ELSEVIER

Biochemical Pharmacology 63 (2002) 1143–1147

Biochemical Pharmacology

Short communication

Characterization of anthracenediones and their photoaffinity analogs

Kai-Ming Chou*, A. Paul Krapcho[1], David Horn[2], Miles Hacker[3]

Department of Pharmacology, School of Medicine, University of Vermont, Burlington, VT 05405, USA

Received 12 June 2001; accepted 19 September 2001

Abstract

In an attempt to overcome the cardiotoxicity and cross-resistance problems caused by the anticancer drugs anthracyclines and anthracenediones during chemotherapy, we have developed a series of aza-anthracenedione compounds by modifying the chromophore and the side arms of anthracyclines and anthracenediones. One of these aza-anthracenediones, 6,9-bis[(2-aminoethyl)amino]benzo[*g*]isoquinoline-5,10-dione (BBR 2778), which is currently under phase II clinical trials, showed remarkable antitumor activity and appeared to lack a cardiotoxic effect in preclinical studies. However, it was still cross-resistant against multidrug resistance (MDR) cells expressing P-glycoprotein (P-gp). In contrast, another aza-anthracenedione, 6,9-bis[[2-(dimethylamino)ethyl]amino]benzo[*g*]isoquinoline-5,10-dione, which has side arm structures different from those of BBR 2778, was highly active against MDR cells. In this study, BBR 2778, BBR 2378, and an anthracenedione compound, 1,4-bis[(2-aminoethyl)amino]-5,8-dimethyl-9,10-anthracenedione, were used to assess the relationship between the chemical structures of these drugs and their interactions with DNA and P-gp. In addition, the biological and pharmacological influences of photoaffinity labeling were also studied for BBR 2778 and DEH. As the results indicate, the photolabeled analogs of BBR 2778 and DEH were less DNA-reactive and less cytotoxic. The more lipophilic compound, BBR 2378, and the photolabeled analogs of BBR 2778 and DEH inhibited P-gp labeling by azidopine better than did the more hydrophilic parental compounds. These studies suggested that the DNA binding affinity of BBR 2778 and DEH could be important in determining their cytotoxicity, and that the chemical structure of the side arms and the lipophilicity of these drugs are critical in determining their cross-resistance. © 2002 Published by Elsevier Science Inc.

Keywords: Anthracycline; Anthracenedione; Photoaffinity labeling; P-glycoprotein; Multidrug resistance

1. Introduction

The anthracyclines daunorubicin and doxorubicin have long been used as effective anticancer drugs against a broad spectrum of tumors [1,2]. This class of anticancer drugs causes free radical-induced DNA damage [3,4], stabilization of the DNA–topoisomerase II complex [5,6], disruption of lipid bilayers [7], and inhibition of DNA and RNA synthesis [8,9]. Although the anthracyclines are clinically important, there exist a number of disadvantages, such as cardiac toxicity [10,11] and tumor resistance [12,13], which limit their clinical potential. In an attempt to improve the therapeutic usefulness of such drugs, we developed a carbocyclic compound DEH (1,4-bis[(2-aminoethyl)amino]-5,8-dimethyl-9,10-anthracenedione), and a series of heterocyclic drugs—the aza-anthracenediones (Fig. 1) [14,15].

Our previous reports have shown a dramatic difference in biological activity between 1-aza and 2-aza compounds—only the 2-aza compounds have marked antitumor activity *in vivo* [14–17]. Among these 2-aza-anthracenediones, BBR 2378 (6,9-bis[[2-(dimethylamino)ethyl]amino]benzo[*g*]isoquinoline-5,10-dione) and BBR 2778 (6,9-bis[(2-aminoethyl)amino]benzo[*g*]isoquinoline-5,10-dione) [18] were selected for advanced studies in this report. The chemical difference between these two compounds is that BBR 2378 has a terminal tertiary amine on both side arms whereas BBR 2778 has a terminal primary amine on both side arms (Fig. 1). These two compounds cause similar DNA mobility shifts in agarose gel electrophoresis and

* Corresponding author. Present address: Department of Pharmacology, School of Medicine, Yale University, 333 Cedar St., SHM B-315, New Haven, CT 06520, USA. Tel.: +1-203-785-7119; fax: +1-203-785-7129.

E-mail address: kai-ming.chou@vtmednet.org (K.-M. Chou).

[1] Present address: Department of Chemistry, University of Vermont, Burlington, VT 05405, USA.

[2] Present address: Department of Chemistry, Goucher College, Towson, MD, USA.

[3] Present address: Department of Health Sciences, Grand Valley State University, Allendale, MI, USA.

Abbreviations: MDR, multidrug resistance; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; P-gp, P-glycoprotein; PMSF, phenylmethylsulfonyl fluoride.

0006-2952/02/$ – see front matter © 2002 Published by Elsevier Science Inc.
PII: S0006-2952(02)00855-9

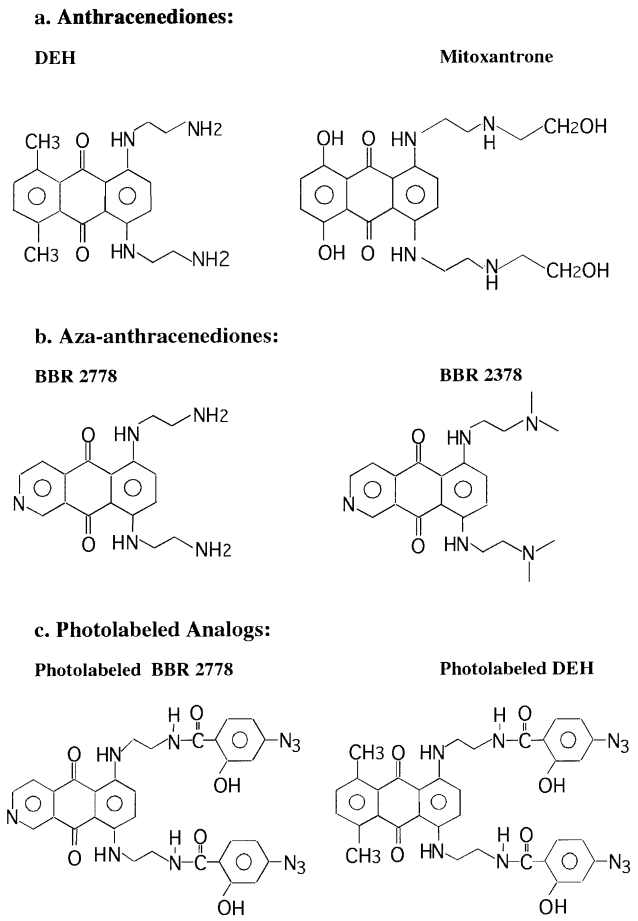

Fig. 1. Chemical structures of compounds.

have similar cytotoxicity profiles [17]. A biological difference between these two drugs is that BBR 2778 is cross-resistant against MDR-expressing cells, whereas BBR 2378 is highly active [17].

While MDR phenotypes can be caused by several different mechanisms [19,20], P-gp is one of the most extensively studied [21,22]. P-gp is a membrane-bound protein, which recognizes a variety of compounds and pumps them out of the cells in an energy-dependent process [21,22]. Since BBR 2378 was active against MDR-expressing cells but BBR 2778 was not, the different side arm structures of these two compounds may play a critical role in P-gp–drug interaction.

In addition, the photoaffinity labeling technique has been used widely to investigate the interactions between P-gp and various anticancer drugs, since the photolabeled probe is chemically stable and the analogs retain biological and pharmacological activities similar to those of the original compound [23]. However, the photoaffinity labeling modification could also cause some property changes of the original compound as the structure of the side arms of anthracenediones plays an important role in determining their activities in MDR-expressing cells. To elucidate the influence of the photoaffinity probe, the photoaffinity analogs of BBR 2778 and DEH were synthesized by coupling *N*-hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) through the primary amine side arms of BBR 2778 and DEH. Furthermore, to compare the impact of the different chromophore and side arm structures among anthracenediones and aza-anthracenediones as well as the photoaffinity labeling tag on their interaction with P-gp, competition studies of P-gp labeling by [^{3}H]-azidopine were performed since azidopine is known to have the ability to bind to P-gp [24].

2. Materials and methods

2.1. Drugs

BBR 2778 and BBR 2378 were synthesized at Boehringer Mannheim Italia. DEH was synthesized by David E. Horn, Goucher College. Purity of all the compounds was assured using NMR and TLC. The photoaffinity probe NHS-ASA was purchased from Pierce. The procedure used to synthesize photolabeled DEH and BBR 2778 is described in our recent report [25]. The purity of each photolabeled compound was verified by NMR and liquid chromatography-mass spectrometry.

2.2. Cytotoxicity assay

S180 and its doxorubicin-derived resistant cell line S180/A10 were gifts from T. Tritton (Hanover College). Both cell lines were grown in McCoy's 5A medium supplemented with 5% donor horse serum. The exponentially growing cells were diluted in fresh medium to approximately 50,000/mL and subsequently were treated with different concentrations of drugs for 72 hr. The number of cells were measured using a Coulter cell counter, and the concentration of drug required to inhibit cell growth by 50% for each drug was calculated (IC_{50}).

2.3. DNA unwinding assay

The DNA unwinding caused by the drugs was measured by monitoring the electrophoretic mobility changes of supercoiled pBR322 DNA (16 μM) in a 1.4% agarose gel (Gibco BRL) in the presence of different concentrations of the drugs. The conditions are as described previously [25].

2.4. Cell membrane preparation

The exponentially growing S180 and S180/A10 cells were harvested by centrifugation and resuspended in a hypotonic buffer (5 mM Tris–HCl, pH 7.4, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4° for 30 min before homogenization. The cell homogenates were centrifuged at 1000 *g* for 10 min at 4°. The supernatants were centrifuged at 100,000 *g* for 1 hr at 4°.

The membrane pellets were suspended in a membrane storage buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 20% glycerol, 0.1 mM PMSF) at a protein concentration of 50 μg/mL and stored at −80°.

2.5. Photolabeling P-gp

[^{3}H]-Azidopine was purchased from the Amersham Co. Each reaction contained [^{3}H]-azidopine (1.25 μM) and a 125 μM concentration of different drugs added to 10 μg of the membrane preparation at 25° for 20 min in the dark. The assay conditions were as described previously [25]. The bands were quantified by a PDI Quantity 1 densitometer (model DNA 35, PDI Inc.).

3. Results and discussion

The cytotoxicity values of the anthracenedione compounds mitoxantrone and DEH, the aza-anthracenedione compounds BBR 2778 and BBR 2378, and the photoaffinity analogs of BBR 2778 and DEH against MDR sensitive S180 and resistant S180/A10 cells are summarized in Table 1. BBR 2778 was the most potent drug among these compounds against S180 cells, but was 50-fold less potent against S180/A10 cells. In contrast, BBR 2378, with tertiary terminal amine groups on both side arms, showed only a 2-fold decrease in potency against the resistant cells. Mitoxantrone was very potent against the sensitive S180 cells but it was cross-resistant against MDR cells. The other carbocyclic compound, DEH, was less active against the sensitive cells, as compared with either the aza-heterocyclic compounds or mitoxantrone, and was not active against the S180/A10 MDR cells. Finally, both the photoaffinity analogs of BBR 2778 and DEH lacked significant cytotoxicity against either the sensitive or MDR-resistant cells. These data suggested that the chemical structure of the side arm plays an important role in determining the cytotoxicity and cross-resistance of these compounds.

Table 1
Cytotoxicity of compounds

Compound	Drug (μg/mL)	
	S180	S180/A10
BBR 2778	0.008	0.4
Photolabeled L2778	>10	>10
DEH	0.3	>10
Photolabeled DEH	>10	>10
BBR 2378	0.07	0.14
Mitoxantrone	0.01	0.65

Compounds were added to cells at 37° for 72 hr. The IC_{50} value for each compound was calculated as the drug concentration needed to inhibit 50% cell growth as compared with the untreated cells. The IC_{50} values are averages from three individual experiments, and the error ranges were within 10%.

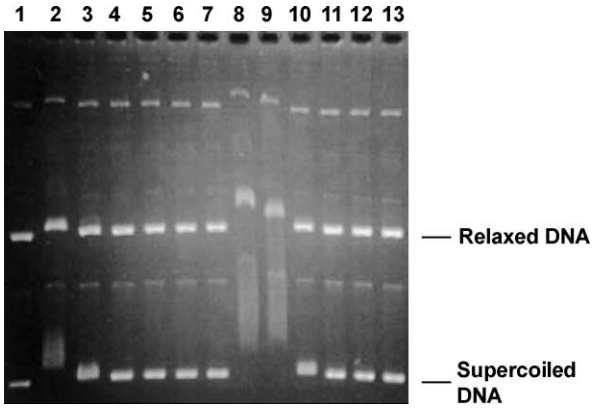

Fig. 2. DNA migration assay. The different concentrations of compounds and pBR322 DNA were incubated in 25 mM HEPES, pH 7.5, 500 mM NaCl, and 1 mM EDTA at 37° for 20 min before loading onto a 1.4% agarose gel for electrophoresis. The gel was stained with ethidium bromide and the photograph was taken under UV light. Lane 1: control DNA; lanes 2–4: BBR 2778 (100, 10, and 1 μM, respectively); lanes 5–7: photolabeled BBR 2778 (100, 10, and 1 μM, respectively); lanes 8–10: DEH (100, 10, and 1 μM, respectively); lanes 11–13: photolabeled DEH (100, 10, and 1 μM, respectively).

One of the major mechanisms of action for anthracycline-like compounds is through DNA intercalation [26]. Intercalation of the drug into supercoiled DNA will unwind the DNA and slow its migration on an agarose gel. As shown in Fig. 2, BBR 2778 showed less impact than DEH on DNA migration which could be due to the nitrogen in the planar ring of BBR 2778. However, both compounds changed the DNA migration at a drug concentration of 10 μM, and neither of their photolabeled analogs had any impact on DNA migration up to a 100 μM concentration. It was reported that the planar ring of the chromophore backbone of anthracyclines intercalates into the DNA double helix and this intercalation is stabilized by interactions between the positively charged amine on the side arm and the negatively charged phosphate group of DNA [27–29]. It is possible that the protonated primary amine on the side arm of BBR 2778 and DEH stabilized the DNA–drug complex through such interactions. The photolabeled analog may interact with DNA through different mechanisms, however, as the result indicated, the photolabeled analogs had much less ability to change the DNA migration, compared with the parent compounds. The DNA intercalating data suggested that the DNA intercalating abilities of BBR 2778 and DEH may have a major influence on their cytotoxicities.

Since S180/A10 cells displayed resistance to mitoxantrone, BBR 2778, and DEH it is possible that all three compounds can be recognized by P-gp. To measure the P-gp binding affinities of the different compounds, the inhibitory effects of these compounds against P-gp labeling by [^{3}H]-azidopine were studied. As shown in Fig. 3 and Table 2, vinblastine inhibited 90% of the azidopine binding to P-gp, which suggested that this azidopine binding is specific. The carbocyclic compound DEH showed 73%

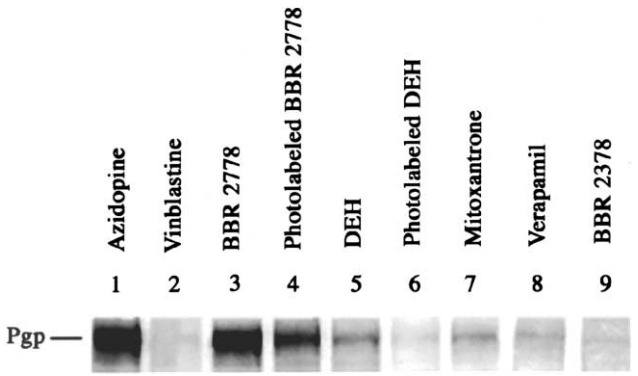

Fig. 3. Competition assay for P-gp binding. [^{3}H]-Azidopine (1.25 μM) was incubated with 10 μg of S180/A10 cell membrane preparation with or without a 125 μM concentration of different compounds at room temperature for 20 min before exposing to UV light for cross-linking reactions. The samples were loaded onto an 8% SDS-PAGE. The gel was subsequently dried after electrophoresis and exposed to a chemoilluminant film. The bands were quantified by a PDI Quantity 1 densitometer (model DNA 35, PDI Inc.).

inhibition while BBR 2778, which has two primary amine side arms like DEH, showed only 2% inhibition. Another carbocyclic compound, mitoxantrone, with secondary amines and terminal hydroxyl groups on both side arms, showed 70% inhibition and the aza-anthracenedione compound BBR 2378 which contains two tertiary amine side arms and is active in MDR-expressing cells [16,17], showed 92% inhibition. The photoaffinity analogs of both BBR 2778 and DEH were more effective in competing with azidopine on P-gp binding (69 and 91% inhibition, respectively) than either parental compound (Table 2).

A previous report suggested that two basic chemical structures are required for P-gp recognition: a single aromatic moiety and a proper lipophilicity [30]. In this report, the more lipophilic compound BBR 2378 was more effective than BBR 2778 at competing against azidopine binding to P-gp. This is a very interesting phenomenon because the cytotoxicity data showed BBR 2778 to be highly cross-resistant in MDR cells whereas BBR 2378 was non-cross-resistant. The other interesting phenomenon is that the parent carbocyclic compound DEH inhibited azidopine binding to P-gp by 73% in comparison to the poor inhibitory effect of BBR 2778, a compound that shares the same side arm structures with DEH but has a different chromophore backbone. It suggested that the structure of the chromophore backbone may be critical for P-gp recognition. It is possible that compounds are recognized by P-gp through binding sites other than azidopine since it has been suggested that there are multiple binding sites in P-gp [31]. It could also be that BBR 2778 is involved with other cellular drug resistance mechanisms in addition to P-gp [19,20]. The photoaffinity analogs of both BBR 2778 and DEH showed better inhibitory effects than their parental compounds, which further suggested the influence of lipophilicity on P-gp recognition.

Table 2
Percentage inhibition of P-gp labeling by compounds

Compound	Inhibition (%)
Vinblastine	89.8 ± 3.94
BBR 2778	1.79 ± 0.3
Photolabeled BBR 2778	68.7 ± 2.46
DEH	72.6 ± 3.55
Photolabeled DEH	91.2 ± 0.78
Mitoxantrone	70.2 ± 4.60
Verapamil	93.0 ± 1.91
BBR 2378	92.1 ± 2.5

The methods are as described in the legend of Fig. 3. The percent inhibition was calculated by dividing the intensity of P-gp band by the total intensity of the whole lane. Values are means ± range, $N = 2$. The percentages of inhibition of different compounds were adjusted using the azidopine labeled P-gp as a 100% standard.

The relationships of chemical charge and lipophilicity to drug resistance and P-gp binding have been discussed [32–34]. It has been shown that P-gp is located on the membrane of the Golgi complex and lysosomes [30]. A microscopic study indicated that BBR 2778 is clustered in the cell nucleus but BBR 2378 is excluded from the nucleus and is trapped in cytoplasmic compartments such as the Golgi apparatus or lysosomes. These subcellular organelles are responsible for a variety of detoxification processes, protein processing and receptor recycling. Compounds like BBR 2378 may exert their action through the disruption of the functions of the subcellular organelles. Further investigations of the relationships between the chemical structure of compounds and their intracellular distribution patterns are necessary.

Acknowledgments

We would like to thank Lori Hazlehurst for insightful discussion and James Bigelow for assistance with the HPLC.

References

[1] Aubel-Sadron G, Londos-Gagliardi D. Daunorubicin and doxorubicin, anthracycline antibiotics, a physicochemical and biological review. Biochimie 1984;66:333–52.
[2] Trouet A, Deprez-De Campeneere D. Daunorubicin–DNA and doxorubicin–DNA: a review of experimental and clinical data. Cancer Chemother Pharmacol 1979;2:77–9.
[3] Powis G. Free radical formation by antitumor quinones. Free Radic Biol Med 1989;6:63–101.
[4] Quillet-Mary A, Mansat V, Duchayne E, Come MG, Allouche M, Bailly JD, Bordier C, Laurent G. Daunorubicin-induced internucleosomal DNA fragmentation in acute myeloid cell lines. Leukemia 1996;10:417–25.
[5] Zunino F, Capranico G. DNA topoisomerase II as the primary target of antitumor anthracyclines. Anticancer Drug Des 1990;5:307–17.
[6] Jensen PB, Sorensen BS, Sehested M, Demant EJ, Kjeldsen E, Friche E, Hansen HH. Different modes of anthracycline interaction with topoisomerase II. Separate structures critical for DNA cleavage, and

for overcoming topoisomerase II-related drug resistance. Biochem Pharmacol 1993;45:2025–35.
[7] Tritton TR. Cell surface actions of adriamycin. Pharmacol Ther 1991;49:293–309.
[8] Chuang LF, Chuang RY, Acton EM, Israel M, Yu M. Effect of morpholinyladriamycin analogs and adriamycin on the activities of DNA polymerase α and RNA polymerase II of chicken leukemia cells. J Pharmacol Exp Ther 1987;242:372–7.
[9] Müller I, Jenner A, Bruchelt G, Niethammer D, Halliwell B. Effect of concentration on the cytotoxic mechanism of doxorubicin—apoptosis and oxidative DNA damage. Biochem Biophys Res Commun 1997;230:254–7.
[10] Ferrans VJ. Overview of cardiac pathology in relation to anthracycline cardiotoxicity. Cancer Treat Rep 1978;62:955–61.
[11] Von Hoff DD, Rozencweig M, Layard M, Slavik M, Muggia FM. Daunomycin-induced cardiotoxicity in children and adults: a review of 110 cases. Am J Med 1977;62:200–8.
[12] Booser DJ, Hortobagyi GN. Anthracycline antibiotics in cancer therapy. Focus on drug resistance. Drugs 1994;47:223–58.
[13] Friche E, Skovsgaard T, Nissen NI. Anthracycline resistance. Acta Oncol 1989;28:877–81.
[14] Krapcho AP, Petry ME, Getahun Z, Landi Jr. JJ, Stallman J, Polsenberg JF, Gallagher CE, Maresch MJ, Hacker MP, Giuliani FC, Beggiolin G, Pezzoni G, Menta E, Manzotti C, Oliva A, Spinelli S, Tognella S. 6,9-Bis[(aminoalkyl)amino]benzo[*g*]isoquinoline-5,10-diones. A novel class of chromophore-modified antitumor anthracene-9,10-diones: synthesis and antitumor evaluations. J Med Chem 1994;37:828–37.
[15] Krapcho AP, Landi Jr. JJ, Hacker MP, McCormack JJ. Synthesis and antineoplastic evaluations of 5,8-bis[(aminoalkyl)amino]-1-azaanthracene-9,10-diones. J Med Chem 1985;28:1124–6.
[16] Hazlehurst LA, Krapcho AP, Hacker MP. Comparison of azaanthracenedione-induced DNA damage and cytotoxicity in experimental tumor cells. Biochem Pharmacol 1995;50:1087–94.
[17] Hazlehurst LA, Krapcho AP, Hacker MP. Correlation of DNA reactivity and cytotoxicity of a new class of anticancer agents: azaanthracenediones. Cancer Lett 1995;91:115–24.
[18] Pezzoni G, Beggiolini G, Manzotti C, Spinelli S, Tognella S, Guiliani FC. BBR 2778, a novel aza-analog of anthacenediones endowed with preclinical anticancer activity. Proc Am Assoc Cancer Res 1993;34:373.
[19] Larsen AK, Escargueil AE, Skladanowski A. Resistance mechanisms associated with altered intracellular distribution of anticancer agents. Pharmacol Ther 2000;85:217–29.
[20] Naito S, Yokomizo A, Koga H. Mechanisms of drug resistance in chemotherapy for urogenital carcinoma. Int J Urol 1999;6:427–39.
[21] Nielsen D, Skovsgaard T. P-glycoprotein as multidrug transporter: a critical review of current multidrug-resistant cell lines. Biochim Biophys Acta 1992;1139:169–83.
[22] Robert J. Multidrug resistance and its reversal. General review of fundamental aspects. Ann Biol Clin (Paris) 1996;54:3–8.
[23] Safa AR, Mehta ND, Agresti M. Photoaffinity labeling of P-glycoprotein in multidrug-resistant cells with photoactive analogs of colchicine. Biochem Biophys Res Commun 1989;162:1402–8.
[24] Safa AR, Glover CJ, Sewell JL, Meyers MB, Biedler JL, Felsted RL. Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. J Biol Chem 1987;262:7884–8.
[25] Chou K-M, Krapcho AP, Hacker MP. Impact of the basic amine on the biological activity and intracellular distribution of an azaanthrapyrazole: BBR 3422. Biochem Pharmacol 2001;62:1337–43.
[26] Osheroff N, Corbett AH, Robinson MJ. Mechanism of action of topoisomerase II-targeted antineoplastic drugs. Adv Pharmacol 1994;29(B):105–26.
[27] Patel DJ, Canuel LL. Anthracycline antitumor antibiotic nucleic acid interactions. Structural aspects of the daunomycin poly(dA-dT) complex in solution. Eur J Biochem 1978;90:247–54.
[28] Patel DJ, Kozlowski SA, Rice JA. Hydrogen bonding, overlap geometry, and sequence specificity in anthracycline antitumor antibiotic·DNA complexes in solution. Proc Natl Acad Sci USA 1981;78:3333–7.
[29] Wang AJ, Ughetto G, Quigley GJ, Rich A. Interactions between an anthracycline antibiotic and DNA: molecular structure of daunomycin complexed to d(CpGpTpApCpG) at 1.2 Å resolution. Biochemistry 1987;26:1152–63.
[30] Dellinger M, Pressman BC, Calderon-Higginson C, Savaraj N, Tapiero H, Kolonias D, Lampidis TJ. Structural requirements of simple organic cations for recognition by multidrug-resistant cells. Cancer Res 1992;52:6385–9.
[31] Shapiro AB, Ling V. The mechanism of ATP-dependent multidrug transport by P-glycoprotein. Acta Physiol Scand Suppl 1998;643:227–34.
[32] Lampidis TJ, Shi YF, Calderon CL, Kolonias D, Tapiero H, Savaraj N. Accumulation of simple organic cations correlates with differential cytotoxicity in multidrug-resistant and -sensitive human and rodent cells. Leukemia 1997;11:1156–9.
[33] Lampidis TJ, Kolonias D, Podona T, Israel M, Safa AR, Lothstein L, Savaraj N, Tapiero H, Priebe W. Circumvention of P-gp MDR as a function of anthracycline lipophilicity and charge. Biochemistry 1997;36:2679–85.
[34] Stein WD. Kinetics of the multidrug transporter (P-glycoprotein) and its reversal. Physiol Rev 1997;77:545–90.