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Preclinical report

Diazene JK-279: potential anticancer drug

Maja Osmak, Tatjana Bordukalo, Branimir Jernej, Janez Košmrlj[1] and Slovenko Polanc[1]
Department of Molecular Genetics, Ruder Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia.
[1]Faculty of Chemistry and Chemical Technology, Aškerčeva 5, 1000 Ljubljana, Slovenia.

The aim of this study was to examine the cytotoxic effect of 10 newly synthesized diazenecarboxamides (diazenes). Using a modified colorimetric MTT assay, their cytotoxicity was determined on 10 human cell lines: cervical carcinoma parental and cisplatin-resistant cells, laryngeal carcinoma parental and cisplatin- and vincristine-resistant cells, glioblastoma parental and cisplatin-resistant cells, breast adenocarcinoma parental and doxorubicin-resistant cells, and mammary carcinoma cells. Results show that diazene JK-279 was most effective, reducing significantly the cell survival of all 10 cell lines examined, including five drug-resistant cell lines. A cytotoxic effect was observed also on nine from 10 cell lines for diazene JK-835. A small reduction in cell survival was obtained (mainly for highest drug concentrations) for diazenes LV-57 and MG-19 on two cell lines, and JK-429 and JK-913 on one cell line. Other diazenes did not demonstrate any cytotoxic activity. The results encourage further research on diazene JK-279 as a potential anticancer drug. [**]**

Key words: Anticancer drugs, diazenes, drug resistance, tumor cells.

Introduction

Glutathione (GSH) is an ubiquitous non-protein thiol essential for cellular homeostasis.[1–4] It plays an important role in protecting cells against free radical-induced oxydant injury, as well as in the detoxification of numerous endogenous and foreign electrophilic compounds. It has also been strongly implicated in the resistance of tumor cells to certain anticancer drugs, including alkylating agents.[5–11] GSH is required for the synthesis of several cellular compounds and is involved in immune modulation.[2,3]

Correspondence to M Osmak, Department of Molecular Genetics, Ruder Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia.
Tel: (+385) 1 4560 939; Fax: (+385) 1 4561 177;
E-mail: osmak@rudjer.irb.hr

It is known that a particular type of diazenes, i.e. diazenecarboxylic acid esters, can easily oxidize thiols to disulfides. Thus, it is not surprising that such compounds have already been studied in several biological systems. Unfortunately, they are unstable.[12] Considering the previous experiments,[13–15] diazenecarboxamides (shortly diazenes) were synthesized.[16] They are stable in both the solid state and in solutions, even over an extended period of time. Therefore, diazenecarboxamides may be more convenient compounds for therapeutic application than diazenecarboxylic acid esters.

So far the anticancer activity of such diazenes has not been investigated. The aim of the present study was to examine whether some of 10 newly synthesized diazenes possess cytotoxic activity. Their cytotoxic effect (at the maximal achievable concentration limited by their solubility, and four subsequent dilutions) was examined on 10 human cell lines: cervical carcinoma, laryngeal carcinoma glioblastoma, breast adenocarcinoma cells and mammary carcinoma parental cells, as well as on their drug-resistant sublines.[17–21]

Materials and methods

Diazenes

1,4-Disubstituted semicarbazides were transformed to the corresponding diazenecarboxamides (diazenes) using ceric ammonium nitrate as an oxidant.[16] Ten such new synthesized compounds were selected and used in this study: JK-279, JK-429, JK-802, JK-835, JK-913, JK-925, LV-35, LV-57, MG-19 and VZ-19. Their structures are presented in Figure 1. Diazenes were dissolved in water:ethanol (1:1) solution. Solutions were sterilized by filtration and stored at −20°C.

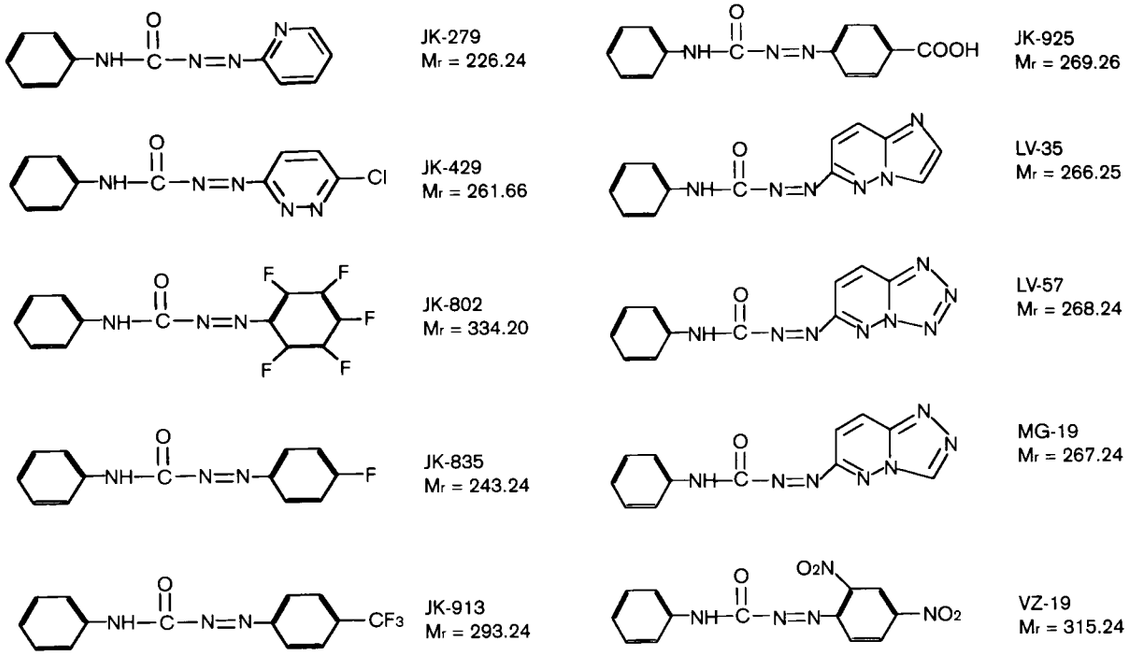

Figure 1. Structures of diazenes JK-279, JK-429, JK-802, JK-835, JK-913, JK-925, LV-35, LV-57, MG-19 and VZ-19.

The solubility of the examined diazenes was markedly different. The maximal concentrations obtained were: 32.4 mM for JK-279, 1.42 mM for JK-429, 0.07 mM for JK-802, 3.92 mM for JK-835, 1.03 mM for JK-913, 1.91 mM for JK-925, 1.83 mM for LV-35, 0.51 mM for LV-57, 1.82 mM for MG-19 and 0.15 mM for VZ-19. These stock solutions were diluted in growth medium to the final concentrations from 1:30 up to 1:3000. The cytotoxic effect of these five concentrations was examined on 10 cell lines. A simultaneous study of the cytotoxic effect of solvent (ethanol + water) was performed.

Other chemicals

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, St Louis, MO) was dissolved in phosphate-buffered saline at 4°C. It was diluted with the medium just before use.

Cell lines

Human cervical carcinoma cells (HeLa), laryngeal carcinoma cells (HEp2), glioblastoma cells (A1235), breast adenocarcinoma cells (SK-BR-3) and mammary carcinoma cells (MCF-7), as well as their drug-resistant sublines, were used in this study. The details concerning the development of drug resistance have been reported earlier.[17–21] Briefly, drug-resistant cell lines were obtained by exposing: (i) human cervical carcinoma cells to the gradually increasing concentrations of cisplatin (HeLa CA cells; treatment through 1 h, final drug concentration 48 μM),[17] (ii) laryngeal carcinoma cells to cisplatin (CK2 cells; treatment through 24 h, final drug concentration 2.4 μM),[18] (iii) laryngeal carcinoma cells to vincristine (VK2 cells; treatment through 24 h, final drug concentration 0.4 μM),[19] (iv) glioblastoma cells to cisplatin (CT cells; treatment through 24 h, final drug concentration 0.289 μM)[20] and (v) breast adenocarcinoma cells to doxorubicin (SC-6 cells; treatment through 24 h, final drug concentration 0.207 μM).[21] The cell lines obtained following treatment with cisplatin are designated cisplatin resistant (although they are also cross-resistant to some other cytostatics). Similarly, other drug-resistant cell lines are designated doxorubicin resistant or vincristine resistant. Some characteristics of these resistant cell lines are given in Table 1.

The cells were grown as a monolayer culture in Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and antibiotics in a humid atmosphere containing 5% CO_2.

Cytotoxicity assay

The chemosensitivity of parental and resistant cells to diazenes was determined using a modified colorimetric MTT assay.[23] The cells were plated into tissue culture 96-well microtiter plates at 1×10^4 cells in 0.18 ml medium/well. The next day the appropriate concentrations of different diazenes were added in 0.02 ml to each well. Each concentration was tested in quadruplicate. Diazenes were present continuously for 48 h at 37°C. Thereafter the medium was aspirated and 20 μg of MTT dye/0.04 ml medium was added to each well. Following 4 h incubation at 37°C, formazon cristals were disolved in dimethylsulfoxide (0.17 ml/well), the plates were mechanically agitated for 5 min and the optical density at 540 nm was determined on the microtiter plate reader. Each experiment was repeated 3 times.

Statistical analysis

Significance of the difference in cell survival between control and diazene treated cells was assessed by Student's *t*-test. The level of significance was set at 0.05.

Table 1. Characteristics of human drug-resistant cell lines

Cell line	Cell origin	Drug[a]	Cross-resistance	Reference
HeLa CA	cervical carcinoma (HeLa) cells	cisplatin	VCR, DOX, ETO, MTX, 5-FU	17
CK2	laryngeal carcinoma (HEp2) cells	cisplatin	VCR, 5-FU, MMC	18, 22
VK2	laryngeal carcinoma (HEp2) cells	vincristine	DOX, 5-FU, MTX	19
CT	glioblastoma (A1235)	cisplatin	VCR, DOX, ETO	20
SC-6	breast adenocarcinoma (SK-Br-3) cells	doxorubicin	VCR, MMC, 5-FU, cisplatin	21

[a]Drug used for resistance development: VCR, vincristine; DOX, doxorubicin; ETO, etoposide; MTX, methotrexate; 5-FU, 5-fluorouracil; MMC, mitomycin C.

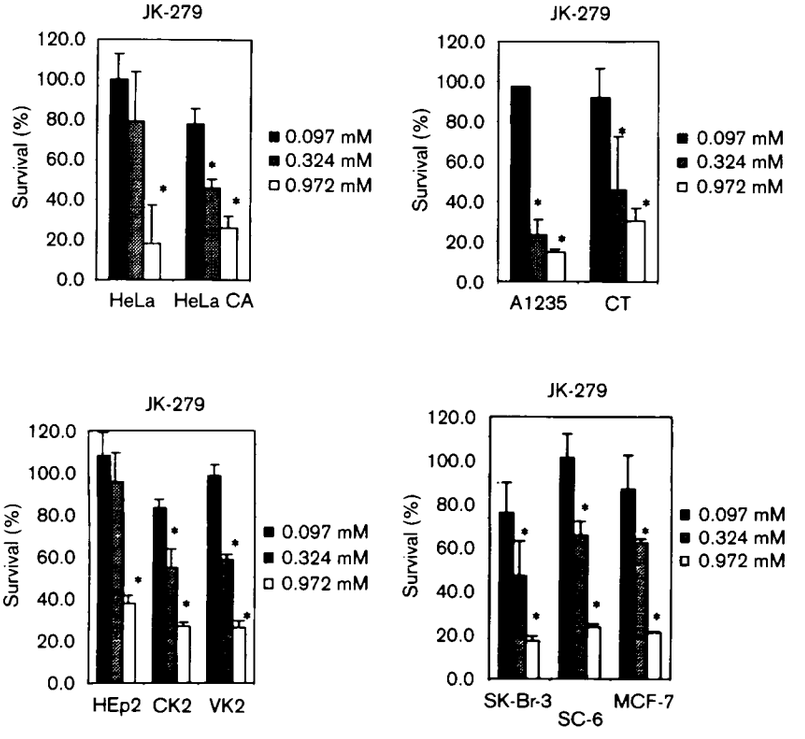

Figure 2. Survival of 10 cell lines following 48 h incubation with diazene JK-279. Pooled data from three experiments (mean ± SD). *Statistically different from control.

Results

The cytotoxicity of five concentrations of each diazene was determined in a 48 h MTT assay. The results are presented in Figures 2–5. Solutions of all diazenes at dilutions of 1:1000 and 1:3000 were not toxic for any of the cell lines, and are not shown in the figures. The solvent diluted in the growth medium according to the same protocol did not demonstrate any toxicity.

As shown in Figure 2, diazene JK-279 reduced the cell survival of all 10 cell lines examined. Given at the highest achievable concentration (0.972 mM), it reduced the cell survival to about 20% (only in HEp2 cells to about 40%). The lower concentrations were less effective in cell killing.

Diazene JK-835 reduced the cell survival to a smaller extent as compared to JK-279 (Figure 3) and diazene LV-57 even less, and that only in two cell lines (Figure 4).

Diazene JK-429 was cytotoxic for HeLa cells, diazene JK-913 for CK2 cells, and diazene MG-19 for HeLa and CK2 cells (Figure 5). The remaining diazenes, JK-802, JK-925, LV-35 and VZ-19, were not toxic to any cell line (data not shown).

Discussion

In order to improve the effectiveness of cancer treatment, new anticancer drugs have been synthesized. In the present study we examined 10 diazenecarboxamide (diazene) compounds that would be expected, according to their structure and biochemical properties, to reduce the survival of tumor cells. It should be kept in mind, as mentioned in Materials and methods, that solubility of these compounds was markedly dissimilar with the consequent difference in maximal concentrations studied. Therefore the potentially stronger pharmacodynamic activity of some compounds cannot be excluded in the case of improvement of its solubility.

As shown in Results, diazene JK-279 was the most active of all examined diazenes in reducing cell survival. Further, it was cytotoxic to all cell lines used in this study (Figure 2). Compared to JK-279, JK-835 reduced cell survival to a lower extent. Even less active were LV-57, JK-429, JK-913 and MG-19, and that only towards some of the cell lines.

The differences in the biological activity of diazenecarboxamides do not follow their oxidative

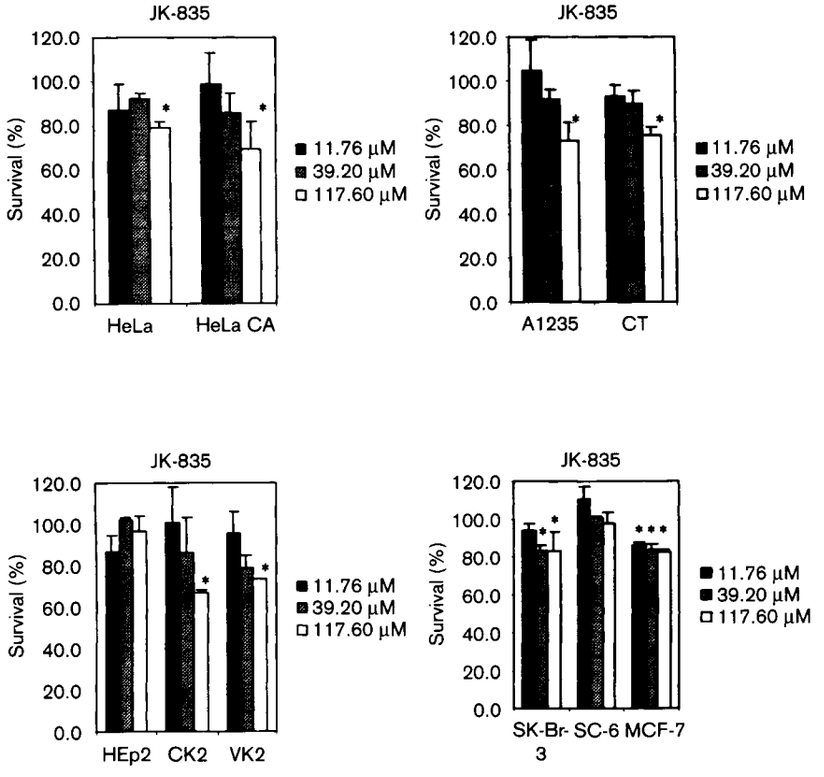

Figure 3. Survival of 10 cell lines following 48 h incubation with diazene JK-835. Pooled data from three experiments (mean ± SD). *Statistically different from control.

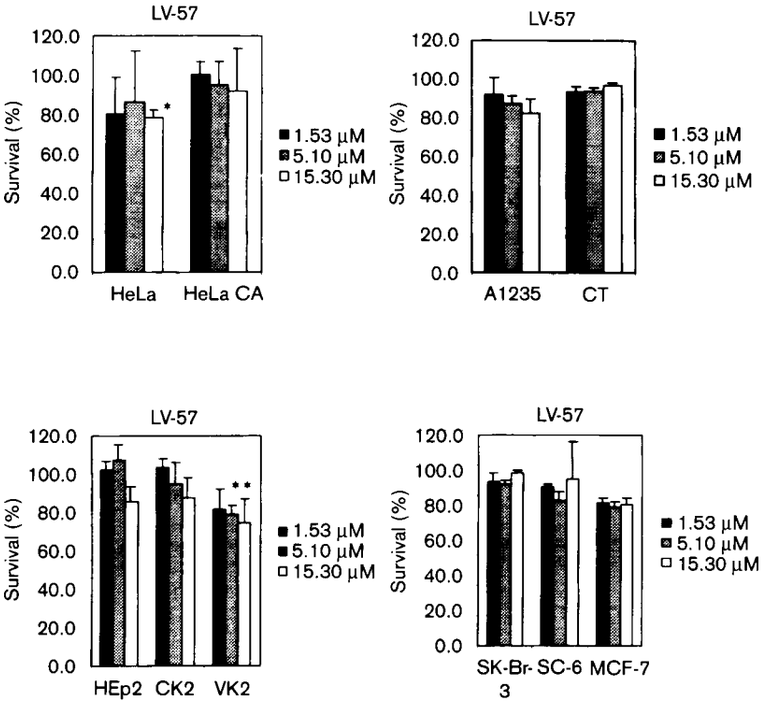

Figure 4. Survival of 10 cell lines following 48 h incubation with diazene LV-57. Pooled data from three experiments (mean ± SD). *Statistically different from control.

efficiency towards thiols and therefore may not be evaluated taking into account their cathodic peak potentials.[24] Comparing heterocyclic moieties, attached to the double bond N=N of the diazenecarboxamides, one can find the connection between the basicity of the heterocyclic ring and the biological activity of the particular compound. Namely, the basicity of the pyridine ring in JK-279 is higher than those of the pyridazine in JK-429,[25] imidazo[1,2-*b*]pyridazine in LV-35,[26] 1,2,4-triazolo[4,3-*b*]pyridazine in MG-19[27] and tetrazolo[1,5-*b*]pyridazine in LV-57.[28] The most basic pyridine ring, present only in JK-279, is expected to be more effective for hydrogen bond formation than other heterocycles. Furthermore, diazenecarboxamides JK-802, JK-835, JK-913, JK-925 and VZ-19 do not possess any basic nitrogen. Stronger interactions, due to hydrogen bond fromation with several important targets in the cells, may be responsible for the higher activity of JK-279 in comparison with other diazenecarboxamides.

The drug resistance of tumor cells is the major obstacle for successful treatment of cancer patients with chemotherapy. Therefore, in our study we also examined the cytotoxic effect of diazenes on drug-resistant cells and compared it with that obtained with parental cells.

The most effective diazene JK-279 was more cytotoxic to cisplatin-resistant HeLa CA cells than parental cervical carcinoma cells (Figure 2). The same was true for cisplatin (CK2)- and vincristine (VK2)-resistant laryngeal carcinoma cells. JK-279 was similarly active in breast adenocarcinoma cells and its doxorubicin-resistant SC-6 cell line, but slightly more effective in parental glioblastoma cells as compared to the cisplatin-resistant cell line (CT). JK-835 exerted a similar effect on parental and cisplatin-resistant cervical carcinoma and glioblastoma cells (Figure 3). It reduced the survival of cisplatin- and vincristine-resistant laryngeal carcinoma cells to a greater extent and in doxorubicin-resistant breast adenocarcinoma cells to a lower extent in comparison to parental cells. LV-57 reduced the survival only in vincristine-resistant laryngeal carcinoma cells (Figure 4) and MG-19 in cisplatin-resistant laryngeal carcinoma cells (Figure 5). JK-429 and MG-19 were cytotoxic only for parental and not cisplatin-resistant cervical carcinoma cells. These data suggest that some of the examined diazenes, but primarily JK-279, could also be active against drug-resistant tumors.

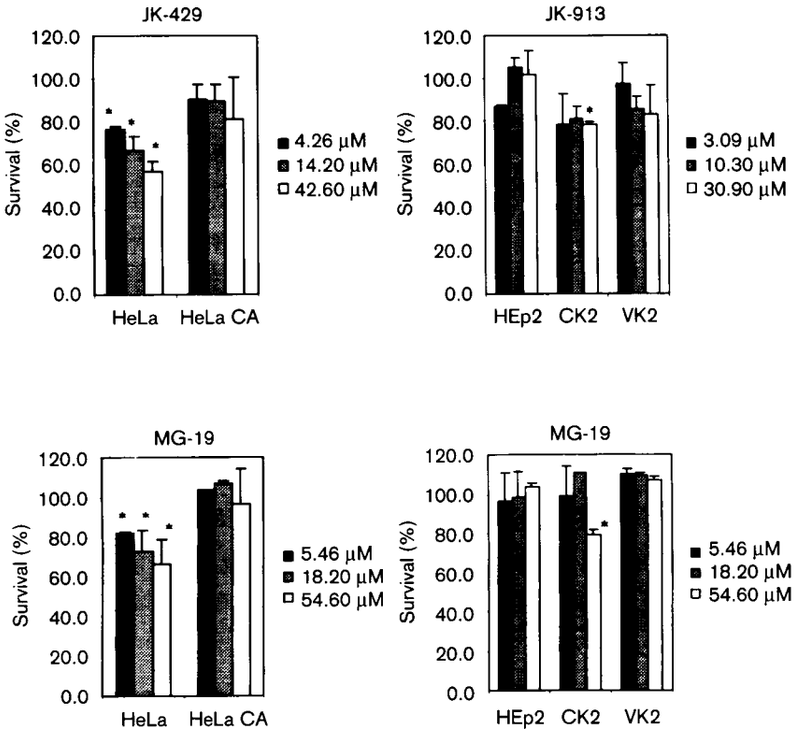

Figure 5. Survival of HeLa and HeLa CA cells treated with diazenes JK-429 and MG-19, and HEp2, CK2 and VK2 cells treated with diazenes JK-913 and MG-19. Pooled data from three experiments (mean ± SD). *Statistically different from control.

Conclusion

Ten new diazenes have been synthesized. Their solubility was markedly dissimilar with the consequent difference in maximal achieved (and used) concentrations. Cytotoxic activity of diazenes was examined on 10 human tumor parental and drug-resistant cell lines. From all diazenes, JK-279 was the most active in reducing the cell survival, with the effect on all examined cell lines. It is important to emphasize that this diazene also reduced the survival of drug-resistant cells. Other diazenes were less toxic or non-toxic at all. The cell killing induced by JK-279 can be explained by its chemical structure. The most basic pyridine ring (only in JK-279) could be more effective for hydrogen bond formation than other heterocycles and therefore more active in reducing the survival of tumor cells. The data obtained with JK-279 encourage further studies.

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