Cytotoxic effect of a new 1,3,4-thiadiazolium mesoionic compound (MI-D) on cell lines of human melanoma

A Senff-Ribeiro1, A Echevarria2, EF Silva2, CRC Franco3, SS Veiga3 and MBM Oliveira*1

1Department of Biochemistry and Molecular Biology, Federal University of Parana, Curitiba, PR, Brazil; 2Department of Chemistry, Rural Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil; 3Department of Cellular Biology, Federal University of Parana, Curitiba, PR, Brazil

The structural characteristics of mesoionic compounds, which contain distinct regions of positive and negative charges associated with a poly-heteroatomic system, enable them to cross cellular membranes and interact strongly with biomolecules. Potential biological applications have been described for mesoionic compounds. 1,3,4-Thiadiazolium mesoionic compound (MI-D), a new mesoionic compound, has been demonstrated to be extremely cytotoxic to B16-F10 murine melanoma cells when compared to fotemustine and dacarbazine, drugs of reference in melanoma treatment protocols, describing inhibition of tumours grown in vitro and in vivo. We now evaluate the effects of mesoionic compound MI-D on different human melanoma cell lines. The drug decreased the viability and proliferation of MEL-85, SK-MEL, A2058 and MEWO cell lines in vitro, showing a considerable cytotoxic activity on these human cells. Adhesion of MEL-85 cells was evaluated in the presence of the drug using different extracellular matrix (ECM) constituents. MI-D decreased MEL-85 adhesion to laminin, fibronectin and matrigel. The morphology and actin cytoskeleton organisation of MEL-85 cells were also modified on MI-D treatment. These results on human melanoma cell lines indicate that MI-D is a very encouraging drug against melanoma, a tumour that is extremely resistant to chemotherapy.

British Journal of Cancer (2004) 91, 297–304. doi:10.1038/sj.bjc.6601946 www.bjcancer.com
Published online 15 June 2004
© 2004 Cancer Research UK

Keywords: mesoionic compounds; MI-D; antimelanoma activity; human melanoma cell lines
Antimelanoma effect of MI-D
A Senff-Ribeiro et al

Materials and Methods

Materials
MI-D (4-phenyl-5-(4-nitro-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride (MI-D)) was synthesised in the Department of Chemistry of the Rural Federal University of Rio de Janeiro, Brazil, as described elsewhere (Grynberg et al, 1997). Its structure was confirmed by $^1$H- and $^{13}$C-NMR spectroscopy and mass spectrometry. Dulbecco’s modified Eagle’s medium (DMEM), RPMI1640 medium and fetal bovine serum (FBS) were obtained from Cultilab (Campinas, Brazil); penicillin and gentamycin were commercial products of the highest available purity grade.

Drug solutions
MI-D was dissolved in dimethylsulphoxide (DMSO) for its experimental use. In order to minimise solvent interference, several stock solutions of MI-D were prepared so that at the desired final concentrations of MI-D in the assays, the amounts of DMSO were identical and equal to 0.12%.

Cell lines and culture conditions
Human melanoma cell lines used in this study were kindly provided by the Ludwig Institute for Cancer Research (São Paulo, Brazil). Cells were maintained in liquid nitrogen with a low number of passages. After thawing, the cells were grown in monolayer cultures in the appropriate media containing penicillin (100 U ml$^{-1}$) and gentamicin (50 µg ml$^{-1}$). MEL-85 and MEWO cells were cultured in RPMI containing 10% FBS, SK-MEL cells in RPMI containing 15% FBS and A2058 cells in DMEM containing 10% FBS. The cultures were kept at 37°C under a humidified atmosphere plus 5% CO$_2$. Release of cells was performed by a treatment for a few minutes with a 2 mM solution of ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). After being counted, cells were then resuspended in an adequate volume of the respective medium supplemented with FBS and again plated in the presence or absence of MI-D.

Cell viability assay
Viability assays were carried out on 24-well plates (TPP, Trasadingen, Switzerland). Human melanoma cells (5 x 10$^3$ cells well$^{-1}$) were plated and allowed to adhere and grown for 20 h before incubation with the drug. MI-D was added in varying concentrations up to 75 µM. At each determined interval (24 and 48 h), supernatants and cells were harvested, centrifuged, washed with PBS and the viability was measured by the Trypan blue-exclusion assay (Phillips, 1973). Briefly, Trypan blue (0.4% in PBS, pH 7.4) was added to the cell suspension and the number of viable (unstained) and nonviable (stained) cells was counted using a Neubauer Chamber. For control experiments, the appropriate medium contained adequate amounts of vehicle (0.12% of DMSO). Cell viability of controls was normalised to 100%.

Cell proliferation assay
Human melanoma cells (6 x 10$^3$ cells well$^{-1}$) were grown for 16 h on 96-well plates (TPP) containing an appropriate medium supplemented with an adequate amount of FBS. The medium was then replaced by a serum-free one. After 24 h, this was replaced with the respective medium containing the usual concentrations of FBS plus MI-D, at different concentrations (2.5 – 25 µM) in quadruplicate. Controls consisted of the respective medium alone or in the presence of 0.12% DMSO, which was the MI-D solvent. After 24, 48 and 72 h the number of cells in each well was determined using the MTT method (Mosmann, 1983). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was dissolved in Hanks’ balanced saline solution (HBSS) at 5 mg ml$^{-1}$. At the above intervals, 20 µl of the MTT solution were added to each well and the plates were incubated at 37°C for 3 h. The MTT solution was removed and DMSO was added and mixed thoroughly to dissolve the dark-blue crystals. The plates were then read using a Microelisa Reader (Bio-Rad, Madison, USA) at 550 nm for sample and 655 nm for reference. Results are expressed as the cell number, which was determined using a standard curve of cells against absorbance.

Cell adhesion assay
Native fibronectin was purified from fresh human plasma according to the procedure of Engvall and Ruuslahti (1977). Laminin and matrigel, a reconstituted basement membrane, were purified from a Engelbreth–Holm–Swarm (EHS) tumour as described by Paulsson et al (1987) and Kleinman et al (1986), respectively. The presence and purity of proteins were assessed by both an electrophoretic analysis on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot. Adhesion assays were performed as described by Saiki et al (1989) with some modifications. Briefly, MEL-85 cells (4 x 10$^4$) with or without MI-D (0.5, 1.0, 2.5 and 5.0 µM) were added to microculture wells (96-well plates, TPP) precoated with fibronectin, laminin or matrigel (10 µg ml$^{-1}$) and blocked with bovine serum albumin (BSA) 1%. The cultures were incubated at 37°C for...
2 h. The wells were washed twice with warm PBS to remove unattached cells, and then the attached cells were fixed with methanol and stained with 0.8% crystal violet dissolved in 20% ethanol. After extensive washing with PBS, the dye was eluted with 50% ethanol in 0.05 M sodium citrate and the absorbance was measured at 550 nm using a MicroElisa Reader (BioRad). At the end of the experiment, the plates were photographed (Leica-MPS30) using an inverted microscope (Leica-DMIL). The control contained the same amount of DMSO as the tested wells (0.12% of DMSO) and its cellular adhesion was normalised to 100%.

Cell morphology analysis
MEL-85 cells were cultured on glass coverslides for 16 h at 37°C under a humidified atmosphere plus 5% CO₂. Cells were treated with 25 and 50 μM MI-D for 2 h. After treatment, they were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 30 min at 4°C. For the control, the appropriate medium contained adequate amounts of vehicle (0.12% of DMSO). Coverslides were stained by Giemsa and studied by bright-field microscopy. A morphometric analysis was performed using an ImageTool software 3.00 version (University of Texas Health Science Center in San Antonio, USA). Images were acquired ( × 400) in grayscale using a software Image-Plus 4.0 version (Media Cybernetics, Silver Spring, USA). A morphometric analysis was performed using an ImageTool software 3.00 version (University of Texas Health Science Center in San Antonio, USA). Cell features, such as area, elongation and roundness of about 400 cells were analysed. For analysis, the area of the object, measured as the number of pixels in the polygon was converted to percent. Elongation was measured as the ratio of the length of the major axis to the length of the minor axis. The result was a value of between 0 and 1 that was converted to % for analysis to obtain an analytical value. Roundness was computed as: (4π × area)/perimeter². The result gave a value of between 0 and 1 that was converted to % for analysis.

Immunofluorescence microscopy
MEL-85 cells (5 × 10⁴) were plated on glass coverslides (13 mm diameter), which were prepared for confocal immunofluorescence microscopy. After 16 h, MEL-85 cells were incubated with 50 μM MI-D for 2 h at 37°C under a humidified atmosphere plus 5% CO₂. In the control, the appropriate medium contained adequate amounts of vehicle (0.12% of DMSO). Cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 30 min at 4°C. Cells on coverslides were incubated with 0.1 M glycine for 2 min, washed with PBS and then blocked with PBS containing 1% goat serum. For the control, the appropriate medium contained adequate amounts of vehicle (0.12% of DMSO). Coverslides were stained by Giemsa and studied by bright-field microscopy. After washing thrice with PBS, coverslides were incubated with phalloidine conjugated with fluorescein isothiocyanate (FITC) (Molecular Probes) diluted in PBS (1:200) for 20 min. After washing with PBS (10 × ) and once in water, slides were mounted with Fluormont-G. Cells were observed using a confocal fluorescence microscope (Confocal Radiance 2100, Bio-Rad) coupled to a Nikon Eclipse 800 with plan apochromatic objectives (Science and Technologies Group Instruments Division, Melville, USA). Images were acquired ( × 600) using Radiance 2100 (Bio-Rad).

Statistical analysis
Statistical analysis of data were carried out using analysis of variance (ANOVA) and the Tukey test for average comparison. Mean ± s.d. values were used. Significance was defined as P<0.05.

RESULTS
Effect of MI-D on cell viability
Figure 2 shows the effect of the mesionic compound on the viability of human melanoma cell lines evaluated 24 and 48 h after treatment with different concentrations of MI-D (2.5–75 μM). Time- and dose-dependent effects were shown by MI-D on the four cell lines studied.

The viability of MEL-85 cells was reduced to 56% after a 24 h treatment with 10 μM MI-D (Figure 2A). At this time of incubation, 75 μM MI-D diminished the viability by ~82%. When a treatment of 48 h was used, the maximum cytotoxic effect (100%) was

![Figure 2](image-url)

Figure 2 Effects of MI-D on the viability of different human melanoma cell lines. (A) Effects of MI-D on MEL-85 cells. (B) Effects of MI-D on SK-MEL cells. (C) Effects of MI-D on A2058 cells. (D) Effects of MI-D on MEWO cells. The viability of melanoma cells was measured at indicated intervals and concentrations of MI-D using the trypan blue-exclusion assay (n = 4). Values given are the mean ± s.d. *P<0.001.
reached starting from 50 μM. A measure of 5 μM MI-D reduced the number of viable cells to 27% after 48 h incubation.

SK-MEL cells also had their viability diminished by MI-D treatment (Figure 2B). After 24 h, 25 μM MI-D reduced the viability to ~30% and at 50 μM to 17%. When the concentration was raised to 75 μM, there were no viable cells after 24 h incubation. A volume of 10 μM MI-D reduced the viability to 30% after 48 h. At this time, there was more than 90% of cell death using 50 μM MI-D.

The viability of A2058 cells was decreased to ~30% when the concentration of MI-D was 10 μM and the incubation time was 24 h (Figure 2C). At this time, the concentrations of 25 and 50 μM reduced the number of viable cells to 12 and 7%, respectively. When a 48 h incubation was assayed, 10 μM MI-D reduced the viability to 20% and 25 μM almost gave a maximum cytotoxic effect (97%). At this time, all A2058 cells were dead when the concentration of MI-D was 50 μM.

MEWO cells were also very sensitive to the toxic effects of MI-D (Figure 2D). After 24 h, MI-D at 10 and 25 μM concentrations, diminished the viability to 23 and 8%, respectively. Increasing the drug concentration to 75 μM resulted in only ~1% of viable cells after 24 h. When a 48 h treatment was performed, 10 μM MI-D reduced the number of viable cells to 13% while 50 μM gave rise to ~100% cell death.

Effect of MI-D on cell growth

Evaluation of the effect of MI-D on the growth of human melanoma cell lines was performed using a growth–time kinetic study of up to 72 h (Figure 3). Proliferation of all melanoma cell lines was greatly inhibited by MI-D, even when subcytotoxic concentrations (1–2.5 μM) were tested.

MEL-85 cells (Figure 3A) treated with 5 μM MI-D gave a 50% reduction of cell growth during the 72 h experiment. At a concentration of 10 μM, the proliferation of MEL-85 cells was completely inhibited.

No growth of SK-MEL cells (Figure 3B) was observed when 5 μM MI-D was used. After 24 h, 1 and 2.5 μM MI-D reduced the number of cells to ~65 and ~55%, respectively.

A2058 cells (Figure 3C) had their growth reduced to 55–61% when the concentration was 2.5 μM. When MI-D was used at 5 μM, there was no increase in cell number up to 48 h. However, at 72 h there was a recovery and the number of cells increased, being 46% of the control.

The proliferation of MEWO cells was inhibited by MI-D (Figure 3D). At a low MI-D dose (2.5 μM) a decrease of cell number of a quarter after 48 and 72 h was observed. The concentration of 5 μM allowed cell growth up to 24 h, but after there was no increase in cell number up to 72 h. In all, 10 μM MI-D did not allow MEWO growth. It must be pointed out that all cell lines, when exposed to MI-D at 10 μM or more, did not grow and extensive cell death was observed, confirming the previous data appearing in Figure 2.

Effect of MI-D on MEL-85 adhesion to laminin, fibronectin and matrigel

Figure 4 shows the effects of MI-D on MEL-85 adhesion to extracellular matrix (ECM) constituents, such as laminin, fibronectin and matrigel. Although there were apparent decreases on cell adhesion when 0.5 and 1 μM of MI-D were used, the values were not statistically different from those of the controls. Higher concentrations of MI-D significantly inhibited MEL-85 adhesion to ECM molecules in a concentration-dependent manner. Adhesion to laminin and fibronectin was decreased to 70 and 55% by 2.5 and 5 μM of MI-D, respectively, after 2 h exposure (Figures 4A and B). At 5 μM, MI-D diminished MEL-85 adhesion to laminin to ~65%.
(Figure 4A) and to fibronectin and matrigel to 34 and 71%, respectively (Figure 4B and C). The micrographs of the adhesion on fibronectin (Figure 4D) show that MI-D not only reduced the number of cells adhering to fibronectin, but also altered the morphology of MEL-85 cells, which shrunk and became round. These changes were also observed when cells were assayed for their adhesion on laminin and matrigel (data not shown).

**Effect of MI-D on MEL-85 morphology**

Figure 5 shows micrographs of MEL-85 cells stained by Giemsa. It can be observed with treated cells (Figure 5B and C) that the presence of MI-D gave rise to a disruption of the interactions between cells and substratum. MI-D treated cells are not so spread out as control cells (Figure 5A). The results clearly demonstrate...
significant changes in cell shape, with cells becoming round when compared to those of the control, as was also observed in adhesion assays. The treatment with 25 μM MI-D gave rise to shrinkage of some cells, presenting protrusions of the plasma membrane, which were pinched off to form membrane-enclosed similar apoptotic bodies probably with cytoplasmic and nuclear contents. Intense vacuolisation of cytoplasm was observed with treated cells. The concentration of 50 μM affected the morphology of almost all of the cells. Condensed chromatin masses with intense eosinophilia and pyknotic nuclei were also observed with treated cells. Morphometric analysis permitted quantification of morphological changes and showed a reduction of 35% in the area of cells treated with 50 μM MI-D. This concentration reduced cell elongation to 56% and increased cell roundness by 112%.

Effect of MI-D on MEL-85 cytoskeleton organisation

In order to evaluate if the changes on cell shape were due to alterations on the actin organisation, an experiment using phalloidin-FITC conjugate was performed (Kusano et al., 2000). As can be observed in Figure 6, organisation of actin cytoskeleton was completely disturbed by MI-D treatment. Fluorescence labelling of MEL-85 cells treated with MI-D did not give rise to the overall pattern that was observed in control cells, which showed a considerable organisation of F-actin fibres all over the cell body. MI-D treatment induced a reorganisation of the F-actin cytoskeleton architecture. Cells in the presence of MI-D did not spread and the filamentous structures disassembled or were reorganised to accumulate in the submembranous area. Actin filaments turned into actin granules mainly localised on the edge of MEL-85 cells. These results point to a disruption of interactions between cells and the extracellular matrix.

DISCUSSION

We are now able to show that MI-D has considerable cytotoxic and antiproliferative effects on human melanoma cell lines. These effects seem to be even more aggressive on human melanoma cells than those observed with B16-F10 murine melanoma cells (Senff-Ribeiro et al., 2003, 2004). For example, a dose of 10 μM after 48 h incubation, on B16-F10 melanoma cells diminished their viability to ~80% (Senff-Ribeiro et al., 2003, 2004) and in all the four human melanoma cell lines the viability was lower than 30% under these conditions. Interestingly, MI-D at a low concentration (5 μM), gave rise to effects of a different magnitude on the viabilities of
human melanoma cells. Under this condition, MEL-85 and MEWO cell lines (Figure 2A and D) were more sensitive to MI-D than SK-MEL and A2058 (Figure 2B and C). Inhibitions of different magnitude on the cell growth of different human melanoma cell lines were also observed. The concentration of 5 μM allowed MEL-85 growth at a lower rate than that of the control (Figure 3A), but completely inhibited the growth of SK-MEL cells (Figure 3B). The results on viability and proliferation are compatible. As one can observe in Figures 2 and 3, MI-D was very cytotoxic to human melanoma cells and had also an antiproliferative effect. The antiproliferative effect of MI-D was present at both noncytotoxic and cytotoxic doses. The effects of MI-D on the viability and proliferation of the various human melanoma cell lines show MI-D to be a toxic drug for human melanoma cells.

Since tumour cell adhesion to ECM constituents is a fundamental step in tumour development and invasion (Mishima et al., 1998), we investigated the effect of MI-D on MEL-85 melanoma cells adhesion to laminin, fibronectin and matrigel. Low MI-D doses were used in order to minimise its extensive metabolic effects, so that the drug was used at subcytotoxic concentration to evaluate MEL-85 adhesion to ECM. Overall, 5 μM MI-D caused a slight decrease of ~5% on MEL-85 viability after 2 h. Under such an experimental condition, MI-D was able to inhibit cell adhesion when it was incubated with the cells for 2 h. Analysing the images of experiments (Figure 4D), we can observe that MI-D treatment promoted loss of cell spreading, which became round, suggesting a possible activity of MI-D on cytoskeleton organisation. These observations were confirmed by morphological and F-actin analyses. Loss of overall cell shape accompanied by cell shrinkage, membrane blebbing and chromatin condensation, besides disturbance of the cytoskeleton organisation, are often observed on apoptotic cells (Hengartner, 2000). The effects of MI-D on MEL-85 adhesion on ECM and morphology may be related to its cytotoxic activity. Although the four different cell lines showed slightly different sensitivities to MI-D, all of them responded to MI-D in a very similar way, so that the MEL-85 results on adhesion, morphology and cytoskeleton organisation probably represent an action of MI-D on human melanoma cell lines.

MI-D is an uncoupler of mitochondrial phosphorylation (Cadena et al., 1998), whose effects on membranes have been recognised (Cadena et al., 2002). Touching on this question, MI-D is an inhibitory uncoupler (Cadena et al., 1998) and according to Skulachev (1998) and others (Colombo et al., 2001), in some instances, an uncoupling action could explain the therapeutic effect of a drug. This seems true for the antitumour action of carbonylcyanide-m-chlorophenylhydrazone (CCCP) (Newell and Tannock, 1989) and other anticancer drugs (Keller et al., 1992). The induction of apoptosis could be a consequence of the cytotoxic activity of MI-D, as it is already known that drugs, which affect mitochondrial metabolism, might facilitate the induction of cell death and overcome apoptosis resistance in cancer cells (Susin et al., 1998; Colombo et al., 2001). Moreover, since membrane proteins are highly dependent on membrane organisation and fluidity (Lopez and Kosk-Kosika, 1997), the effects of MI-D on membranes could disturb recognition sites, which are necessary for adhesion.

In conclusion, MI-D is a potent drug against human melanoma cells and this is the first study on its effects against these cells, as well as for a mesoionic compound of the 1,3,4-thiadiazolium class.
Besides its cytotoxic properties, MI-D also interferes in cell features involved in tumour development, such as adhesion to ECM components. These results, combined with previous data (Senff-Ribeiro et al., 2003, 2004) that showed MI-D to be a potent drug against B16-F10 murine melanoma both in vitro and in vivo, emphasise that MI-D could be a promising drug for the treatment and study of melanoma.

ACKNOWLEDGEMENTS

We thank Professor Dr RR Brentani, Ludwig Institute for Cancer Research (São Paulo, SP, Brazil), for providing the human melanoma cell lines used in our study. This study was supported by PRONEX, CAPES, FUNDAÇÃO ARACUÁRIA and CNPq.

REFERENCES

Badachikar SV, Tikare RK, Puranik GS (1986) Synthesis, reactions and biological activity of 3-[(p-[(N-methyl(ethyl-N-phenylcarbamoyl)] phenyl) syndiones. Indian J Chem 25B: 1079–1080

Cadena SMSC, Carnieri EGS, Echevarria A, Oliveira MBM (1998) Effect of MI-D, a new mesionic compound, on energy-linked functions of rat liver mitochondria. FEBS Lett 440: 46–50

Cadena SMSC, Carnieri EGS, Echevarria A, Oliveira MBM (2002) Interference of MI-D, a new mesionic compound, on artificial and native membranes. Cell Biochem Funct 20: 31–37

Cheung KK, Echevarria A, Galembek S, Maciel MAM, Miller J, Rumjaneck VM, Simas AM (1992) Mesionic compounds 3. Structure of the hydrochloride of 5-(4-methoxyphenyl)-1,3,4-thiadiazolium-2-phenylamine. Acta Crystallogr C 48: 1471–1474

Colombo P, Gunnarsson K, Iatroupoulos M, Brughera M (2001) Toxicological testing of cytotoxic drugs (Review). Int J Oncol 19: 1021–1028

Corell T, Pedersen SB, Lissau B, Moilanen E, Mêtsä-Ketela T, Kankaanranta H, Vuorinen P, Vapaatalo H, Rydell E, Andersson R, Marcinkiewicz E, Korbut R, Gryglewski R (1994) Pharmacology of mesionic oxaazatoxazol derivatives in blood, cardiovascular and respiratory systems. Pol J Pharmacol 46: 553–566

Engwall E, Rusoiahi E (1977) Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int J Cancer 20: 1–5

Grynberg N, Gomes RM, Echevarria A, Miller J (1992) Some new aryl sydnones: effects on murine tumors. Anticancer Res 12: 1025–1028

Grynberg N, Santos AC, Echevarria A (1997) Synthesis and in vivo antitumor activity of new heterocycles derivatives of 1,3,4-thiadiazolium-2-amidine class. Anti-Cancer Drugs 8: 88–91

Hengartner MO (2000) The biochemistry of apoptosis. Nature 407: 770–776

Hogg N, Darley-Usmar VM, Wilson MT, Moncada S (1992) Production of vascular endothelial growth factor in ischemic tissues. Br J Pharmacol 102: 281: 419–424

Kankaanranta H, Rydell E, Peterson AS, Holm P, Moilanen E, Corell T, Karup G, Vuorinen P, Pedersen SB, Wennmalm A, Mêta-S-Ketela T (1996) Nitric oxide-donating properties of mesionic 3-aryl substituted oxaazatoxazole-5-imine derivatives. Br J Pharmacol 117: 401–406

Keller BJ, Marsans DS, Popp JA, Thurman RG (1992) Several nongenotoxic carcinogens uncouple mitochondrial oxidative phosphorylation. Biochim Biophys Acta 1102: 237–244

Kier LB, Roche EB (1967) Medicinal chemistry of the mesionic compounds. J Pharm Sci 56: 148–169

Kleinman HK, McGarvey ML, Hassel JR, Star VL, Cannon FB, Laurie GW, Martin GR (1986) Basement membrane complexes with biological activity. Biochemistry 25(2): 312–318

Kusano Y, Oguri K, Nagayasu Y, Munesue S, Ishihara M, Saiki I, Yonekura H, Yamamoto H, Okayama M (2000) Participation of syndecan 2 in the induction of stress fiber formation in cooperation with integrin a5f1: Structural characteristics of heparan sulfate chains with avidity to COOH-terminal heparin-binding domain of fibronectin. Exp Cell Res 256: 434–444

Lopez MM, Kook-Kosika P (1997) Entropy-driven interactions of anesthetics with membrane proteins. Biochemistry 36: 8864–8872

Majid PA, DeFeyter PJF, Van der Wall EE, Wardeh R, Ross JP (1980) Molsidomine in the treatment of patients with angina pectoris. N Engl J Med 302: 1–6

Mishima T, Murata J, Toyoshima M, Fujii H, Nakajima M, Hayashi T, Kato T, Saiki I (1998) Inhibition of tumor invasion and metastasis by calcium spirulan (Ca-SP), a novel sulfated polysaccharide derived from blue-green alga, Spirulina platensis. Clin Exp Metastas 15: 541–550

Montanari CA, Beezer AE, Sandall JP, Montanari MLC, Miller J, Giesbrecht AM (1992) On the interaction of some mesionic compounds with Saccharomyces cerevisiae by biological microcalorimetry. Rev Microbiol 23: 274–278

Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63

Moustafa MAA, Eisna HM (1991) Synthesis and antimicrobial activity of 3-[(substituted-phenyl)-sydnones. Arch Pharmacol 325: 397–401

Newell KJ, Tannock IF (1989) Reduction of intracellular pH as a possible mechanism for killing cells in acidic regions of solid tumors: effects of carbonylcyanide-3-chlorophenylhydrazone. Cancer Res 49: 4477–4482

Newton CG, Ramsden CA (1982) Meso-iond heterocycles. Tetrahedron 38: 891–901

Ollis WD, Ramsden CA (1976) Meso-iond compounds. Adv Heterocycl Chem 19: 1–121

Paulusson M, Aumailey M, Deutzmann R, Timpl R, Beck R (1987) Laminin—nidogen complex: extraction with chelating agents and structural characterization. Eur J Biochem 166: 11–19

Phillips HI (1973) Dye exclusions tests for cell viability. In Tissue Culture, Methods and Applications, Kruse JR, Patterson JR (eds) pp 406–408, New York: Academic Press

Rehse K, Ciborski T, Müller B (1995) Platelet aggregation inhibiting and anticoagulant effects of oligaalone. XXVII: inhibition of leucocyte adherence to endothelium by oligaalone RE 1492C and the NO-donor RP 2047. Arch Pharm (Weinheim) 328: 125–126

Rehse K, Konig P (1995) New NO-donors with antiatherothombotic and vasodilating activities. XII. Mesionic oxaazatiazole and related monocyclic nitrosohydrazine derivatives. Arch Pharmacol 328: 137–142

Rudolph W, Derschinger J (1991) Clinical comparison of nitrates and sydnones. Eur Heart J 12: 33–41

Saiki I, Iida J, Murata J, Ogawa R, Nishi N, Sugimura K, Tokura S, Azuma I (1989) Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequences of cell-adhesive molecules. Cancer Res 49: 3815–3822

Satyanarayana K, Rao MNA (1995) Synthesis and anti-inflammatory, analgesics and antipyretic testing of 4-[1-oxo-(3-substituted aryl)-2-propyl]-3-phenylsydnones and of 3-[4-3-(substituted aryl)-1-oxo-2-propyl][phenyl]sydnones. J Pharmacol Sci 84: 263–266

Senff-Ribeiro A, Echevarria A, Silva EF, Veiga SS, Oliveira MBM (2003) Effect of a new 1,3,4-thiadiazolium mesionic compound (MI-D) on B16-F10 murine melanoma. Melanoma Res 13(5): 465–472

Senff-Ribeiro A, Echevarria A, Silva EF, Veiga SS, Oliveira MBM (2004) Antimelanoma activity of 1,3,4-thiadiazolium mesoionics: a structure–activity relationship study. Anti-Cancer Drugs 15(3): 269–275

Shinzato TO, Gryglewski R, Gomes RM, Echevarria A, Miller J (1989) Antitumor activity of new mesionic compounds against three murine tumors. Med Sci Res 17: 865–866

Skulachev VP (1998) Uncoupling: new approaches to an old problem of bioenergetics. Biochim Biophys Acta 1363: 100–124

Süsän SA, Zamzami N, Kroemer G (1998) Mitochondria as regulators of apoptosis: doubt no more. Biochim Biophys Acta 1366: 151–165