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Electromicroscopic Observations on Gliotoxin-induced Apoptosis of Cancer Cells in Culture and Human Cancer Xenografts in Transplanted SCID Mice

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Abstract. *Background: Gliotoxin belongs to a group of compounds produced by fungi, all of them having a bridged polysulfide piperazine ring in their chemical structure. This internal polysulfide bridge enables them to carry out various biofunctions, but so far, the toxicity of these compounds limited them to be used as medicines in clinic. However, the toxicities of these compounds are quite different and determined by their different part of chemical structures. Therefore, it is still possible to find a suitable low toxic compound for drug use. As for anticancer drug developing, the first need is to confirm the anticancer activity in vivo. Materials and Methods: The morphological changes of human breast cancer MCF-7 cells affected by gliotoxin in culture, and the structural damages of human cancer xenograft tissue in SCID mice after intra-tumor injection of gliotoxin were observed after histological stain and transmission electromicroscopic treatment. The DNA changes of the human colon cancer xenograft were observed in 1.2% agarose gel electrophoresis. Results: Gliotoxin 1 or 5 μM in medium for 24 hours induced typical apoptotic structural changes to MCF-7 cells, the cell surface membrane showed blebbing clearly. Injection of 1 mg gliotoxin into the tumor tissue directly did not induce noticeable side-effects to the host mice but induced complete damage of the cell structure, the cell surface membran broken down and the components of the nuclei segmented. The whole cancer tissue shrinked and finally formed a dark color scab which came off from the skin few days later. The cured mice showed no tumor recurrence in the six months following observation. The apoptotic DNA damage was also found in human colon cancer xenograft C1-2 tissues after gliotoxin was injected inside the tumor tissue. Conclusion: The anticancer activity of gliotoxin is confirmed in vivo.*

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Gliotoxin is an epipolythiodioxopiperazine compound produced by various *Penicillium* and *Aspergillus fungi* (1), as well as the Gliocladium (2), Sporidesmin (3) and Candida (4). The main feature of this group of compounds is the internal bridge of disulphide piperazinedione six-membered ring (Figure 5). Gliotoxin attracted attention because of its antibacterial (1, 5), antiviral (6, 7), and antiangiogenic activity (8), as well as its potential to be used as an anticancer agent (9-11). Gliotoxin allows the survival of primitive precursor stem cells while inhibiting the proliferation of T-lymphocytes and macrophages (12-14). Lately gliotoxin has been considered for colitis preventation (15, 16) also the autoimmune diabetes (17).

In this report, we studied the action of gliotoxin against human breast cancer MCF-7 cells in culture and on several solid human cancer xenograft tissues in SCID mice.

Materials and Methods

Materials. Gliotoxin, bisdethio-di(methylthio)-gliotoxin and the other regular chemical compounds unless otherwise stated, were purchased from Sigma (St. Louis, MO, USA). The DMEM/F12 (Dulbecco's Modified Eagle's Medium-Nutrient Mixture F12) culture medium, L-glutamine and pyridoxine hydrochloride were obtained from Gibco BRL (Carlsbad, CA, USA). The fetal bovine serum was purchased from Gemini Bio-Products, Inc (West Sacraments, CA, USA).

Human breast cancer MCF-7 cells in culture. Human breast cancer MCF-7 cells were cultured in DMEM/F12 medium with 10% (v/v) heat-inactivated fetal bovine serum and a final concentration of 50 units/ml penicillin G and 50 μg/ml streptomycin. For electron microscopy, cells were planted onto small round microscope cover glasses (1 cm in diameter). In six well culture plate, four pieces of cover glass and two ml of culture medium were placed in one well. A total number of $2x10^5$ MCF-7 cells were planted into each well and the plates were kept in 5% CO_2 air phase incubator at 37°C for 48 hours. The confluence of the cells on the cover glass at that time was about 80%.

Gliotoxin was dissolved in 100% dimethyl sulphoxide (DMSO) as a stock solution and diluted with culture medium before use. The final concentration of DMSO in the culture system was always less than 0.1%.

Human cancer tissue xenograft models in SCID mice. The method for developing primary human cancer xenograft tissue models in SCID mice was basically as follows: human cancer tissues obtained directly from surgery, were cut into 1 mm^3 size and transplanted into SCID mice behind the fat pat through a surgery procedure. After few weeks, the size of the cancer xenograft tissues became 0.5 to 0.8 cm in diameter, and the mice were then used in the experiments. All the procedures for animal treatments followed the directions of The Committee of the Animal Control at The City of Hope National Medical Center. For injection, the ground gliotoxin suspension in saline was 1 mg per 0.1 ml, and it was directly injected into the center of the cancer xenograft tissue (18).

Histological and electron microscopical observations. For histological analysis, the cancer tissue samples were collected at special times after gliotoxin suspension in saline was injected and fixed immediately in 5% pH 7.0 buffered formalin solution. The other procedures for paraffin embedding and slice cutting were processed routinely. Hematoxylin and eosin stained sections were used for reviewing the morphological changes. For electron microscopic observations, the MCF-7 human breast cancer cells and the cancer xenograft tissues were fixed in freshly made 1% glutaraldehyde in 0.01 M cacodylate buffer at 4˚C overnight. The cells or tissues were then fixed in 1% OsO_4 at 4˚C for 60 minutes, washed three times with cacodylate buffer, then dehydrated in 60, 70, 80, 95, 100% ethanol. The dehydrated cells and tissues were washed twice with propylene oxide and immersed in propylene oxide/eponate 1:1 (v/v) mixture overnight at room temperature. Then the propylene oxide/eponate was replaced with 100% eponate, and polymerized at 70˚C. Samples were cut into sections of 50 nm thickness with a diamond knife, and then were mounted on copper grids and stained for 10 min with aqueous 5% uranylacetate, and followed with 1 min Sato's lead staining. The samples were examined with transmission electron microscopy under Philips CM 10 microscope.

DNA extraction of human colon cancer xenograft tissues and analysed in agarose gel. Human colon cancer xenograft tissue (0.3 g) was homogenized in 5 ml lysis buffer containing 50 nM tris.HCl, 2 mM EDTA and 1 nM NaCl at pH 8.0, added 0.5 ml 10% SDS and after mixed up a final concentration of 100 μg/ml proteinase K was added. Incubated the sample overnight in a water bath at 48˚C. Then the sample was extracted twice with phenol/ chloroform/ isoamylalcohol (25:24:1 by volume) and once with chloroform. An aliquot of 7.5 μl of boiled bovine pancreatic RNase A (20 mg/ml stock solution in H_2O) was added to the sample and then incubated for 3 hours in a 37˚C water bath. Finally DNA was collected by ethanol precipitation and resuspended in 40 μl pH 7.4 TE buffer which containing 10 mM Tris. HCl and 1 mM EDTA. Twenty microliters of DNA from each sample were loaded onto 1.2% agarose horizontal slab gels and electrophoresis was performed in TBE buffer for 12-15 h at 1.5 V/cm. Following electrophoresis, DNA was visualized using ethidium bromide staining.

Results

Morphological changes of cultured human breast cancer MCF-7 cells induced by gliotoxin. Human breast cancer MCF-7 cells were cultured in the full medium with 1 μM, 5 μM and 10 μM glotoxin for 24 hours. At the end of culture, the cells with 10 μM gliotoxin were already damaged, while those cells cultured with 1 μM and 5 μM gliotoxin showed typical changes on the cell surface and the cell nuclei. The cell surface membrane was convoluted and blebby, the chromatins in the cell nucleus were compacted and segmented (Figure 1).

Human cancer xenograft tissues in SCID mice destroyed by injection of gliotoxin. Several human cancer xenograft tissues growing in SCID mice were used for this observations. The anticancer effect of gliotoxin started showing at the time about 24 hours after injection. The tumor tissue graduatly turned dark and became soften, and the size was contracted. This phenomenon will be continuously showing for up to one week. At the end, the cancer tissue became a black scab and which came off from the skin days later. In case if the size of the tumor was larger, a second injection of 0.5 mg or 1 mg gliotoxin will help to damage the rest of the cancer tissue.

In this paper we only showed the damaging results of human colon adenocarcinoma xenograft (Figures 2 and 3), similar results have been seen in human poorly differentiated bladder carcinoma, and human malignant melanoma xenograft in SCID mice. The cured mice survived healthfully over six months, no recurrences were found during that following observation. On the other hand, if the drug dosage used was not enough, the residue cancer tissue started re-growing in about two weeks. The cancer tissue showed no change in control mice with saline injection. In another group of control, the gliotoxin saline suspension was injected into normal SCID mice subcutaneously or in muscle. There were no serious side effects showed in those mice. Sometimes, local skin damage occurred, but the mice recovered quickly.

The histological changes of human colon cancer xenograft tissue in SCID mice, 36 hours after 1 mg gliotoxin injection is showed in Figure 2. The cells in control cancer tissue which was injected with 0.1 ml saline only were still growing happily, while in the gliotoxin treated cancer tissue, the cell nuclei have been obviously damaged. This same tissue was also observed under electron microscope, the result is showing in Figure 3. From the photo we can see that at the time 36 hours after gliotoxin injection, the surfaces of the cells were broken down already, the cytoplasm of the cells were no longer integral and the components of the cell nuclei were segmented.

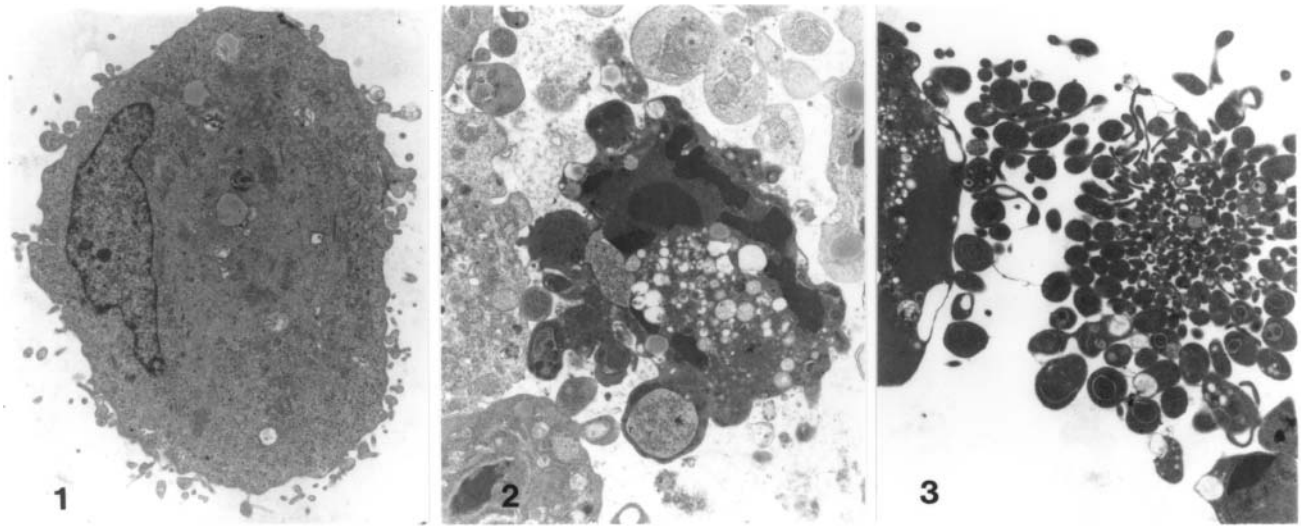

Figure 1. *Structural changes in cultured MCF-7 cells after gliotoxin treatment as observed under electron transmission microscopy. 1. Control. 2; 3. Typical cell surface bobbing, apoptosic changes happened after 1 μM gliotoxin treatment for 24 hours.*

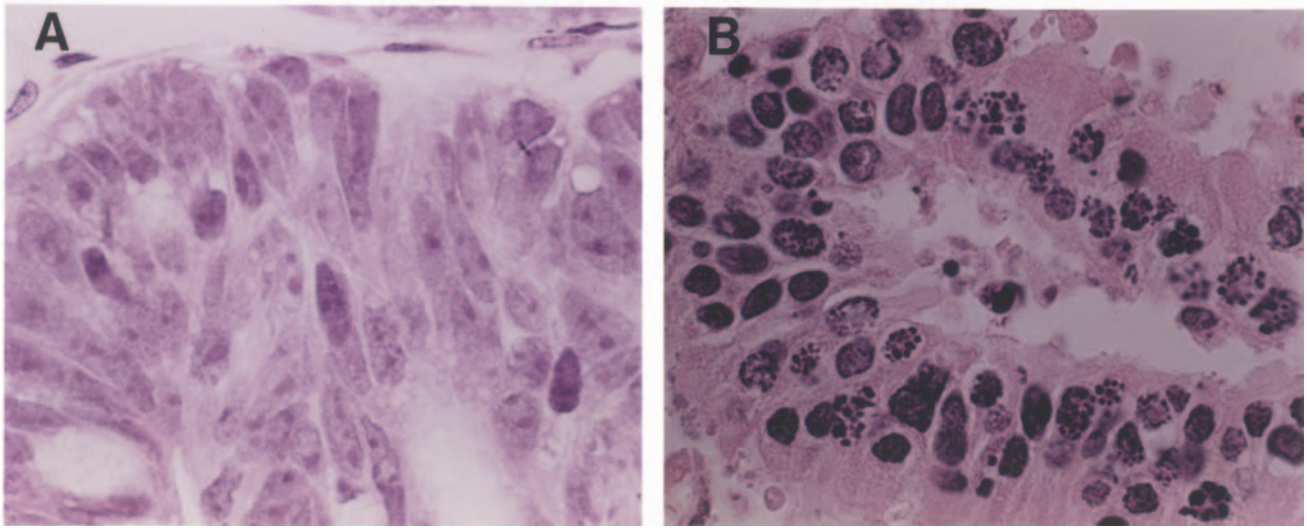

Figure 2. *The histological change in human adenocarcinoma colon cancer C1-2 xenograft tissue in SCID mouse. The samples were collected at 36 h after intra-tumor injection. H&E stained. (A) Injected with saline as a control. (B). Injected with 1 mg gliotoxin suspension in 0.1 ml saline.*

The comparison of the DNA pattern in agarose gel electrophoresis between gliotoxin treated human colon cancer xenograft and the control. The photo of the DNA patterns after running electrophoresis in 1.2% agarose gel is depicted in Figure 4. Since necrosis had occurred in the solid cancer xenografts, the DNA samples extracted from saline injected control human colon cancer xenograft tissues (Figure 4 line 6 and 7) showed as a smear. The DNA samples extrated from gliotoxin treated cancer tissue (Figure 4, line 2, 3, 4 and 5) showed the typical apoptosis ladder type fragmentation.

Discussion

The biological functions of gliotoxin and other epipolythiodioxopiperazine compounds have been studied in different areas (1, 19-24). Evidences indicated that the antiviral function is closely related to the disulfide bridge (25). Epicorazines A and B inhibited the gram-positive bacterium, but not the gram-negative bacterium (26, 27). Gliotoxin has been considered to use in bone marrow and other organ transplantation to prevent the graft-*versus*-host disease, as gliotoxin can offer a fast and convenient

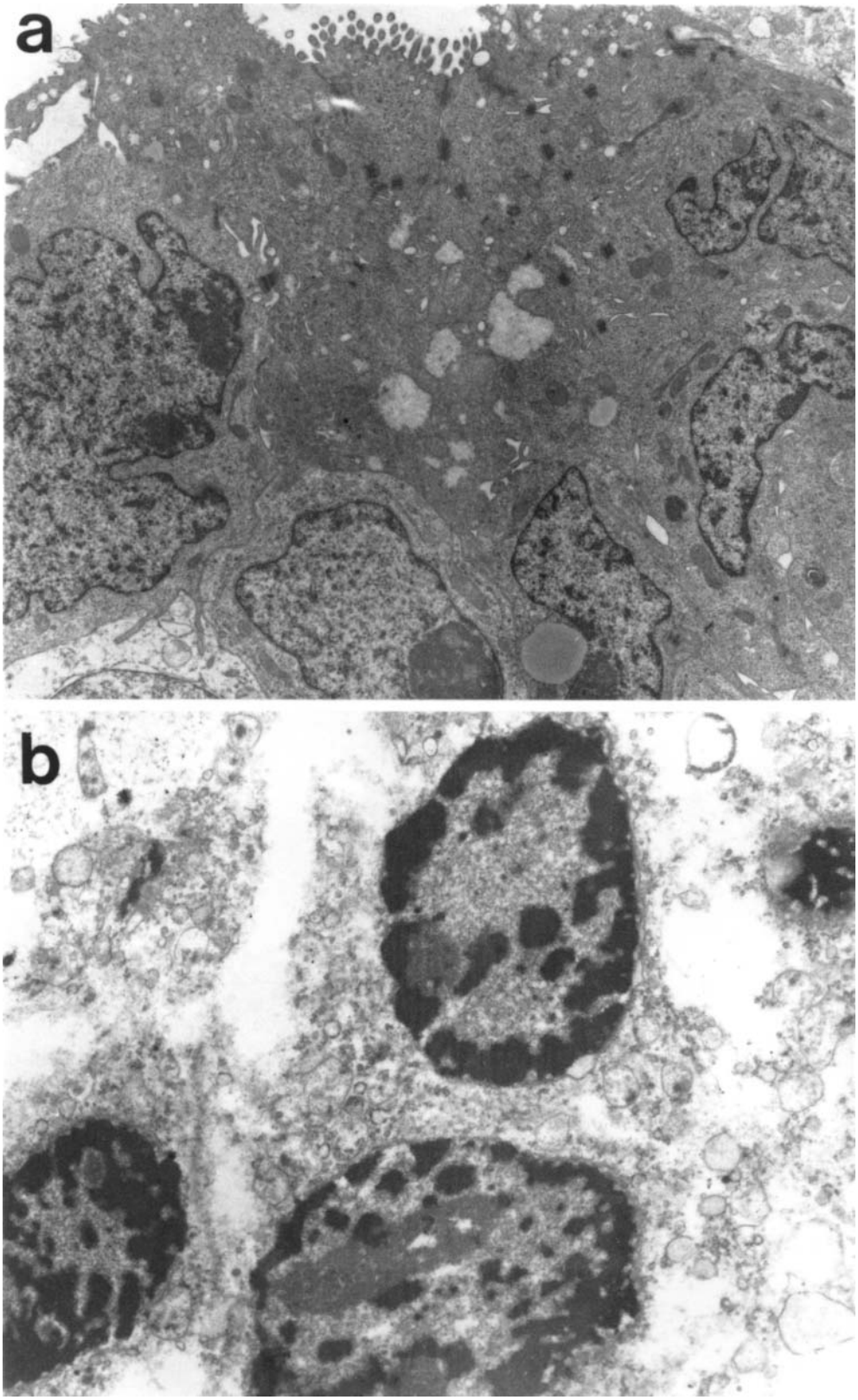

Figure 3. *The ultra-structural changes of human adenocarcinoma colon cancer C1-2 xenograft tissue in SCID mouse after injection. (a) The control, injected with saline only. (b) Injected with 1 mg gliotoxin suspension in 0.1 ml saline.*

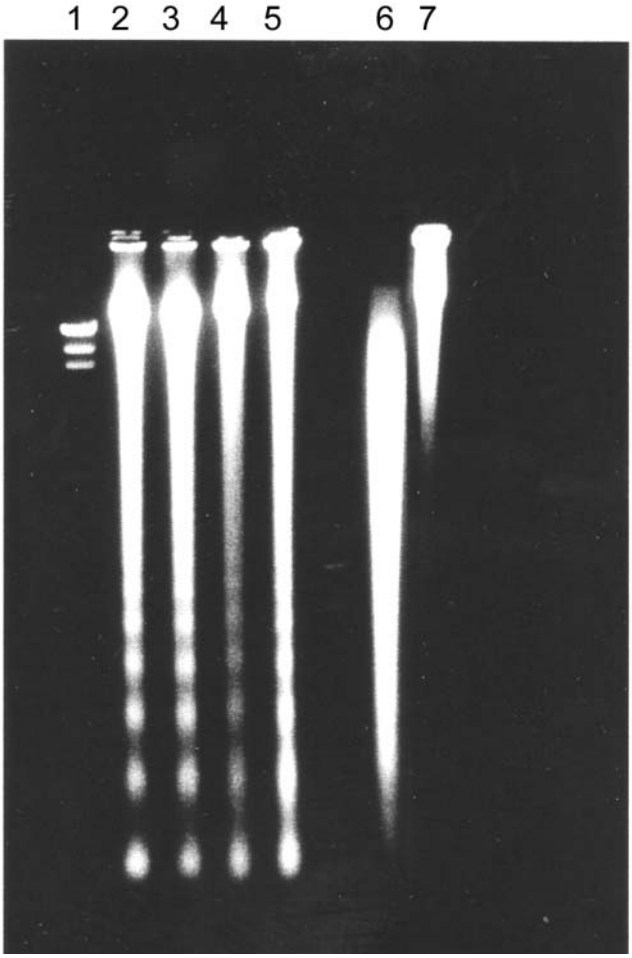

Figure 4. *DNA electrophoresis in 1.2% agarose gel. Line 1, is different size standard DNA molecular weight control. Number 2, 3, 4, 5 are the DNA samples extracted from four different human colon cancer C1-2 xenograft tissues. Sample were collected at the time 36 h after 1 mg gliotoxin suspension was injected. Number 6, 7, are the DNA samples extracted from two human colon cancer C1-2 xenograft control tissues, 36 h after 0.1 ml saline only was injected.*

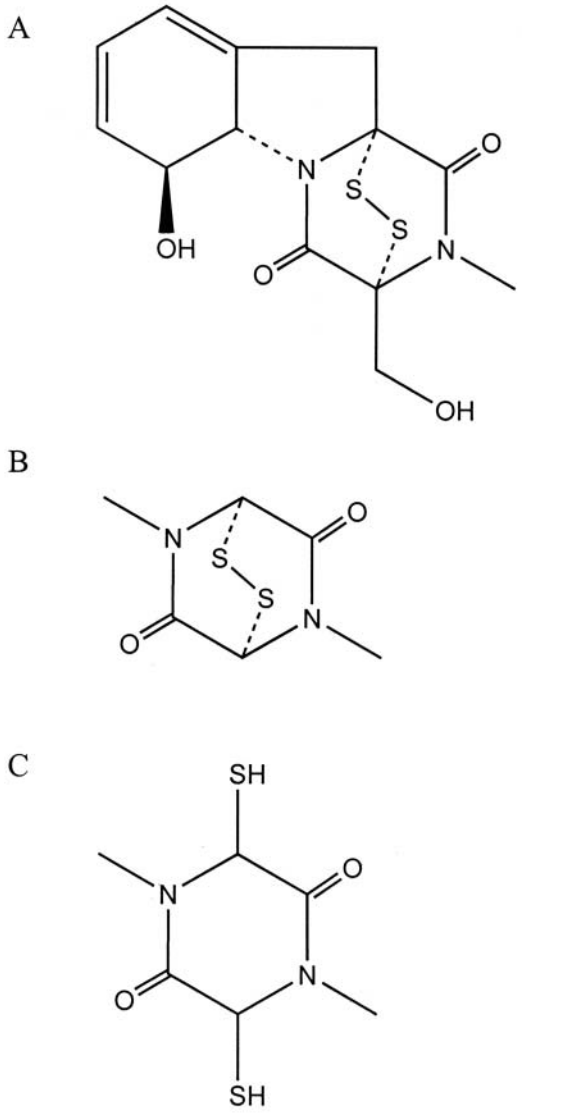

Figure 5. *The chemical structure of (A) Gliotoxin. (B) 3,6-dithio bridge-1,4-dimethyl-2,5-piperazinedion and (C) 3,6-dithiol-1,4-dimethyl-2,5-piperanedion.*

way to deplete the existing T-cell in the tissue sample (12, 28, 29). Gliotoxin has been reported to block Ras farnesylation but not disturbed lamin farnesylation (30, 31), based on that observation, it has been tested for mouse lung tumor chemoprevention (32), but the result was negative.

The chemical structure of gliotoxin is shown in Figure 5A. Since the anti-malarial drug Artimisinin contains an internal oxygen bridge –o-o- chain, and then killed malarial parasites through free radicals reaction (33), the possibility mechanism of free radical of gliotoxin which may be initiated from the structure of –S-S- bridge was also considered (34-35). But since the free radical scavenger compounds could not inhibited the toxic effect of sporidesmin (36). This mechanism possibly is not the one supposed to be responsible to the anticancer function of gliotoxin. With the computer molecular modeling method (37), we calculated the distance between two SH groups in gliotoxin after the polysulfide bridge was reduced. It is about 2.88 A for the cis-form. These two chemical SH groups with this special distance possibly is the active center through which gliotoxin molecules reacted to the cancer cell surface. Gliotoxin has been reported to induce apoptosis (38, 39), interestingly, glutathione intensifies the toxicity of gliotoxin while the inhibitor of glutathione synthesis attenuates the cytotoxic effect of gliotoxin (40).

In this observation, gliotoxin was given through intra cancer tissue injection. An anticancer treatment through intra-tumor tissue injection will certainly not be considered in the clinic, but at the beginning, for the testing of the drug activity, practically when the source of the material is limited, it seems still considerable.

Upreti and Jain (41) suggested that the difference of lipophilicity of the compounds in this group, was responsible for their different distribution in the cell, and determined their different effects. Yoshida pointed out that their inhibitory function on platelet-activating factor were related to the diketopiperazine structure (42). In our observations, it was shown that few derivatives with larger molecular size and more complicated structure showed higher toxicity and lower effectiveness. In a previous paper which compared the anti-RNA virus activity of gliotoxin and several relative compounds (43), the simplest compound N,N-dimethyl-epidithiodioxo-piperazine (Figure 5B) exhibited the stronger antiviral activity and lower toxicity, while compound 3,6-dithioacetate (Figure 5C) was even more active. It will be worth to check their anticancer functions in future.

Conclusion

The anticancer function of gliotoxin was confirmed in culture and in mice. Apoptosis seems to be the way that the cancer cells undergo. It may be worth to test the gliotoxin derivatives with lower toxicities.

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