Antimelanoma activity of 1,3,4-thiadiazolium mesoionics: a structure–activity relationship study

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The effect of a series of 4-phenyl-5-(2′-Y, 4′-X or 4′-X-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides was evaluated against B16-F10 murine melanoma cells *in vitro* and against tumors resulting from implanted B16-F10 cells in C57BL/6 mice. These compounds differ from each other only at the cinnamoyl ring substituent (MI-J, X=OH; MI-2,4diF, X=Y=F; MI-4F, X=F and MI-D, X=NO_2). The results were compared with those obtained for MI-D, which has already been shown to be a potent and promising drug against melanoma. On exposure of B16-F10 cells to MI-D, MI-2,4diF and MI-4F, all of them at the same micromolar concentration (50 μM) decreased the cell viability to 8, 50 and 22%, respectively, while MI-J did not show any significant effect under the same conditions. However, low doses such as 10 μM MI-D were sufficient to impair cell growth over 72 h, but for MI-2,4diF and MI-4F the effect on B16-F10 proliferation was only observed at a concentration of 25 μM. Furthermore, MI-4F had a slightly better effect than MI-2,4diF *in vitro*; its effect on tumor growth *in vivo* was not significant. MI-D inhibited tumor growth by 77%. The greater effectiveness of MI-D compared with MI-2,4diF, MI-4F and MI-J against B16-F10 melanoma cells is probably due to its stronger electron-withdrawing group (NO_2), which increases the positive charge on the mesoionic ring and allows extensive conjugation of the side-chain with the exocyclic moiety. This seems to be important for degree of anti-tumor activity of these compounds. *Anti-Cancer Drugs* 15:269–275 © 2004 Lippincott Williams & Wilkins.

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Keywords: 1,3,4-thiadiazolium mesoionics, anti-melanoma activity, structure–activity study

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Introduction

Mesoionic compounds such as sydnones, sydnonimines, isosydnones and 1,3,4-thiadiazoles are members of a distinctive group of heterocycles, which have received considerable attention and have been extensively studied because of their unique structures, biological activity and pharmaceutical use [1–4]. Mesoionic compounds have an interesting structural feature and can be considered as belonging to a class of mesoionic heterocyclic betaines [5,6]. These are planar five-membered heterocycles with at least one side-chain whose α atom is also in the plane having a dipole moment in the order of 5D [7]. In addition, they possess a five-membered heterocyclic ring associated with a sextet of p and π electrons, giving a positive charge counter-balanced by a formal negative charge on the α atom of the chain, which cannot be represented satisfactorily either by covalent or polar structures (Fig. 1) [5,6,8,9]. The association of these characteristics with the small polyhetero-atomic system suggests a high probability of a strong interaction with biomolecules such as DNA and proteins [10]. Although the molecules are internally charged, they are overall neutral and, therefore, can cross *in vivo* biological membranes [10].

Several biological activities have been described for mesoionic compounds showing their potential pharmaceutical use. Anti-inflammatory, analgesic, anti-bacterial and anti-fungical activities have been demonstrated [1,3,11,12]. Effects such as potent anti-platelet, fibrinolytic, trombolytic, broncholytic [13,14], anti-cancer [15–17] or even on the cardiovascular system [4,18–20] are intimately related with the presence of specific substituent groups in the ring [3,13,17] or to the ability of releasing nitric oxide [21] from their structures.

We have synthesized various salts of mesoionic compounds belonging to the 1,3,4-thiadiazolium-2-aminide class [17,22,23]. Four derivatives, 4-phenyl-5-(4′-X-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chlorides, where X is OH (MI-J), NO_2 (MI-D), F (MI-4F) and 4-phenyl-5-(2-Y-4-X-cinnamoyl)-1,3,4-thiadiazolium-2-phe-

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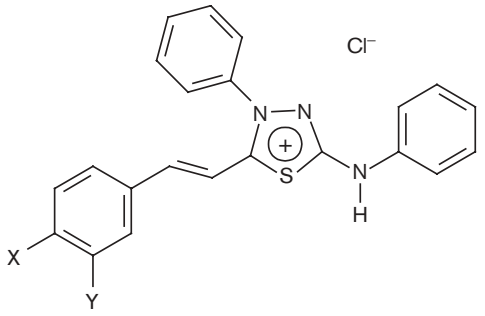
Fig. 1



Chemical structure of various 4-phenyl-5-(2′-Y-4′-X-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride derivatives: MI-D (X = NO_2; Y=H), MI-J (X=OH; Y=H), MI-4F (X=F; Y=H) and MI-2,4diF (X=Y=F).

nylamine chloride, where Y = X = F (MI-2,4diF) (Fig. 1), have been detailed. MI-D and MI-J were able to enhance the survival of Ehrlich carcinoma and Sarcoma-180 tumor-bearing mice, preventing the growth of the tumor, with no significant concomitant alterations of hematological parameters in the test animals, at a dose of 25 mg/kg [17].

Recently, some derivatives of this class were reported as new anti-leishmanial agents, including MI-4F [23]. It is relevant that a number of anti-trypanosome compounds have been found to be effective against experimental tumors, reinforcing that chemotherapeutic agents may modify some common metabolic pathways in different cell models, which replicate rapidly [24,25].

Among these 1,3,4-thiadiazolium mesoionics, MI-D has been the most studied; it was shown to be able to inhibit the respiratory chain between complexes II and III, collapse the transmembrane potential, and stimulate ATPase activity in intact mitochondria [26]. Alterations were also reported in membrane permeability and fluidity, which are related to the MI-D effect on the energy-linked functions of mitochondria [27].

Recent studies in our laboratory showed MI-D to be a potent drug against melanoma [28]. Compared with two other anti-neoplastic agents (fotemustin and dacarbazine), it was effective against the B16-F10 murine melanoma model both *in vitro* and *in vivo*, under the same experimental conditions and concentrations [28]. However, no studies concerning anti-tumor activities have been performed with MI-4F and MI-2,4diF.

We now evaluate the effects of MI-J, MI-2,4diF and MI-4F on B16-F10 mouse melanoma cell in terms of *in vitro* viability and proliferation, and compare the results with those obtained for MI-D. The *in vivo* effect of the mesoionic compounds on B16-F10 tumor-bearing mice (C57BL/6) was also investigated using a single-dose protocol. These data give rise to a structure–activity relationship (SAR) study that can be used as a background for design and synthesis of new molecules.

Materials and methods

Materials

All 1,3,4-thiadiazolium mesoionic compounds were synthesized in the Department of Chemistry of the Rural Federal University of Rio de Janeiro, Brazil, as described elsewhere [17,22,23]. Their structures were confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry. Modified Eagle's medium (MEM) and fetal bovine serum (FBS) were from Cultilab (Campinas, Brazil); penicillin and gentamycin were both purchased from Gibco (Bethesda, MD). All other reagents were commercial products of the highest available purity grade.

Drug solutions

For *in vitro* experiments, mesoionic derivatives were prepared in dimethylsulfoxide (DMSO; purchased from Merck, São Paulo, Brazil). In order to minimize solvent interference in the experiments, several stock solutions of the mesoionic compounds were prepared so that at the desired final concentrations of the drugs in the assays the amount of DMSO was the same and equal to 0.12%. For *in vivo* experiments, mesoionic compounds were dissolved in commercial sunflower oil in order to avoid the toxic effect of DMSO.

Animals

These were C57BL/6 mice (male and female, 8–12 weeks old) from the Central Animal House of Federal University of Paraná, which received a standard laboratory diet (Purina). All recommendations of the national law (no. 6638, 05/11/1979) for Scientific Management of Animals were respected.

Cell line and culture conditions

B16-F10 was kindly provided by the Ludwig Institute for Cancer Research (São Paulo, Brazil). The mouse melanoma cell line was maintained in liquid N_2 at low number of passages. After thawing, they were grown in monolayer cultures in MEM containing 7.5% FBS, penicillin (100 U/ml) and gentamycin (50 μg/ml). 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 EDTA in phosphate-buffered saline (PBS). After being counted, cells were then resuspended in an adequate volume of MEM supplemented with 7.5% FBS and again plated in the presence or absence of the compound under study.

Cell viability assay

Viability assays were carried out in 24-well plates (TPP, Trasadingen, Switzerland), as previously described [28]. Briefly, B16-F10 cells (5×10^5 cell/well) were plated and

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allowed to adhere, and then grown for 20 h before incubation with the drug. Mesoionic compounds were then added in varying concentrations (2.5, 5, 10, 25, 50 and 75 μM). At each time interval (1–72 h), supernatants and cells were harvested, centrifuged, washed with PBS and their viability was measured by the Trypan blue exclusion assay [29]. Briefly, Trypan blue (0.4% in PBS, pH 7.4) (Sigma-Aldrich, St Louis, MO) was added to the cell suspension, and the number of viable (unstained) and non-viable (stained) cells was counted using a Neubauer Chamber. In control experiments, the MEM contained adequate amounts of vehicle: 0.12% DMSO (v/v) at final concentrations. Cell viability of controls was normalized to 100%.

Cell proliferation assay

Cell proliferation assays were performed as previously described [28]. Briefly, B16-F10 cells (5×10^3 cells/well) were grown on 96-well plates (TPP) in MEM containing 7.5% FBS for 16 h. The medium was then replaced by serum-free MEM. After 24 h, this was replaced with MEM and 7.5% FBS containing the mesoionic compound at different concentrations (2.5–25 μM) in quadruplicate. Controls consisted of MEM alone or in the presence of 0.12% DMSO. After 24, 48 and 72 h, the number of cells in each well was determined using the MTT method [30]. MTT (Sigma) was dissolved in HBSS at 5 mg/ml. At the above intervals, 20 μl of MTT solution was 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, WI) at 550 nm for the sample and 655 nm for the reference. Results were expressed as the cell number, which was determined using a standard curve of cells against absorbance.

In vivo tumor growth

B16-F10 cells (5×10^5 cells/animal) were s.c. implanted in C57BL/6 mice so that a tumor developed at the injection site. Animals were i.p. treated with a single dose of mesoionic compound at 57 μmol/kg (in 100 μl), 24 h after cell injection. Control groups received the vehicle (commercial sunflower oil) under the experimental conditions described for the treated group. All mice were kept under observation for the following 17 days and then killed with ether anesthesia for final evaluation. Tumors were excised and their weights were determined. Inhibition of tumor growth was determined as previously described [31,32] and calculated as: Inhibition Ratio (%) = 100 $(A–B)/A$; where A is the average weight of tumors from control animals and B is that of tumors from treated animals.

Statistical analysis

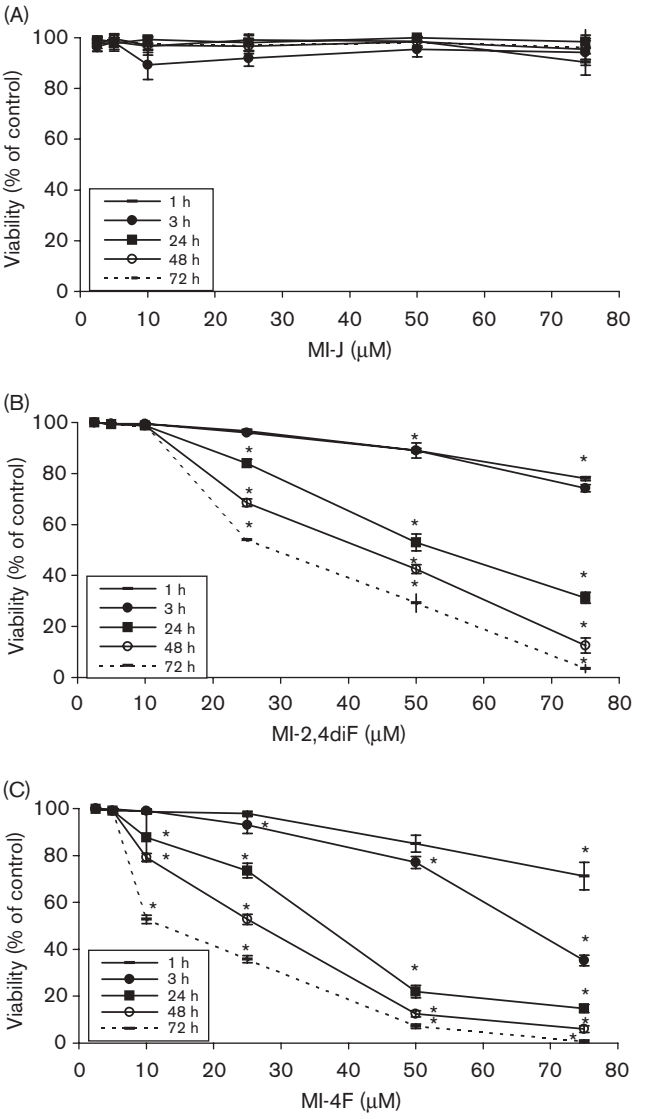
Statistical analysis of the *in vitro* data was carried out using ANOVA and the Tukey test for average comparison. Mean ± SD values were used. Significance was defined as $p < 0.05$. *In vivo* data were analyzed using Student's *t*-test. Mean ± SEM values were used and significance was again defined as $p < 0.05$.

Results

Effects on B16-F10 viability *in vitro*

Figure 2 shows the effect of the mesoionic compounds on B16-F10 viability. MI-J did not alter cell viability at the concentrations tested even after 72 h of treatment (Fig. 2A). The mesoionic compound substituted with two fluor atoms, MI-2,4diF, presented a cytotoxic effect at

Fig. 2



Effect of 1,3,4-thiadiazolium derivatives on the viability of B16-F10 cells. (A) Effect of MI-J. (B) Effect of MI-2,4diF. (C) Effect of MI-4F. The viability of B16-F10 melanoma cells was measured by the Trypan blue exclusion assay at indicated intervals and concentrations of each compound ($n=4$). Results are expressed as mean ± SD values. *$p<0.05$.

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Table 1 B16-F10 viability (% of control) after 24 h of treatment with 1,3,4-thiadiazolium derivatives

Concentration (μM)	Derivative			
	MI-D	MI-J	MI-2,4diF	MI-4F
2.5	97 ± 3.0	98 ± 2.1	100 ± 0.2	100 ± 0.5
5	93 ± 6.0	98 ± 3.2	99 ± 1.0	99 ± 1.0
10	85 ± 5.5[a]	99 ± 1.4	99 ± 1.5	88 ± 10.2
25	20 ± 5.0[a]	98 ± 3.3	84 ± 3.3[a]	74 ± 3.1[a]
50	8 ± 2.5[a]	100 ± 0.5	53 ± 2.2[a]	22 ± 2.7[a]
75	2 ± 1.5[a]	99 ± 2.5	31 ± 2.6[a]	15 ± 1.7[a]

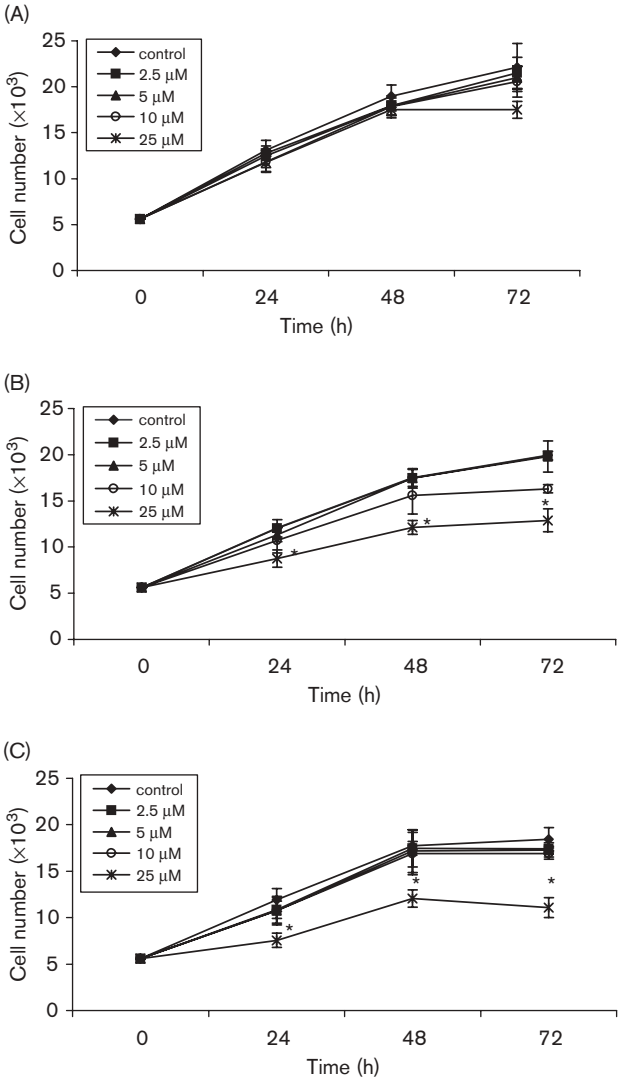
Assays were carried out as described in Materials and methods. Results are expressed as mean ± SD values.
[a] $p < 0.05$.

25 μM after 24 h incubation. This concentration was able to reduce the cell viability to 50% after 72 h (Fig. 2B). A maximum cytotoxic effect was obtained at 75 μM after 72 h of treatment. At concentrations lower than 25 μM, MI-2,4diF did not give rise to any effect on B16-F10 viability. The MI-4F derivative showed a higher effect on B16-F10 viability when compared to MI-2,4diF (Fig. 2C). The viability was reduced to 80% using 10 μM at a 48-h incubation. This concentration resulted in 50% viability after 72 h. A 3-h incubation with 75 μM caused a decrease of viability of 65% and using a 24-h incubation only 15% of cells remained viable. In order to compare the effect of these mesoionic compounds to that exerted by MI-D, different concentrations and a 24-h treatment were used. MI-D at 50 μM lowered the cell viability to only 8% after treatment (Table 1), while MI-J at the same molar concentration had no effect, and MI-2,4diF and MI-4F reduced cell viability to 53 and 22%, respectively. For MI-D, the results were in accordance with those previously obtained for this compound [28] and confirm its accentuated cytotoxicity on the murine melanoma cells. Although the fluorine-substituted mesoionic derivatives showed cytotoxic effects on B16-F10 melanoma cells, none of them gave better results than those obtained for the NO_2-substituted compound (MI-D) (Table 1) and those described before [28].

Effects on B16-F10 proliferation *in vitro*

The effects of the mesoionic compounds on B16-F10 growth are shown in Fig. 3. MI-J did not significantly affect cell growth over 72 h, since treated and non-treated cells grew at the same rate (Fig. 3A). MI-2,4diF affected cell growth only at the higher concentration (25 μM) (Fig. 3B). Incubation with 25 μM with this difluoro derivative was able to reduce the number of cells to around 70% after 24 and 48 h, this value being diminished to 65% after 72 h. MI-4F as MI-2,4diF reduced cell growth only at the higher concentration (25 μM). As was observed in the cell viability assay, MI-4F had a slightly greater effect than MI-2,4diF on melanoma cells on assessment of B16-F10 growth (Fig. 3C). At 24 and 48 h there were around 35% less cells than in the control. At 72 h this increased to

Fig. 3



Effect of 1,3,4-thiadiazolium derivatives on the proliferation of B16-F10 cells. (A) Effect of MI-J. (B) Effect of MI-2,4diF. (C) Effect of MI-4F. The proliferation rate was measured by the MTT method at indicated times and concentrations (2.5–25 μM). $n = 3$. Results are expressed as mean ± SD values. *$p < 0.05$.

40%. The effect of MI-D on B16-F10 proliferation was evaluated using a concentration of 10 μM (Table 2). Cell growth was completely inhibited during the 72 h of the experiment at this concentration, confirming results previously obtained for this compound [28]. As was observed with the viability results, none of the tested mesoionic compounds was shown to be more effective than MI-D in inhibiting cell growth at 10 μM (Table 2) or other concentrations [28].

Effects on an *in vivo* melanoma model

Figure 4 permits comparison of the *in vivo* effects of MI-D, MI-J, MI-2,4diF and MI-4F against B16-F10 melanoma

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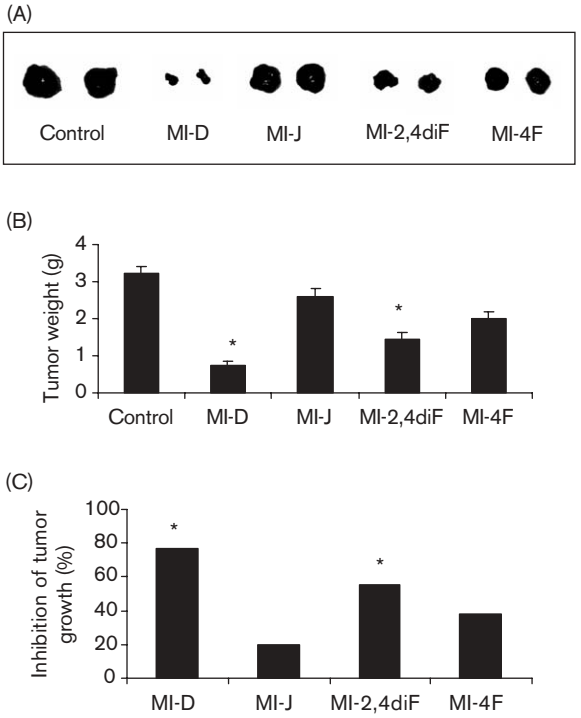
Table 2 B16-F10 growth (% of control) during 1,3,4-thiadiazolium derivatives treatment at 10 μM

Derivative	Time (h)		
	24	48	72
MI-D	0 ± 2.1[a]	0 ± 3.0[a]	1 ± 3.9[a]
MI-J	90 ± 4.6	94 ± 3.5	93 ± 12.9
MI-2,4diF	89 ± 10.5	89 ± 15	82 ± 3.8
MI-4F	91 ± 12.8	95 ± 19	92 ± 5.1

Assays were carried out as described in Materials and methods. Results are expressed as mean ± SD values.
[a]$P < 0.05$.

Fig. 4



Effect of 1,3,4-thiadiazolium derivatives on inhibition of tumor growth. (A) Representative excised tumors from control animals and treated animals. (B) Tumor weight of mesoionic derivative-treated mice. (C) Inhibition (%) of tumor growth. B16-F10 melanoma cells were injected s.c. into C57BL/6 mice, each receiving a single dose of 57 μmol/kg of the drug i.p., 24 h following cell injection. Control groups received the drug vehicle with the same volume as for treated animals. Animals were sacrificed after 17 days and the tumor weight was determined. The inhibition of tumor growth was determined as previously described [31,32]. (MI-D, $n=5$; MI-J, $n=7$; MI-2,4diF, $n=5$; MI-4F, $n=6$). Results are expressed as mean ± SEM values. *$p < 0.05$.

cells growing on C57BL/6 mice, as evaluated as a single i.p. dose of 57 μmol/kg administrated 24 h after cell inoculation. Animals from the control group received the same volume of solvent (sunflower oil) as that used for the drug treatment injection. Figure 4(A) represents tumors excised on the 17th day after treatment with mesoionic compounds. The mean weight of tumors and inhibition of tumor growth are shown in Figure 4(B and C, respectively). The *in vivo* development and growth of B16-F10 melanoma were not modified by the treatment using MI-J. For MI-4F, a reproducible tendency to inhibit *in vivo* tumor growth was observed in spite of not being statistically significant (Fig. 4C). The *in vivo* tumor growth responded to MI-2,4diF and MI-D. The results of *in vivo* assays involving MI-2,4diF showed an inhibition of tumor growth of 55 and 77% for MI-D. This experiment demonstrated that as has been already shown for MI-D [28], MI-2,4diF has a significant anti-tumor activity against melanoma. The results are in accord with those under *in vitro* conditions that pointed to MI-D as being the most active and MI-J as being ineffective.

Discussion

We have studied a series of 1,3,4-thiadiazolium compounds in search of anti-melanoma activity and performed a SAR analysis. The requirement is that for a set of congeneric molecules, which incorporated a small substructural modification with the same pharmacophoric group, it must be capable of disclosing any structure activity relationship [33]. This approach often involves the synthesis of analogs containing a range of substituents on aromatic or heteroaromatic ring or accessible functional groups. Thus, the drug effects obtained using SAR for MI-D, MI-J, MI-2,4diF and MI-4F (Fig. 1) were determined against B16-F10 melanoma cells. It has been recently demonstrated that MI-D is a potent drug against the murine melanoma model B16-F10, showing a better activity than fotemustin and dacarbazine, which are antineoplastics used for treatment of melanoma patients [28]. Compounds that differ from MI-D at the cinnamoyl substituent, such as MI-2,4diF and MI-4F, are shown to have activity against B16-F10 melanoma cells, although none of them are as effective as MI-D.

Comparing the cell viability results, MI-D at a concentration of 50 μM showed a maximum cytotoxic effect (around 100%) after 24 h of treatment (Table 1); MI-2,4diF, under these conditions resulted in 50% of viable cells (Fig. 2B). MI-D showed 100% of the cytotoxic effect with 25 μM after 48 h [28]. At this concentration and time of incubation, MI-2,4diF and MI-4F gave rise to a viability of 70 and 53%, respectively (Fig. 2B and C).

Observing the growth of B16-F10 cells in the presence of MI-2,4diF and MI-4F (Fig. 3), the values obtained for MI-D could not be surpassed (Table 2). Only a concentration of 25 μM was able to inhibit the growth of melanoma cells when the fluorine-substituted compounds were tested (Fig. 3B and C). MI-D at 10 μM did not allow cell growth over the 72 h of the experiment (Table 2); and when it was used at 2.5 and 5 μM, a large inhibition of cell growth was also observed [28].

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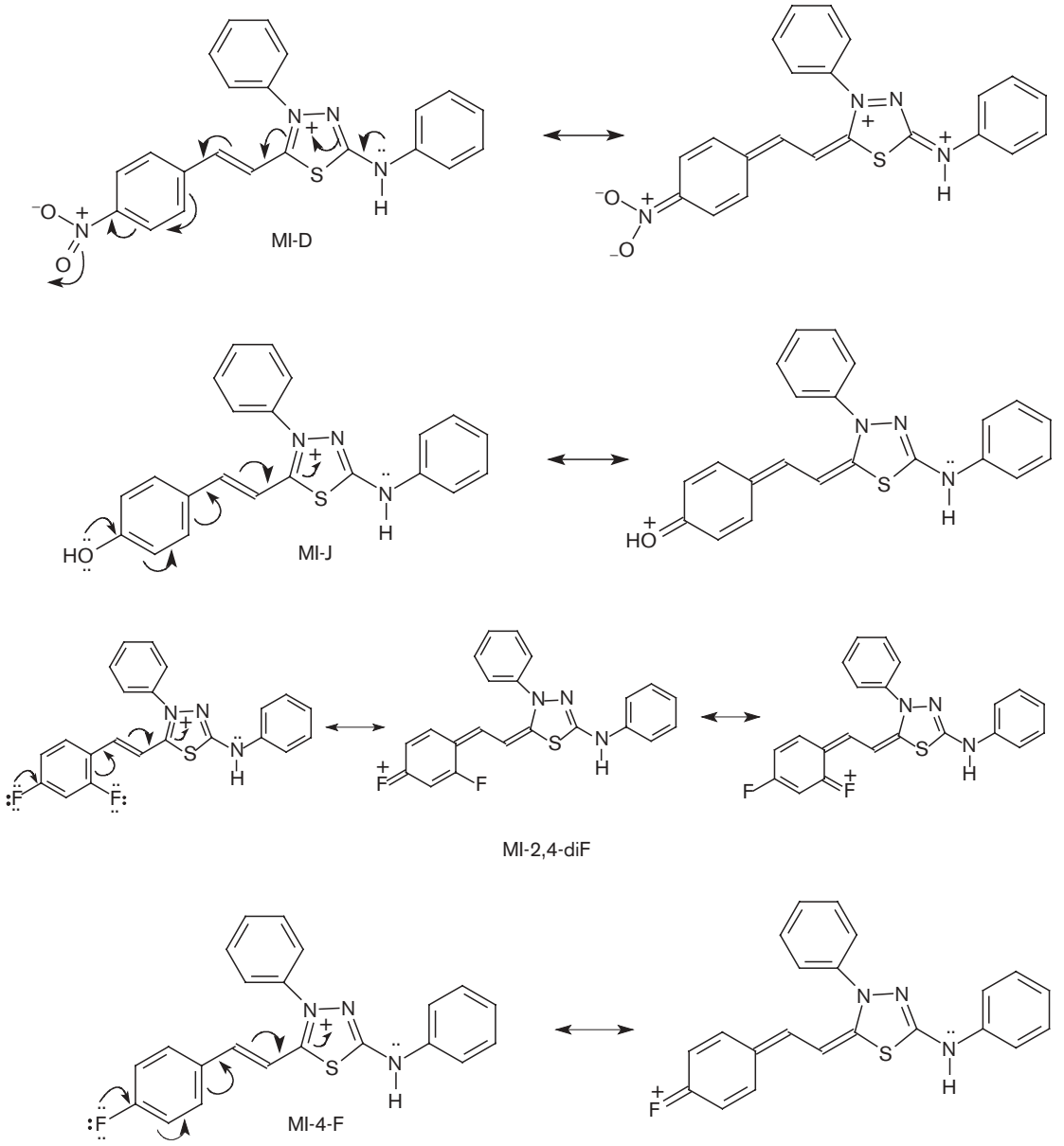
Fig. 5



Resonance structures of derivatives of 1,3,4-thiadiazoles: MI-D, MI-J, MI2,4diF and MI-4F.

When the mesoionic compounds were tested *in vivo*, the *in vitro* effectiveness of MI-D was confirmed. MI-4F had a slightly better effect than MI-2,4diF *in vitro*, but *in vivo* MI-2,4diF was able to inhibit tumor growth and MI-4F did not shown a significant effect (Fig. 4). This result may be due to the effects of the presence of two fluorines that can make a more hydrophobic molecule allowing it to cross membranes easily.

Although the mesoionic compound MI-J has a structure related to that of MI-D, differing only at the cinnamoyl substituent, it did not show any effect on melanoma cells both *in vitro* and *in vivo* under the experimental conditions. The electronic effects influence the charge distribution of the heterocyclic ring, so that when the electronic nature of substituents is considered, MI-J is the only one that has an electron-release group (OH), which reduces the positive charge on the heterocyclic ring (Fig. 5). On the other hand, MI-D, MI-4F and MI-2,4diF, contain electron-withdrawing groups (NO_2 and F) which increase the positive charge on the mesoionic ring (Fig. 5). However, the F substituent gives

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rise to an electron-donor effect by resonance and, since it is an electron-withdrawing group by an inductive effect, these characteristics result in F being a less effective electron-acceptor group when compared to NO_2. Moreover, the combination of the electronic and lipophilic parameters, expressed by Hammet substituent (σ) and Hansh hydrophobicity constants (π) [34], may be required for biological activity versus structure. The most active derivative MI-D has $\sigma_{NO_2} = 0.78$ and $\pi_{NO_2} = -0.28$, whereas MI-J $\sigma_{OH} = -0.37$ and $\pi_{OH} = -0.67$. MI-2,4diF shows $\sigma_{diF} = 0.40$ and $\pi_{diF} = 0.28$, indicating a different combination of these effects.

It was shown for this class of mesoionic compounds that electron-withdrawing groups allow an extensive conjugation of the side-chain with the exocyclic moiety [22]. As the compound substituted with the stronger electron-withdrawing group (MI-D) gave more desirable biological effects, we now suggest that this conjugation is important for anti-tumor activity of these compounds.

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