

Sulfane sulfur deficiency in malignant cells, increasing the inhibiting action of acetone cyanohydrin in tumor growth¹

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ABSTRACT

PURPOSE: To demonstrate the irreversible poisoning action of the acetone cyanohydrin (AC) in malignant cells.

METHODS: Thirty male Swiss mice were inoculated with 1×10^3 Ehrlich tumor (ET) cells. The mice were divided into three groups (n=10): CG (saline); ACG1 (1.864 mg/Kg of AC) and ACG2 (2.796 mg/Kg of AC), treated every 48 hours from day 3 until day 13. On day 15 the mice were euthanized and the number of viable cells in ascites was determined. In the meantime, ET cells were incubated with AC (0.5, 1.0, 2.0 $\mu\text{g/mL}$). Cell viability and percentage of growth inhibition (PGI) were checked after one, two, three, four, 18 and 24 hours.

RESULTS: There was reduction in volume and number of viable cells in ACG1 and ACG2 compared to CG. In ACG1 one of the animals did not present ascites. In ACG2 two mice did not present ascites and in CG none of the mice present ascites. The action of AC was dose and time dependent and there was no significant difference among the three doses.

CONCLUSION: The acetone cyanohydrin promoted reduction of the tumor and also prevented tumor development in 20% of the treated animals.

Key words: Antineoplastic Agents. Sulfur Compounds. Thiosulfate Sulfurtransferase. Mice.

Introduction

Cancer study is being conducted by numerous scientists around the world and their findings in oncology make it one of the greatest and most rapidly evolving areas in modern medicine, causing great technological advances and a growing understanding of what cancer is.

Although cancer has many heterogeneous characteristics, malignant tumors have developed a feature which causes them to grow beyond the imposed limits of the regular cells. The clonal expansion of a transformed cell depends on its uncontrolled proliferative capacity and an increasing inability to die by apoptosis. Therefore, even though there is a great variety of cancer types, evidence has shown that resistance to apoptosis is one of the most remarking characteristics of the majority of malignant tumors¹.

A feature of neoplastic cells is a complete lack of Cystationase (CST) activity, while cysteine aminotransferase, 3-mercaptopyruvate sulfurtransferase (MpsT) e rhodanese activity is only residual. As a result, the biosynthesis and transport of compounds from the sulfane sulfur set do not occur in these cells. Toohey suggests that the uncontrolled proliferation of neoplastic cells is a result of the deficiency of sulfane sulfur and hyperactivity of these enzymes, which would be inhibited in regular cells by that active form of sulfur^{2,3}.

The term sulfane sulfur refers to the set of compounds that contain one sulfur atom covalently bonded to another reduced sulfur atom. Proteins associated to sulfur, bonded sulfur and refer to specific compounds with sulfane sulfur. Thus, sulfane sulfur should not be used to relate to any of the terms mentioned above^{3,4}.

Sulfane Sulfur is generated from various metabolic pathways already described, and it is common to find carrier proteins with the ability to stabilize and transport it. Compounds that contain sulfane sulfur are able to regulate the activity of several enzymes and also have antioxidant properties. This deficiency in the anaerobic metabolism of sulfur is related to neoplastic processes, viral infections and immunodeficiency, suggesting that the sulfane sulfur may exert a regulatory function in cells by modifying the -SH groups and this high activity and short half-life characteristics make it an efficient regulator⁴.

Currently, there are some reviews in the literature carrying information such as characteristics of the sulfane compounds and its affinity to react with cyanide, evidence of metabolism in the malignancy processes, cystathionase deficiency in many malignant cells, and differences in homocysteine metabolism between normal and malignant cells³. It was also possible to gather

important information regarding the defective sulfur metabolism in cancer cells with antitumor effects in sources of sulfane sulfur. This fact seems to show that the uncontrollable proliferation of malignant cells may be related to sulfane sulfur deficiency and to an uncontrollable action of the enzyme set, which is normally inactivated by such substance. Tumors with inherently high growth rates may have lower sulfur-transferase activities (rhodanese, for example) than the normal tissue and tumors with low growth rates^{3,5,6}.

The action of acetone cyanohydrin is based on its cyanide metabolite that comes from its spontaneous degradation in slightly alkaline pH, which acts in lethal malignant cells taking advantage of a flaw in the cyanide metabolism in these cells, due to a suppressed action of the rhodanese enzyme, responsible for their detoxification. This residual action of the rhodanese enzyme would be triggered by a lack of substrate for the reaction, known as sulfane-sulfur, responsible for providing sulfur for disposal of cyanide as thiocyanate⁷.

The advances in various fields of knowledge have led to increased insights about cancer biology, offering by far more means to fight the disease, its prevention, early diagnosis and new forms to treat it. However, it is known that there is still much to learn. Based on that, the present study seeks to highlight the action of cyanide from acetone cyanohidrin in alkaline pH, exploring the sulfane sulfur deficiency in malignant cells, which promotes on them irreversible poisoning.

Methods

The research project was approved by the Ethics Committee in animal use of the Federal University of Mato Grosso do Sul (UFMS), under protocol number 227/2009.

A total of 30 male Swiss mice weighing an average of 35g from the animal colony of the Federal University of Mato Grosso do Sul were used in this study. The animals were kept in the experiment for seven days prior the procedures. Randomly, the animals were divided into three groups: Control (CG), n=10, saline treated; Acetone cianohydrin 1 (ACG1), n=10, treated with 1.864 mg/Kg of Acetone cianohydrin; and Acetone cianohydrin 2 (ACG 2), n=10, treated with 2.796 mg/Kg of Acetone cianohydrin.

Tumor transplant

The cells were kept in mice by the intraperitoneal transplantation of 2×10^6 EAT cells. Every ten days the formed intraperitoneal fluid was removed and transplanted in another

animal.

Donor animals were euthanized with an overdose of anesthetic. After that a peritoneal wash was performed with 3 mL of saline, and then performed again the same 3 mL withdrawal by suction. The obtained cell suspension was centrifuged, the supernatant discarded and the pellet resuspended in 3 ml of saline. In the suspension, cell viability test was performed through the Trypan blue exclusion. Next, it was determined the number of cells in Newbauer chamber.

All three groups of mice received injections containing 0.4 ml of cell suspension of Ehrlich ascites tumor (containing 1.0×10^3 viable cells) in the peritoneal cavity

Treatment program

The day of tumor inoculation in the animals was regarded as day 0. Treatment was initiated on day 3 and repeated every 48 hours until day 13. The CG received 0.4 ml of saline intraperitoneally; ACG1 received 0.4 ml of acetone cyanohydrin 1.864 mg/kg intraperitoneally and, the ACG2 received 0.4 ml of acetone cyanohydrin 2.796 mg/kg intraperitoneally. 48 hours after the last treatment, the animals were euthanized by an overdose of anesthetic to measure the antitumor activity of acetone cyanohydrin.

EAT cell viability

EAT cell viability was checked Trypan blue test. The cells were stained with Trypan blue (0.4% in normal saline); those that did not incorporate the dye were considered viable, while others were unfeasible. These viable and non-viable cells were counted.

In vitro experimental design

Cell preparation and culture

The EAT cells suspension obtained was washed two times and resuspended in Dulbecco's Modified Eagle's (DMEM) supplemented with 5% fetal bovine serum and the suspension adjusted to 1.0×10^5 cells/mL. After adjusting the concentration of 1.0×10^5 EAT cells the suspensions were separated into aliquots of 1.0 mL and cultured with 0 (control), 0.5, 1.0 and 2.0µg of acetone cyanohydrin in DMEM supplemented with 5% fetal serum, with a pH between 7.0 and 7.2. After one, two, three, four, 18 and 24 hours cell viability tests were performed and the percentage of

growth inhibition (PGI) was determined, and this procedure was repeated twice in duplicate.

To check significant differences among groups, the results were analyzed with one-way ANOVA followed by Tukey's multiple comparison test. The significance level was 5% ($p < 0.05$).

Results

In vitro experiment

The AC intraperitoneal administration at dose of 1,864 and 2,796 mg/kg body weight led to a reduction in tumor volume and viable cell count of tumor-bearing mice compared to control mice EAT (Table 1).

TABLE 1 - Effect of Acetone cyanohydrin in volume and count of viable tumor cells in mice with EAT.

	Total cells (10 ⁷)	Volume (mL)
CG	56.15±21.5	8.6±2.24
ACG1	24.42±12.51*	3.8±1.69*
ACG2	14.45±9.81*	3.12±1.79*

Each value represents the average ±DP. (n=10 mice per group)

* $p < 0.0001$, when treated and compared to the control group

In ACG1, only one animal showed no ascites at the end of 15 days, not presenting tumor cells after peritoneal wash with saline. In ACG2, two animals showed ascites and after the wash showed no peritoneal tumor cell counting. In the CG, all animals developed ascites and showed viable cells counting.

In vitro experiment

With the results shown in Figure 1, concerning the percentage of inhibition of EAT cell growth after treatment with AC, it was found that doses of AC used showed the same pattern, where the inhibition was dose-and time-dependent.

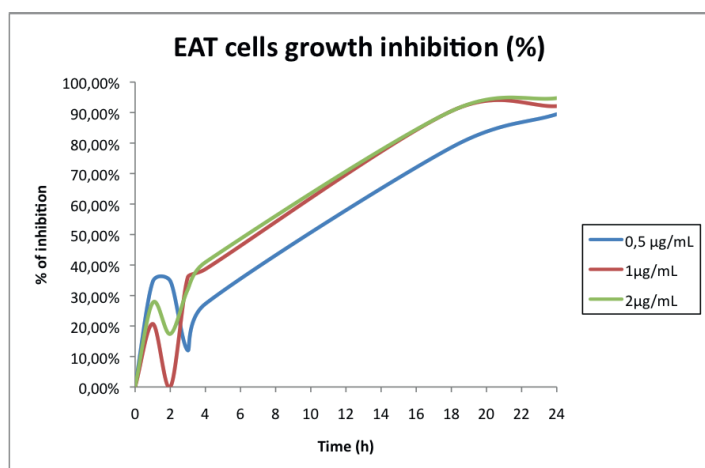


FIGURE 1 - inhibition growth curve after treatment with in vitro acetone cyanohydrin compared to control group.

The evaluation of the inhibition of the AC showed no significant difference between the three doses, 0.5, 1 and 2 mg / mL, 24 hours after reaching 89.47, 92.11 and 94.74% respectively.

Discussion

The use of Acetone cyanohydrin was motivated by the fact that its action in the organism is similar to its molar equivalent of cyanide. For example, the intraperitoneal lethal dose (LD50) in mice to Acetone cyanohydrin is equivalent to 2.65 mg of cyanide ion/kg which is similar to that of sodium cyanide which is 2.54 mg/kg cyanide ion and has an average five minute-time to death for the two compounds. The LD50 of acetone cyanohydrin is 8.5 mg/kg weigh^{8,9}. In this experiment we used doses under the lethal values, 1.864 and 2.796 mg of acetone cyanohydrin/Kg.

The tumor inhibition without causing acute poisoning in animals was only possible due to cyanide act promoting the inhibition of cytochrome C oxidase, an enzyme directly involved in mitochondrial respiration, thus promoting a decrease in ATP levels and resulting in cellular injury or establishing cell death, depending on its concentration^{8,10}. However, due to unavoidable exposure to cyanide, living organisms have developed a self-defense mechanism by conversion of cyanide to thiocyanate by the transfer of sulfur from the sulfane sulfur group (sulfur donors) for the cyanide by the rhodanese mitochondrial enzyme^{10,11}. A feature present in cancer cells is the residual activity of rhodanese¹², due to a deficiency of sulfane sulfur, which makes transport and biosynthesis of sulfur from sulfane not occur satisfactorily in such cells. This suggests that the uncontrolled proliferation of cancer cells is a result of deficiency of this compound, and that this active form of sulfur promotes inhibition of regular cell proliferation^{13,14}.

The sulfane sulfur is generated from multiple metabolic pathways and from protein which are capable of stabilize and transport it. The activity of a number of enzymes is regulated by compounds containing sulfane sulfur. It is believed that this substance can exert a regulatory activity through changes in -SH groups, and disability in the anaerobic metabolism of sulfur is related to neoplastic processes and immunodeficiency virus infections, and such high activity and short half-life confer characteristics of an efficient regulator⁴. As a result, cells with sulfane sulfur deficiency have a significantly diminished rhodanese activity^{2,6}.

The action of acetone cyanohydrin in both experiments, and , demonstrates the ability of tumor cells to irreversibly poison the action of cyanide, while normal cells are resistant to small doses of cyanide due to the protection afforded by mitochondrial rhodanese with its ability to detoxify cyanide to a certain extent. Once the limit is exceeded, however, the toxicity is expressed. The absence of this intracellular mechanism in tumor cells may explain their inability to eliminate cyanide efficiently. This provides a dose and time dependent effect¹⁵⁻¹⁷, as occurred in the experiment and previous study⁷. Cyanide toxicity occurs in two ways: ATP depletion caused by blockage of oxidative phosphorylation and production of reactive oxygen¹⁷.

The acetone cyanohydrin led to a significant decrease in tumor volume, leading to inhibition of the tumor in 3 animals, one from ACG1 and two from ACG2, which means that the higher dose in the cure rate was approximately 20%. The literature also reports the success of therapy with cyanide using suicide gene, based on the transfer of an encoding B-glucosidase (linamarase) gene to tumor cells which, in the presence of an innocuous substrate (linamarin), produces cyanide, causing these cells to die proportionally to the concentration of linamarin¹⁷⁻²⁰.

Evidence observed in the present study suggests that the selective action of cyanide is due to a deficiency in its elimination, which is observed in tumor cells as widely discussed in the literature. We believe that further studies are necessary before it is possible to fully understand the mechanism of action involved in the process.

Conclusions

Acetone cyanohydrin, through its molar equivalent of cyanide, was able to promote a reduction in tumor volume, a decrease in the total number of tumor cells, as well as prevent tumor development in 20% of the treated animals. In the experiment the acetone cyanohydrin presented a dose and time dependent action.

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Acknowledgement

To Profa. Isabella Saliba Pereira for revising the English language.

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Received: June 12, 2013

Review: Aug 14, 2013

Accepted: Sept 12, 2013

Conflict of interest: none

Financial source: none

¹Research performed at Laboratory of Experimental Carcinogenesis, Federal University of Mato Grosso do Sul (UFMS), Campo Grande-MS, Brazil. Part of PhD degree thesis, Health and Development Postgraduate Program. Tutor: Ricardo Dutra Aydos.