Scanning Microscopy

Volume 3 | Number 2 Article 30

4-11-1989

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Scanning Microscopy, Vol. 3, No. 2, 1989 (Pages 681-693)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/89$3.00+.00

MORPHOLOGICAL EFFECTS OF LONIDAMINE ON TWO HUMAN-TUMOR CELL CULTURE LINES

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(Received for publication January 25, 1989, and in revised form April 11, 1989)

Abstract

Lonidamine, 1-(2-4-dichlorobenzyl)-1-H-indazol-3-carboxylic acid, is an anticancer drug that has its primary action on cellular metabolism rather than cell division. Since lonidamine is not effective in all tumor cells, we have tested it in two human-tumor cell culture lines: MOLT-4, a T-leukemia and U-87 MG, a glioma. Lonidamine exposure of MOLT-4 cells at 50 μg/mL and pH 6.7 disrupted the mitochondria within 1 h of treatment. The mitochondria were swollen and the cristae were disrupted. When the treated cells were re-incubated in fresh medium at pH 7.4 the mitochondria rapidly returned to their normal morphology. The U-87 MG glioma cells did not show ultrastructural disruption after 1-h treatment with lonidamine at concentrations up to 200 μg/mL at pH 6.7

In the concentration range of 25 μg/mL to 200 μg/mL, lonidamine did not produce any cell killing in MOLT-4 after a 1-h exposure at pH 7.4, although the drug had some limited effectiveness at pH 6.7. Compared to sham-treated controls, long exposures to 100 μg/mL of lonidamine at pH 6.7 reduced survival in MOLT-4 to 92% and 53% after 6-h and 24-h exposures, respectively. Survival of U-87 MG glioma cells was also strongly pH dependent, a 2-h exposure to 50 μg/mL lonidamine at pH 7.4 did not cause cell death; however, survival dropped to 84% of the control at pH 6.65.

Key words: lonidamine, ultrastructure, mitochondria, morphology, hyperthermia, cell killing, MOLT-4, glioma, cancer therapy, tumor cells, U-87 MG.

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Introduction

Lonidamine, 1-(2,4-dichlorobenzyl)-1-H-indazol-3-carboxylic acid, is a novel anticancer drug, which has been undergoing Phase I and Phase II clinical trials by itself (Band et al. 1984; Evans et al. 1984; Gulisano et al. 1987) and in conjunction with radiotherapy (Cripps et al. 1988). It is reported to act by interfering with respiration in the mitochondria rather than as a mitotic poison, like most other anticancer drugs (Caputo and Silvestrini 1984). Lonidamine inhibits aerobic and anaerobic lactate production (Floridi et al. 1981a,b; De Martino et al. 1984), inhibits mitochondrially bound hexokinase (De Martino et al. 1984) and is reported to produce ultrastructural changes in the mitochondria and the plasma membrane (Floridi et al. 1981a,b; De Martino et al. 1984; Arancia et al. 1988; Floridi et al. 1987).

Since lonidamine acts on the cell's metabolism rather than cell division, it has the potential to cause tumor regression in new ways. In addition, since the treated cells are in a weakened state, synergism between lonidamine and ionizing radiation, hyperthermia, or other chemotherapeutic agents is possible (Kim et al. 1984; Starace et al. 1987; Bagnato et al. 1987; Floridi et al. 1987; Hahn et al. 1984; Silvestrini et al. 1983; Kim et al. 1986).

Although effects of lonidamine have been reported in a number of cell systems, it is not effective in all (De Martino et al. 1984). Thus, it is important to test various types of tumor cells to further understand the mode of action of lonidamine. Its effect on two human-tumor cell lines, MOLT-4 and U-87 MG, are reported here.

We have used transmission and scanning electron microscopes to observe the amount of structural damage in MOLT-4 and U-87 MG glioma cells immediately after lonidamine exposure and after a short incubation time. The level of cell killing induced by the drug has also been measured in order to correlate survival with morphological changes. It is important to observe morphological changes after

lonidamine exposure because it may become clinically useful in conjunction with other drugs, heat or radiation. Thus identification of organelles, which are the targets of lonidamine, will offer clues to its mode of action and may suggest further combinations of treatments to expand its use. In addition, different cell types from different tissues may react differently to the drug. Therefore, it is important to build up a body of experience about the morphological and toxicological effects of lonidamine.

The MOLT-4 cell line is derived from a T-cell leukemia and has many interesting properties: it is radiation-sensitive; it has a large number of pycnotic nuclei when it dies, similar to the characteristic apoptotic death seen in circulating lymphocytes; it does not repair sublethal damage; and it does not show synergistic cell killing by heat and radiation (Raaphorst et al. 1983; Szekely and Lobreau 1985). The apoptotic death of MOLT-4 cells allows cell killing to be measured by trypan blue dye exclusion or flow cytometry after radiation or drug treatment (Szekely et al. 1985).

The gliobastoma cells, U-87 MG, are grade III human astrocytoma cells. The morphology and growth characteristics of glioma cell lines have been studied extensively (Collins et al. 1979, 1980). Since human gliomas are difficult to treat and are often malignant (De Ridder et al. 1987), agents such as lonidamine, which may be useful on their own or with radiation or hyperthermia, may offer increased tumor cell killing.

Materials And Methods

Cell Culture

The MOLT-4 cell line was established by Minowada et al. (1972). The cells resemble relatively mature T-lymphocytes; they form rosettes with unsensitized sheep red blood cells, and they carry surface antigens present in normal human T-cells and thymus leukemia (Minowada et al. 1972; Szekely et al. 1987b).

MOLT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. They were maintained in suspension in loosely capped T75 Falcon plastic flasks at 37°C in a 5% CO_2 humidified atmosphere. They were maintained in log-phase growth by subculturing every 3 to 4 days and their doubling time ranged from 24 h to 32 h. They were seeded at 10^5 cells/T75 flask and grew to approximately 2.5 x 10^6 cells/T75 flask at the time of subculture.

The U-87 MG glioma cells grow as a monolayer of epithelia-like cells in DME/F-12 medium with 15% heat-inactivated fetal bovine serum, and 10 mM $NaHCO_3$ and 20 mM HEPES buffering in a 2% CO_2 humidified atmosphere. For glioma cells used in microscopy, the medium was supplemented with glutamine to 1.83 g/L, sodium pyruvate to 0.11 g/L and glucose to 4.5 g/L. Cells were seeded at 10^5 cells/T75 flask and were subcultured weekly by trypsinization. For SEM and TEM the cells were grown to confluence at approximately 3 x 10^6 cells/flask.

Lonidamine Treatment

For the MOLT-4 cells, T25 culture flasks containing 15 mL of medium were placed overnight in incubators containing a humidified CO_2 atmosphere of ~ 25% for pH 6.7 and 5% for pH 7.4 to equilibrate pH and temperature. The pH was measured with a Markson 5650 pH meter. The 5 x 10^6 cells were pelleted from a stock culture and resuspended in the pH equilibrated medium. Lonidamine was added at the test concentration from a stock solution at 10 mg/mL in dimethyl sulfoxide (DMSO). The DMSO was used to increase the solubility of lonidamine and decrease the amount of solvent added to the culture media. The flasks were then returned to their respective incubators.

For the glioma cells, the pH was adjusted by adding HCl to the HEPES buffered medium before the flasks with cells were placed in an incubator containing a 2% CO_2 humidified atmosphere. The pH remained constant during the treatment.

Electron Microscopy

For scanning electron microscopy (SEM), lonidamine-treated MOLT-4 cells were fixed by adding 3 mL of sample to 9 mL of 4% glutaraldehyde in Hank's buffer with 0.04 mol/L sucrose. The fixation time was 30 min. The sample was then rinsed twice in Hank's buffer with 0.04 mol/L sucrose, and it was allowed to settle on poly-L-lysine-coated glass coverslips. Since the U-87 MG cells grow attached to a surface, they were grown and fixed on glass coverslips placed within the flasks. Both types of cells on the coverslips were dehydrated through a graded series of ethanol and then amyl acetate before being critically point-dried with CO_2. Cells were viewed in an ISI microscope operated at 15 kV.

For transmission electron microscopy (TEM), the U-87 MG cells were scraped off the flask. The MOLT-4 or U-87 MG cells were pelleted from the medium, and then fixed and dehydrated in acetone by Hayat's (1972) rapid procedure, using Millonig's phosphate buffer before being embedded in Spurr's epoxy mixture. Gold sections were stained with 2% uranyl acetate and Reynold's lead citrate, and viewed in a Philips EM 300 electron microscope at 80 kV.

Cell Survival

MOLT-4 survival was measured by either flow cytometry, using a method developed for radiation and

glucocorticoid-induced killing, or by trypan blue dye exclusion (Szekely et al. 1985; 1987a). Briefly, for trypan blue staining, one part of 1% trypan blue in distilled water was added to five parts of cells in medium. After a 5 min equilibration period, the cells were scored under an optical microscope and cells that had taken up the stain were scored as dead cells. For flow cytometry a 4 mL sample of MOLT-4 cells in medium was analysed in an Ortho Cytofluorograf 50 H interfaced with a 2150 Data General Micro Nova computer. The flow rate was approximately 200 cells/s. A 0.8 mW He-Ne laser was used as the light source. Axial light-loss and right-angle scatter were collected for analysis. The three-dimensional scattergram generated by plotting the axial light-loss versus the right-angle scatter was used to determine the percentage of live and dead cells. Survival was measured at various times following the Ionidamine exposure. Immediately following the treatment, the MOLT-4 cells were resuspended in fresh medium and kept at pH 7.4 until survival was measured.

The glioma cells were plated in dishes immediately following the drug exposure. Survival was determined by counting the number of colonies produced by the treated cells, as previously described (Raaphorst and Azzam 1981). Briefly, cells were trypsinized after treatment, resuspended in fresh medium counted and plated at a concentration chosen to give approximately 10 to 150 colonies per dish. Colonies were counted at 12 to 14 days after plating. The plating efficiency averaged 27% for the series of experiments. Untreated controls were plated after exposure to media or media plus DMSO at the concentration used in the treatment series. No difference was seen.

Results

MOLT-4 T-lymphocytes were exposed to Ionidamine for 1 h at pH 7.4 and pH 6.7 followed by a 24-h incubation in fresh medium at pH 7.4. Since survival measured by trypan blue staining or flow cytometry gave similar results, the experiments using both methods were pooled.

Table 1 gives the relative survival levels of MOLT-4 cells after 1-h exposure to various concentrations of Ionidamine. Measurements of survival immediately after the exposure (not shown) showed no decrease in survival at either pH or Ionidamine concentration tested. High drug concentrations, up to 200 μg/mL, did not cause very much cell death at 24 h after the exposure. At 200 μg/mL, cell survival averaged 90% in cells treated at pH 6.7. The exposures were carried out at low pH because work by Kim et al. (1984) has shown that in some cell systems Ionidamine is much more effective in cell killing at low pH.

TABLE 1. MOLT-4 survival 24 h after a 1-h Ionidamine exposure

Treatment	% Survival*	Number of experiments
Control pH 7.4	100	7
Control pH 6.7	99 ± 0.3	7
5 μg/mL pH 6.7	95	1
25 μg/mL pH 6.7	93	1
50 μg/mL pH 6.7	97 ± 2.2	7
100 μg/mL pH 6.7	85 ± 13.9	7
200 μg/mL pH 6.7	90 ± 2.0	6

* Normalized to pH 7.4 control

In clinical practice, serum concentrations of 10 μg/mL to 40 μg/mL are found during treatments that last for several days. Since a 1-h exposure did not produce much cell death, longer exposure times were tried. Although longer exposures produced more cell killing, (Tables 2 and 3), there still was a high survival rate. A 24-h exposure at 100 μg/mL gave approximately 50% survival and the percentage of survivors increased to an average of 77% by 24-h posttreatment.

Although a large concentration of Ionidamine and a long exposure time is required to decrease survival in MOLT-4 cells, samples viewed in the transmission electron microscope show morphological damage at doses that do not cause significant cell killing. The cells exposed to 50 μg/mL (Fig. 1) and 200 μg/mL (Fig. 2) for 1 h show disrupted mitochondria. The swollen mitochondria are near the cell surface and, in some cases, they break through the surface. The treated cells have debris on their surface (arrows in Figs. 1 and 2). The cell surface also has a swollen appearance with fewer surface ruffles and more microvilli than the controls. Fig. 3 shows an untreated cell after 1 h in medium at pH 6.7. The mitochondria are well formed with visible cristae. The surface is not as smooth as in the treated cells. When the MOLT-4 cells are treated at pH 7.4 (Fig. 4) there is some slight swelling of the mitochondria (arrow), but most are similar to those seen in the controls.

TABLE 2. MOLT-4 survival after a 6-h lonidamine exposure

Treatment		No posttreatment incubation		24-h incubation	
		% Survival*	Number of experiments	% Survival	Number of experiments
pH 6.7	Control	99 ± 0.6	6	99 ± 1.1	6
	50 μg/mL	98 ± 1.0	6	83 ± 9.6	6
	100 μg/mL	92 ± 3.6	6	72 ± 8.5	6
	150 μg/mL	77 ± 8.1	6	47 ± 12.4	6
pH 7.4	Control	100	6	100	6
	50 μg/mL	100 ± 0.3	3	100	3
	100 μg/mL	99 ± 0.9	3	99 ± 0.6	3
	150 μg/mL	96 ± 2.3	3	89 ± 3.2	3

* Normalized to pH 7.4 control

TABLE 3. MOLT-4 survival after a 24-h lonidamine exposure

Treatment		No posttreatment incubation		24-h incubation	
		% Survival*	Number of experiments	% Survival	Number of experiments
pH 6.7	Control	93 ± 7.0	4	100	2
	25 μg/mL	89 ± 8.7	4	96	2
	50 μg/mL	73 ± 10.3	4	78	2
	100 μg/mL	53 ± 19.7	4	77	2
pH 7.4	Control	100	1	100	2
	25 μg/mL	98	1	100	2
	50 μg/mL	99	1	100	1
	100 μg/mL	100	1	93	1

* Normalized to pH 7.4 control

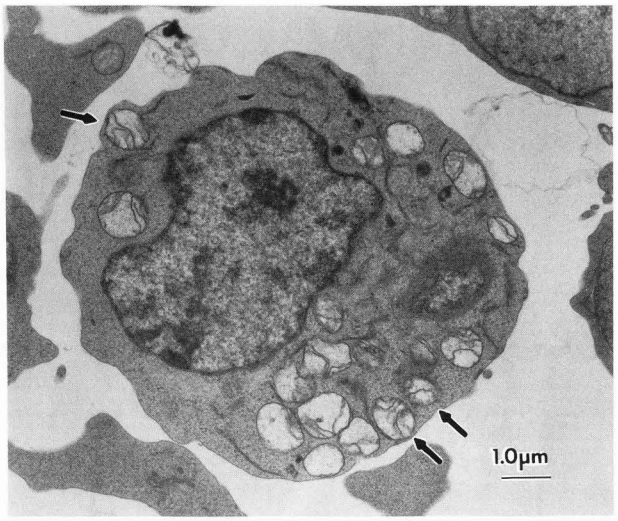


Fig. 1. A transmission electron micrograph of MOLT-4 cells exposed to 50 μg/mL lonidamine for 1 h at pH 6.7. Swollen mitochondria are shown by arrows.

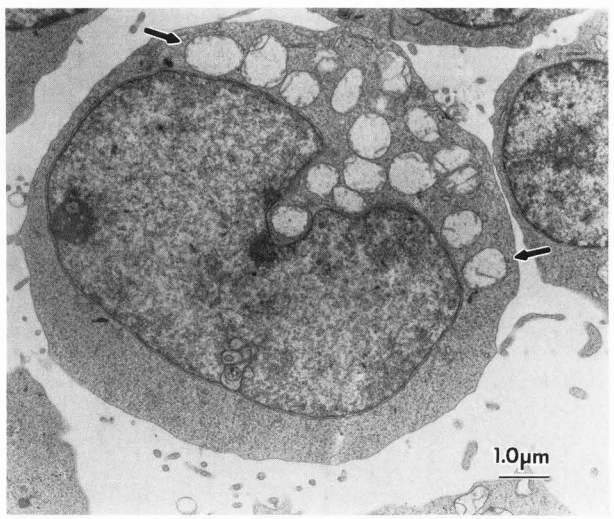


Fig. 2. A transmission electron micrograph of a MOLT-4 cell exposed to 200 μg/mL lonidamine for 1 h at pH 6.7. Swollen mitochondria are shown by arrows.

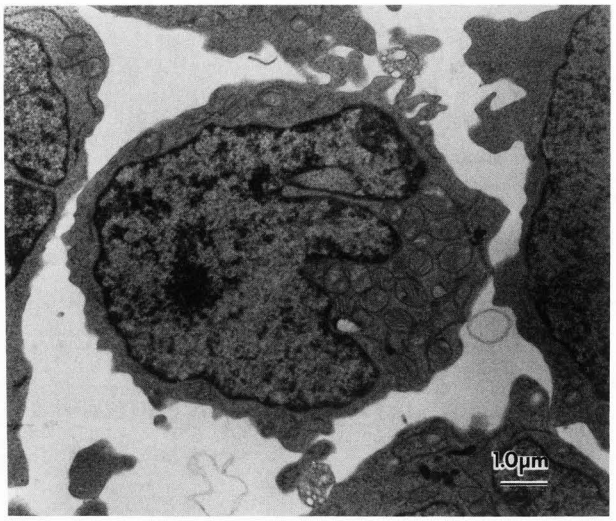


Fig. 3. A control MOLT-4 cell sham-treated with medium at pH 6.7

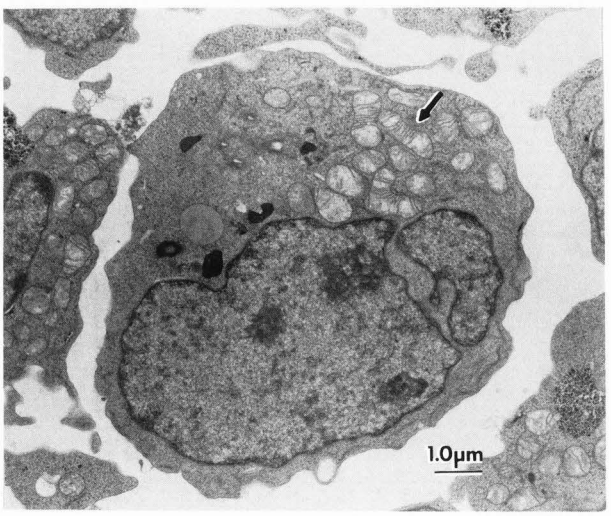


Fig. 4. A MOLT-4 cell exposed to 50 μg/mL lonidamine for 1 h at pH 7.4. The mitochondria (arrow) are slightly swollen, but the cristae remain well formed. These mitochondria may be compared to those shown in Fig. 1. There the cells were given the same treatment except the pH was 6.7.

The cell surface can be effectively observed with the scanning electron microscope. Figs. 5a and 5b are control MOLT-4 cells, which were incubated in medium at pH 7.4 and pH 6.7, respectively. MOLT-4 cells are generally spherical with broad ridges over their surface. Some cells, however, show a uropod structure as described by Bessis (1977) along with multiple ruffles and small filaments. Cells incubated for 1 h at pH 6.7 have more surface projections and ruffles than the pH 7.4 control. After a 1-h exposure to 50 μg/mL lonidamine at pH 6.7 (Fig. 6), the cell surface still looks similar to the pH 6.7 control. In general, the SEM views of treated and pH 6.7 control cells were similar. Only the 200 μg/mL lonidamine-treated sample had extracellular debris and disrupted microvilli on the surface.

When cells that have been exposed to lonidamine are incubated at pH 7.4 in fresh medium

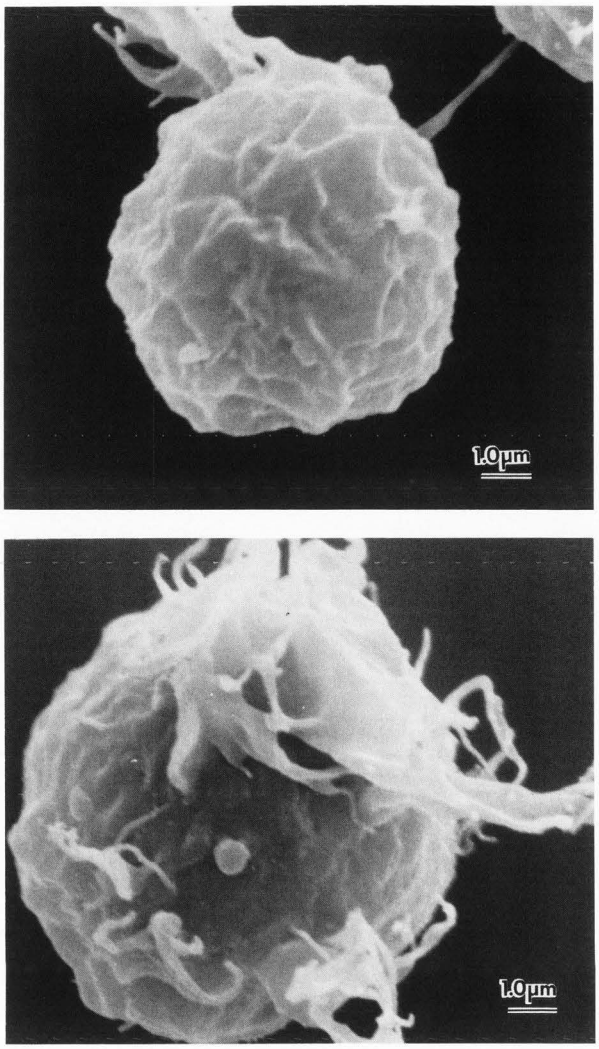


Fig. 5. Scanning electron micrographs of control cells sham-treated with medium at (a) pH 7.4 and (b) pH 6.7.

for 1 h, the cell surface regains the appearance of pH 7.4 control cells with the exception of some long projections. The 200 μg/mL lonidamine-treated sample (Fig. 7) also shows some remaining cellular debris (arrow). In the TEM, the return to a normal morphology is seen more easily. After a 1-h incubation at pH 7.4, the mitochondrial swelling has decreased dramatically and the cristae have reformed to a great extent. The 50 μg/mL lonidamine-treated sample (Fig. 8) looks very similar to the control and the 200 μg/mL-treated sample (Fig. 9) has only a few swollen mitochondria. The numbers of long, thin projections seen in the SEM, are visible in the TEM sections (Figs. 8 and 9) along with some extracellular debris.

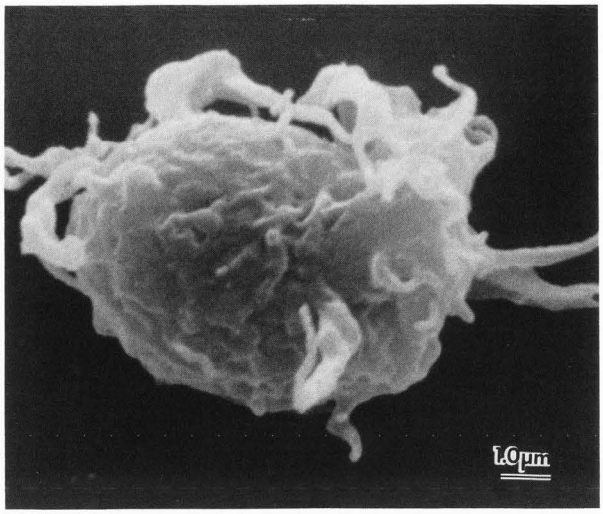


Fig. 6. A MOLT-4 cell exposed to 50 μg/mL lonidamine for 1 h at pH 6.7.

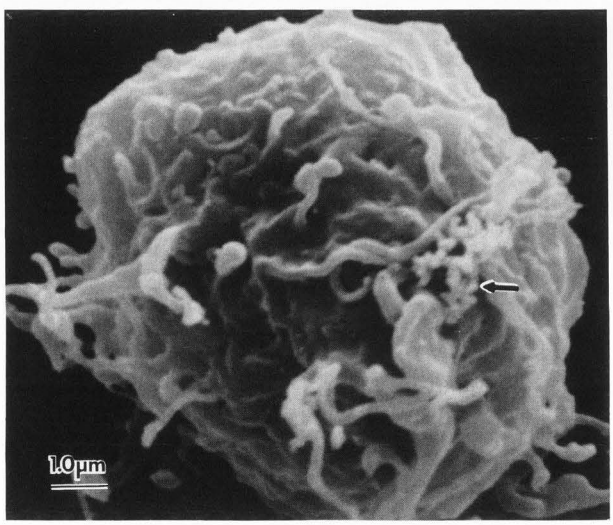


Fig. 7. A MOLT-4 cell exposed to 200 μg/mL lonidamine for 1 h at pH 6.7, followed by 1-h incubation in fresh medium at pH 7.4.

Glioma cells, U-87 MG, have also been tested for lonidamine toxicity. Since they grow as attached cells, the more stringent method of colony counting was used. A summary of U-87 MG survival is shown in Table 4. The values are normalized to the plating efficiency of control cells exposed at pH 7.4. After a 1-h exposure to 50 μg/mL at pH 6.65, the relative survival fell to 91% of the control and decreased to 84% after a 2-h exposure. As with MOLT-4 cells, there is a strong pH dependence of the lonidamine-induced killing. Long exposures of 8 h at

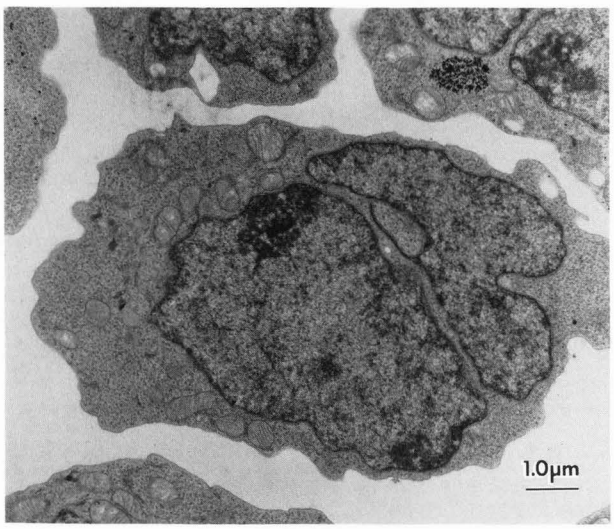


Fig. 8. A transmission electron micrograph of a MOLT-4 cell exposed to 50 μg/mL lonidamine for 1 h at pH 6.7, followed by a 1-h incubation in fresh medium at pH 7.4.

TABLE 4. Survival of U-87 MG glioma cells after a lonidamine exposure

pH	Concentration (μg/mL)	Time (h)	Survival* %
7.4	50	1	100
7.4	50	2	100
7.4	50	8	86 ± 8.6
7.4	10	1	100
7.4	10	2	100
7.4	10	8	84 ± 5.6
7.2	50	2	97 ± 3.1
7.0	50	1	95 ± 2.1
7.0	50	2	84 ± 5.1
6.65	50	1	91 ± 3.6
6.65	50	2	84 ± 8.7

*Normalized to pH 7.4 control

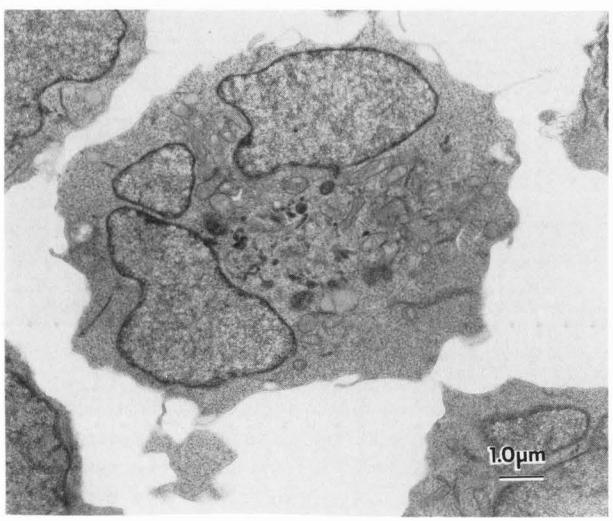


Fig. 9. A MOLT-4 cell exposed to 200 μg/mL lonidamine at pH 6.7 followed by a 1-h incubation in fresh medium at pH 7.4.

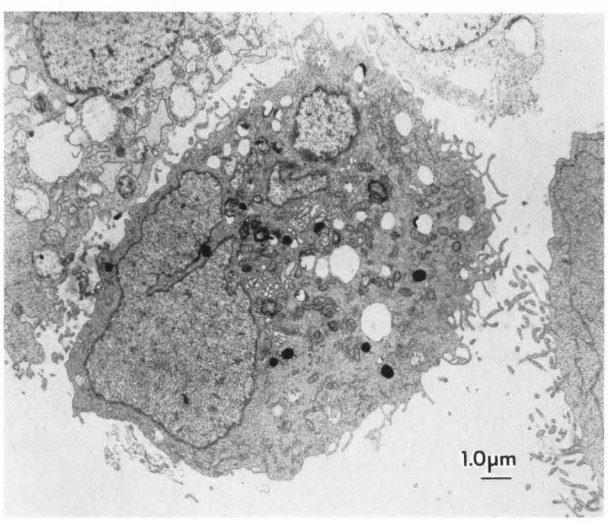


Fig. 10. A transmission electron micrograph of a control U-87 MG glioma cell sham-treated with medium at pH 6.7 for 1 h.

lower concentrations of lonidamine also did not decrease survival greatly. Thus, U-87 MG glioma cells are not very sensitive to lonidamine under these conditions.

At the ultrastructural level the glioma cells are less reactive to the drug than are MOLT-4. The glioma cells are large with many vacuoles, residual bodies and lysosomes. The mitochondria are small and not well defined in the micrographs (Fig. 10).

After a 1-h exposure to concentrations of up to 50 μg/mL lonidamine at pH 6.7, there was no visible difference between treated and control cells. After an 1-h exposure to 200 μg/mL lonidamine at pH 6.6, the endoplasmic reticulum and mitochondria were swollen (Fig. 11), but they returned to their usual morphology after a 1-h incubation in fresh medium at pH 7.4 (Fig. 12).

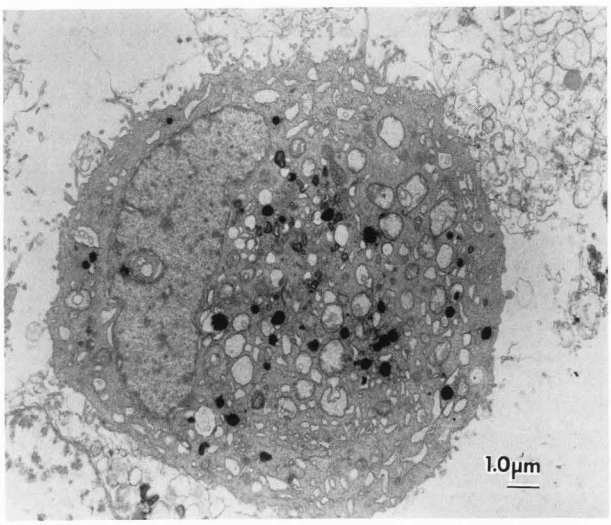


Fig. 11. A U-87 MG glioma cell exposed to 200 μg/mL lonidamine at pH 6.7 for 1 h.

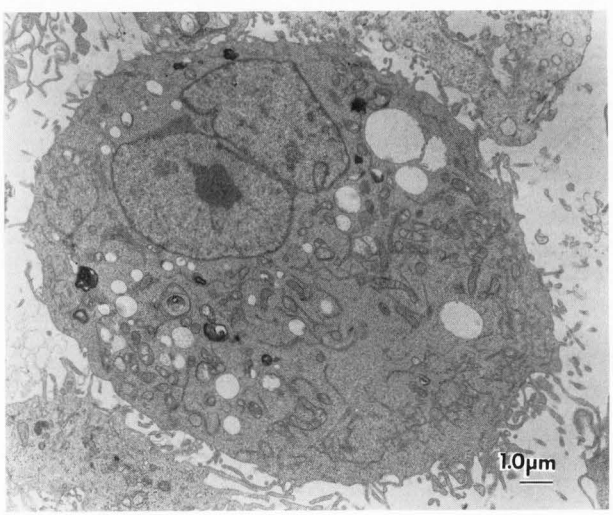


Fig. 12. A U-87 MG glioma cell exposed to 200 μg/mL lonidamine at pH 6.7 for 1 h followed by a 1-h incubation at pH 7.4 in fresh medium.

In the scanning electron microscope views, there were no visible differences noted between the control cells and those treated at pH 7.4 or pH 6.7 (Figs. 13 a-d). The glioma cells were flat, thin cells, with many microvilli on their surface. Thus, by morphological assessment the glioma cells were little affected by lonidamine. This correlated with the small effect of the drug on survival.

Discussion

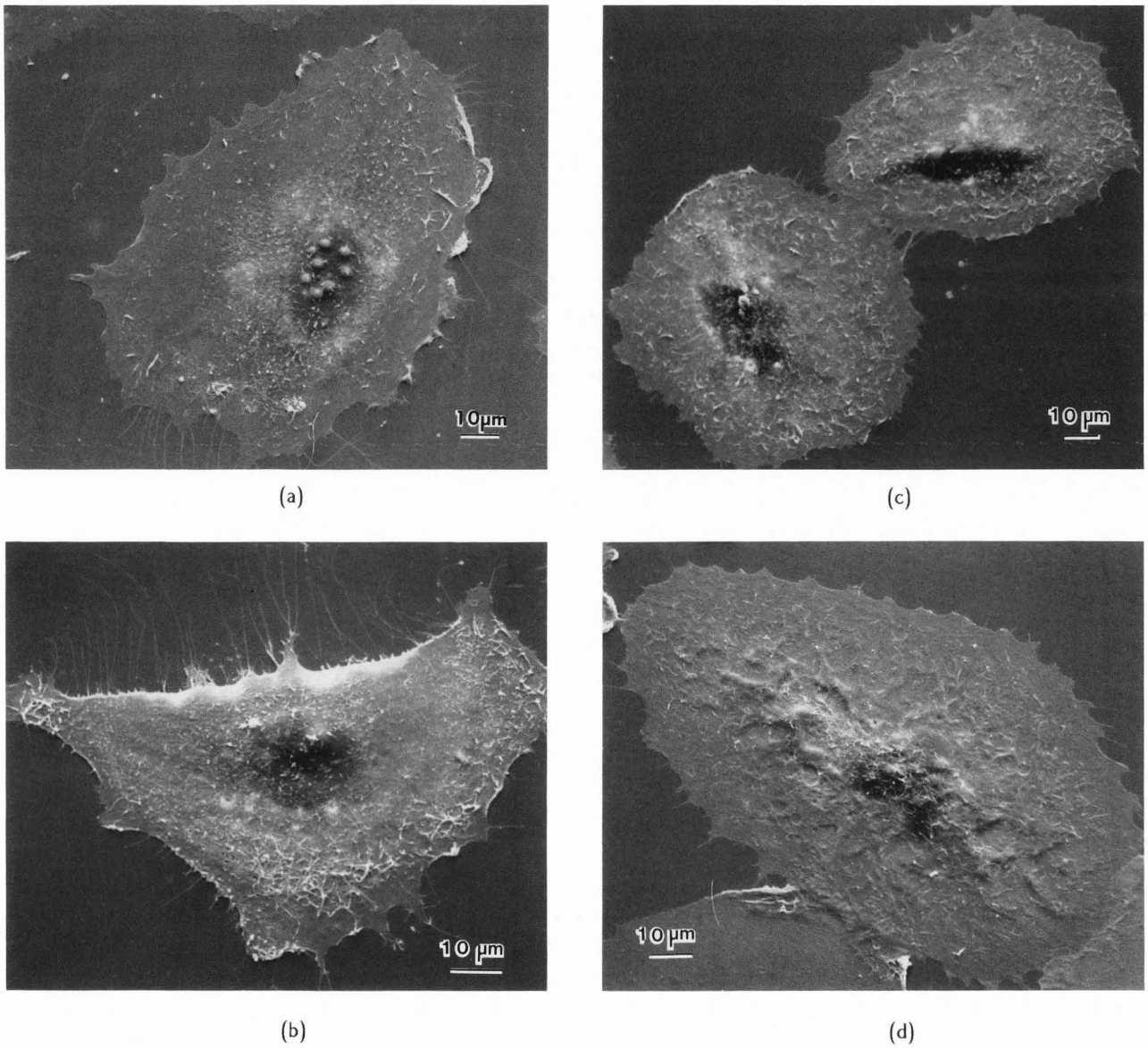
Although lonidamine is being tested as an anticancer agent in humans, it is not equally effective in all in vitro test systems (De Martino et al. 1984). In human and murine lymphocytes, lonidamine is reported to be more effective in B and null leukemia than in T-cell leukemia (Natali et al. 1984; Floridi et al. 1981b). The MOLT-4 cells were selected as a test system for lonidamine because they are a leukemia that carries a number of T-cell markers, including surface markers for sheep red blood cell rosetting. They die by apoptotic death similar to normal lymphocytes and they are radiation sensitive, which makes them of interest for studies involving synergism between radiation, heat or lonidamine (Minowada et al. 1972; Szekely et al. 1987a; Raaphorst et al. 1983).

Since it had been reported that brain cells are not affected by lonidamine (De Martino et al. 1984) and, since brain tumors are difficult to treat, we also looked at the effects of lonidamine on a malignant glioma cell line U-87 MG. These cells grow attached to the flask with an epithelial-like morphology, in contrast to the free-floating MOLT-4 cells. Glioma cells are relatively radioresistant; hence, it is of interest to compare their response to lonidamine with that of the radiosensitive MOLT-4 cells.

The survival results showed many MOLT-4 and U-87 MG cells surviving the lonidamine treatment even when the dose was large compared to the plasma level achievable in humans (Hahn et al. 1984).

Although the MOLT-4 and U-87 MG cells were relatively insensitive to lonidamine, they both showed a strong pH dependence of cell death and morphological change. This increased cell death at low pH agrees with reports on other cell types (Kim et al. 1984; Stryker and Gerweck, 1988). This fact is especially important since one of the suspected mechanisms of hypothermic killing in tumors is due to the lower pH level within the center of the tumor. Combined lonidamine and hyperthermia treatments are being tried and these results suggest a synergism is possible. For both MOLT-4 and glioma cells there was no significant cell killing at pH 7.4 even after 6 h of exposure to the drug.

In the electron micrographs it was clear that in MOLT-4 ultrastructural damage of the mitochondria took place within a 1-h exposure to lonidamine at pH 6.7. There was also some slight damage at pH 7.4. The mitochondria, however, regained a structure very close to the control within 1 h after the cells were returned to normal medium at pH 7.4. Therefore, the relative resistance of MOLT-4 to lonidamine, as compared to lymphocytes or Ehrlich tumor cells (Natali et al. 1984; Arancia et al. 1988; Floridi et al. 1981b), may be explained by the MOLT-4 cells rapid return to a normal ultrastructure after removal of the drug. The rapid return of the ultrastructure to a normal appearance suggests that lonidamine should be present at its maximum concen-



(a) (c)

(b) (d)

Fig. 13. Scanning electron micrographs of U-87 MG cells treated for 1 h. (a) Control cell sham-treated at pH 7.4, (b) control cell sham-treated at pH 6.7, (c) 50 μg/mL lonidamine-treated cell exposed at pH 6.7 and (d) 200 μg/mL lonidamine-treated cell exposed at pH 6.7.

tration to be of use clinically, either alone or as part of a combination treatment.

U-87 MG glioma cells, on the other hand, are not greatly altered by the lonidamine exposure. Although some mitochondria swelling was seen at the highest lonidamine concentration used, there was little difference between control and treated samples in both the SEM and TEM observations. The ultrastructural appearance of cultured glioma cells has been described earlier (Collins et al. 1979; 1980) and in our hands the U-87 MG cells had an ultrastructure similar to that already reported. The only additional factor was the large number of residual bodies we observed in our culture conditions. Since the glioma cells were not greatly altered by lonidamine in the concentrations we used, it is possible that the reduced transport of lonidamine across the plasma membrane is a factor, or that the activation state of the mitochondria was such that they were not affected by the drug. It has been reported that mitochondria in the condensed state (III) are disrupted by lonidamine (De Martino et al. 1984). From our observations, the U-87 MG cells

and MOLT-4 cells do not show the condensed structure characteristic of mitochondria, which are actively producing energy. In our control micrographs at pH 6.7 or pH 7.4, the mitochondria have an expanded morphology with visible finger-like cristae.

Although lonidamine does not dramatically reduce viability in all cell types, such as the MOLT-4 or U-87 MG, the oxidative stress produced by the drug during and shortly after the exposure suggests that lonidamine may be a useful treatment in conjunction with X-irradiation, hyperthermia or chemotherapy with other drugs. Thus, even if tumor cells do not respond to lonidamine directly, they may show synergistic killing in combined treatments. In fact, glioma cells have shown enhanced cell killing during combined lonidamine-hyperthermia treatment (data to be published elsewhere). Although the ultrastructure of the nucleus was not altered by lonidamine, there are reports that it inhibits the repair of sublethal damage in some cell types (Kim et al. 1986; Hahn et al. 1984), but not in all (Danjoux et al. 1988).

The fact that lonidamine is more active at low pH also suggests that it will be valuable in combination with heat or irradiation. Solid tumors frequently have a hypoxic core, which is at a lower pH than the rest of the tumor. The rapid growth of tumor cells also makes the center of solid tumors more acidic. Thus, the likelihood that lonidamine will interact synergistically with hyperthermia or irradiation is increased.

In our observations of MOLT-4 and U-87 MG we have not seen any large blebs or blistering of the plasma membrane as reported by De Martino et al. (1984). Lonidamine does have an effect on molecular transport across the outer mitochondrial membrane, however, as reflected by its ability to increase the response of tumor cells to adriamycin (Floridi et al. 1987; Starace et al. 1987; Bagnato et al. 1987). Differential effects of lonidamine have also been shown by the freeze fracture technique (Arancia et al. 1988). They report a dose-dependent clustering of intramembrane particles in lymphocytes and leukemic cells.

Conclusion

We have shown the effect of lonidamine exposure to cells of two types: human T-leukemia and glioblastoma. Survival of both cell lines was relatively insensitive to the drug at low pH and had no significant response at pH 7.4. MOLT-4, a T-leukemia line, had major ultrastructural damage in the mitochondria after a 1-h exposure; however the mitochondria rapidly regained their ultrastructural appearance after a 1-h incubation in fresh medium at pH 7.4. U-87 MG glioma cells did not show significant ultrastructural damage after the lonidamine exposures. This reinforces the idea that various cell types show a range of response to the drug and that lonidamine efficiency in cancer treatment may be site dependent. These data indicate the necessity of evaluating lonidamine responses for all types of tumors targeted for clinical trials of lonidamine therapy.

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Discussion With Reviewers

U.T. Brunk: Would you please expand on the type of oxidative stress you suggest is produced by the drugs? Are formation of oxygen-derived free radicals involved?

Authors: We concur with the widely held view that lonidamine acts primarily in the condensed mitochondria as an inhibitor of the aerobic glycolytic pathway. In cancer cells there is an increased glycolytic flux due to their higher energy requirements. It is suggested by many workers that lonidamine inhibits aerobic lactate production by inhibiting the action of mitochondrially bound hexokinase, which is present in the outer mitochondria membrane of neoplastic cells. Floridi and Lehninger (1983) also suggest that lonidamine inhibits electron transport in the NAD-linked dehydrogenase system. The drug produces an inhibition of oxygen uptake by cultured cells, which increases dramatically at low pH (Stryker and Gerweck 1988). In conjunction with hyperthermia, lonidamine may act synergistically because condensed mitochondria are produced from the imbalance in energy requirements and the reduced oxygen supply induced by the heat treatment. Evidence suggests that lonidamine acts by biochemical disruption of oxygen utilization and energy production, and not by a free radical mechanism.

H. Gamliel: For the various modes of analysis, U-87 MG cells were grown in flasks, and/or glass coverslips, and/or in dishes. Can you provide data to support your assumption that they grow the same on all these kinds of substrates? Why did you not use the widely acceptable, special type of Petri dish with an inner lining that can serve for SEM, TEM, LM, etc.? Why did you not embed cells while attached to the substrate?
Authors: The flasks and dishes are made from the same material and have the same surface treatment. Large numbers of dishes are required for the survival experiments and hence, specialized dishes would not be practical for that use. Flasks are the most convenient vehicle for long-term culture of the cell stocks. In retrospect, the inner linings you suggest would have been ideal for the microscopy part of the experiment; however, we did not consider them at the time. We are satisfied that, since control and treated cells are being compared, any effect of the glass substrate (if one exists) would be common to both samples.

H.Gamliel: Long exposure to lonidamine seems to be not less important than concentration. Thus, it is not clear why no data is provided on the exposure of U-87 MG for 24 h, the most useful time to test, as indicated with MOLT-4.
Authors: One purpose of the experiments was to compare the survival and morphology of MOLT-4 and U87-MG after lonidamine exposure with results published with other cell lines. The shorter drug exposure times of 1 h to 8 h were chosen to correspond with experiments published by other workers on other cell culture systems (see text reference list). Since in vivo exposures are much longer than this, we decided to extend some MOLT-4 experiments to 24 h.

H. Gamliel: Why is it so important to show sensitivity at pH 6.7? - although briefly discussed, it is not clear how in practice such a low pH can be achieved in-vivo (beside the point that the "center of solid tumors is more acidic")? Why "the likelihood that lonidamine will interact synergistically with hyperthermia or irradiation is increased"? Is there evidence that hyperthermia or radiation increases the acidity of tumor cells?
Authors: Tumor growth relies on the supply of nutrients and drainage of waste by a functional vascular network. The generally higher level of metabolism in the oxygenated portion of the tumor and cell death in the center of a solid tumor produces an acidic environment. Heat or radiation causes vascular damage in tumors, which may lead to a nutritionally deprived, waste-product-rich and hence acidic environment. For example, Rhee et al. (1984) showed that the intrinsically acidic intratumor environment became further acidic for 12 h after heating. Fellenz and Gerweck (1988) have shown that intracellular pH depends on extracellular pH and that thermal sensitivity is closely related to internal pH. Since the vasculature in normal tissues is usually resistant to heat relative to the vasculature in tumors (Song et al. 1980), a synergistic effect between lonidamine and heat is expected.

H. Gamliel: MOLT-4 cells, under our hands displayed villous surfaces, as did many other lymphoid leukemia cell lines. The SEM pictures provided show cells which could be categorized as suffering from air drying (displaying only ridges and undulating surfaces). Why did you have to add sucrose to your fixation solution? If osmolarity is the issue, it is now well established that the osmolarity of the vehicle (Hank's buffer) is more important than the total osmolarity of the fixative solution. Fixation with glutaraldehyde alone is not sufficient, and osmium tetroxide should be used also to prevent massive shrinkage of specimens and other artifacts of CPD. Did you compare your results with other methods using better fixation procedures, e.g., TAO, GTGO, etc.?
Authors: We have tried various buffer-glutaraldehyde combinations and have found that the 4% glutaraldehyde in Hank's balanced salt solution plus 0.04 mol/L sucrose suggested by Abugaber et al. (1981) gave good preservation of MOLT-4 cells. As they and you point out, buffer osmolarity is more important than that of the fixative, since the cell membrane is not very osmotically active to glutaraldehyde. In our hands, the buffer and buffer plus glutaraldehyde fixative had osmolarities of 340 mosm and 775 mosm, respectively. We used this combination as our basic fixative and did not try any other.

M. Albertsson: Are there any measurements available on how lonidamine penetrates into the hypoxic tumor's core when administered orally or intravenously? What about penetration in the brain tumor?
Authors: We are not aware of any human data on the distribution of lonidamine within tumors. One of us (G.P.R.) is collecting biopsy material from patients treated with lonidamine to monitor its concentration in various organs and tumors. In rats lower concentrations with respect to the plasma are observed in most organs. Only kidney and liver concentrations approach the plasma value. The concentration of lonidamine in the brain is generally low.

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