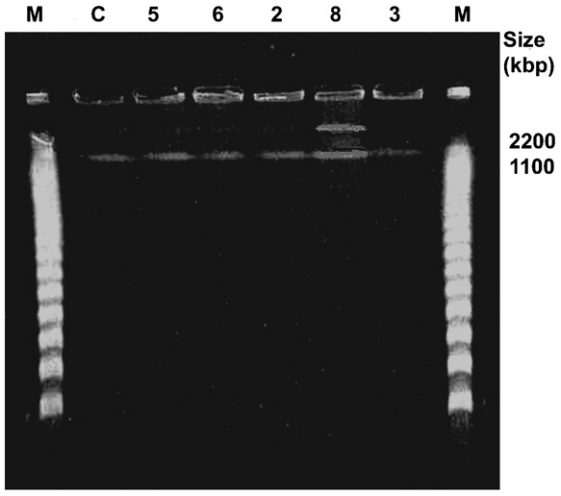

Figure 1. Detection of intracellular DNA cleavage in Jurkat cells treated with heterocycles **2**, **3**, **5**, **6**, and **8**. Cells (1×10^6/ml were treated with 1 μM of each heterocycle **2**, **3**, **5**, **6**, and **8** at 37 °C for 20 h. Cells, prepared as agarose plugs, were lysed and subjected to pulsed field gel electrophoresis through a 1% agarose gel. C, control; M, size marker DNA.

8 induces genotoxicity by stabilizing the DNA helix just like the sequence-selective pyrrolobenzodiazepine dimer SJG-136. The latter is unable to interact covalently with DNA but has significant cytotoxicity in some cell lines.[17] Further experiments are needed for the clarification of the mechanism of action of non-covalent DNA-interactive pyrrolobenzodiazepine derivatives. The results presented augment the repertoire of compounds that have pro-apoptotic properties and may be used as a basis for the design of new non-toxic anti-cancer drugs.

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12. (a) Data for **7**: as an oil; IR (Neat) ν_{max} 3388, 3097, 3068, 2915, 2105, 1597, 1578 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H (ppm) 4.06 (s, 2H, CH_2), 6.04 (m, 1H, H-3), 6.19 (m, 1H, H-4), 6.73 (m, 1H, H-5), 7.14–7.26 (m, 4H, H-3′, H-4′, H-5′, H-6′), 7.99 (br s, 1H, NH pyrrole); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C (ppm) 31.26, 107.75, 109.39, 118.03, 127.40, 128.37, 128.43, 129.07, 130.65, 130.77, 135.20, 137.02; MS (EI), *m/z* 214 (M^+, 100), 186 (28), 181 (31), 154 (12), 80 (8); EI HRMS M^+, found 214.0559 $C_{12}H_{10}N_2S$ requires 214.0565; (b) Data for **8**: as a light yellow powder mp = 183 °C (dec); IR (Nujol) ν_{max} 3165, 3111, 1609, 1583 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ_H (ppm) 3.95 (s, 2H, CH_2), 6.03 (m, 1H, H-1), 6.20 (t, 1H, J = 3.3 Hz, H-2), 7.15 (m, 1H, H-10), 7.20–7.29 (m, 3H, H-7, H-8, H-9), 7.68 (dd, 1H, J = 3.4, 1.9 Hz, H-3), 9.91 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C (ppm) 31.57, 109.32, 112.26, 120.88, 125.61, 127.28, 127.53, 128.03, 132.58, 132.89, 136.40, 180.17; MS (EI), *m/z* 214 (M^+, 100), 186 (33), 181 (32), 149 (36), 135 (20), 97 (15%); EI HRMS M^+, found 214.0556 $C_{12}H_{10}N_2S$ requires 214.0565; (c) Compounds **4**–**6** were characterized by satisfactory elemental analyses, and from their mass, ^1H and ^{13}C NMR spectra.
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14. (a) *Cerebrocortical cultures*—Cortical cultures of mixed neurons and glia were derived from embryonic (fetal day 15 or 16) Wistar rats. Briefly, following dissociation in 0.027% trypsin, cerebral cortical cells were plated either on 96-well multiwell plates or 35 mm dishes that had been previously coated overnight with 15 μg/ml poly-L-lysine and then with DMEM (Sigma) culture medium supplemented with 10% fetal bovine serum. After removal of the final coating solution, cells were seeded (10^6 ml) in a serum free medium composed of a mixture of DMEM, contained 40 μg/ml gentamycin, and 60 μg/ml penicillin. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and were used after 6–7 days; (b) *Jurkat cell culture*—The human Jurkat T cell line (clone E6-1) was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% FBS (Sigma), 2 mM L-glutamine (Life Technologies), and 10 μg/ml gentamycin (Life Technologies). Cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2. Cells were passaged three times weekly and maintained at a density between 0.2 and 1×10^6 cells/ml. Cells used for all experiments were in logarithmic growth phase, and the medium used for experiments had the same constituents as that used for cell passage, unless otherwise indicated; (c) *MTT reduction cell viability assay*—Experiments were performed using glia/neurons and Jurkat cells cultured in 96-well plates. The effects of heterocycles **2**, **3**, **5**, **6** and **8** (1 μM) or/and NMDA (10 μM) on cell viability was assessed by a colorimetric assay based on the cleavage of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-

mide (MTT) into a blue-colored formazan product by mitochondrial succinate dehydrogenase. Additions were made directly to the culture medium for 24 h. Cells then washed twice with HEPES-buffered incubation medium (HBM) (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 1.2 $CaCl_2$, 5.5 mM glucose, and 20 mM HEPES, pH 7.4.) and incubated for 45 min at 37 °C in HBM containing MTT (0.5 mg/ml). After this period, the HBM was carefully removed, and the blue formazan product was solubilized in 300 μl of 100% dimethyl sulfoxide. The absorbance of each well was read at 570 nm. The data were treated by one-way ANOVA analysis. The data from each experiment were analyzed separately. Where a significant effect was observed in the ANOVA analysis, comparisons of those samples were made by the *t*-test.

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