Research articles

Vitamin C and the Red Wine Polyphenol Resveratrol - but not Curcumin and the Glycolysis Inhibitors 2-Deoxyglucose, Dichloroacetate and 3-Bromopyruvate - Induce Selective Cytotoxicity against Lung Cancer Cells

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Abstract

Cancer statistics show that the most commonly diagnosed cancer in the world is lung cancer, that over 50% of patients diagnosed with this cancer have distant metastasis, and that only 4% of these patients manage to survive more than 5 years. The limited selective cytotoxicity of the drugs used for the treatment of these patients probably accounts for these high mortality rates. In this work, we have assessed the selective anticancer activity of several drugs currently undergoing clinical trials by using human A549 lung cancer cells and human MRC5 non-malignant lung fibroblasts. Vitamin C and the red wine polyphenol resveratrol induced selective cytotoxicity towards the cancer cell line. Vitamin C (1 mM) induced higher selective cytotoxicity than the anticancer agents cisplatin, oxaliplatin, etoposide and 5-fluorouracil. A lyophilized red wine extract, but not a hydroalcoholic extract from red grapes, also showed certain selectivity against lung cancer cells. Neither the curry polyphenol curcumin nor the glycolysis inhibitors 2-deoxyglucose, dichloroacetate and 3-bromopyruvate displayed selective cytotoxicity. We also report that A549 lung cancer cells have higher glycolytic rates (higher glucose consumption and higher lactate production) than human MRC5 non-malignant lung fibroblasts, and that the combination of each glycolytic inhibitor with the pro-oxidant agents pyrogallol and hydrogen peroxide does not result in a significant increase in their cytotoxicity or selectivity against the cancer cell line. Our results support the possible evaluation of vitamin C and resveratrol in clinical trial for the treatment of metastatic lung cancers, and suggest that curcumin and the glycolysis inhibitors 2-deoxyglucose, dichloroacetate and 3-bromopyruvate have a limited potential (at least as single agents) for the treatment of patients with this type of cancer.

Introduction

Global cancer statistics show that lung cancer is the most common cancer and the leading cause of cancer death [1]. The latest global cancer statistics show that, in 2008, lung cancer was the most commonly diagnosed cancer and the leading cause of cancer death in males. Among females, lung cancer was the fourth most commonly diagnosed cancer and the second leading cause of cancer death. This type of cancer accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in 2008 [1]. In the United States, the American Cancer Society estimates 228,190 new lung cancer cases and 159,480 lung cancer deaths for the year 2013 [2]. Perhaps the most worrying data are those showing that over 50% of patients diagnosed with lung cancer have distant metastasis, and that only 4% of these patients manage to survive more than 5 years [2]. Many cancer cells in patients with metastatic lung cancers are not localized and, therefore, cannot be eliminated by surgery or radiation therapy. These patients need to be treated systemically with anticancer drugs. Although these drugs can kill lung cancer cells, most of them are also toxic to non-malignant cells, cause severe side effects in patients and, therefore, need to be used at suboptimal concentrations. The low selective cytotoxicity of the drugs used for the treatment patients with metastatic lung cancers probably accounts for the high mortality rates observed in these patients.

Several natural products (e.g., vitamin C [3-7], resveratrol [8-12] and curcumin [13-17]) and several synthetic glycolytic inhibitors (e.g., 2-deoxyglucose, dichloroacetate and 3-bromopyruvate) [18-24] have shown promising anticancer effects in preclinical models. Indeed, vitamin C, resveratrol, curcumin, 2-deoxyglucose, and dichloroacetate have entered clinical trials for the treatment of specific cancers (see http://clinicaltrials.gov/). By using human A549 lung cancer cells and human MRC5 non-malignant lung cells, here we have evaluated the selective anticancer activity of these drugs with the aim of identifying potential new treatments for patients with metastatic lung cancers.

Material and Methods

Chemicals and cell lines

Ascorbic acid (vitamin C; 99%), resveratrol (99%), curcumin (70%), 2-deoxyglucose (2-deoxy-D-glucose; 98%), dichloroacetate (98%), 3-bromopyruvate (97%), etoposide (98%), 5-fluorouracil (99%), oxaliplatin (99%) and cisplatin (99.9%) were purchased from Sigma. Their chemical structures are represented in Illustration 1. The human A549 lung cancer cell line and the human embryo lung fibroblastic MRC-5 cell line were purchased from the European Collection of Cell Cultures. Both cell lines were maintained in DMEM supplemented with 2 mM glutamine, 50 µg/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum, and were cultured at 37°C in a humidified atmosphere containing 5% CO2. Cell culture reagents were obtained from Life Technologies.

Preparation of the extracts

Commercial red grapes were extracted with ethanol:water (1:1) at 60°C for 1 hour by using an ultrasound water bath apparatus. Ethanol was then eliminated in a rotary vacuum evaporator and the remaining water solution was lyophilized. Commercial red wine was directly lyophilized, obtaining an alcohol free-lyophilized red wine extract.

Assay for cytotoxic activity (MTT assay)

The MTT assay is a colorimetric technique that allows the quantitative determination of cell viability. It is based on the capability of viable cells to transform the MTT salt (3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide) into a formazan dye. Exponentially growing cells were seeded into 96-well plates and drugs were added 24 h later. Following an incubation period specified in figure legends (generally 48 h), medium was removed and 125 µL MTT (1 mg/mL in medium) was added to each well for 4 hours. Then, 80 µL 20% SDS in 0.02 M HCl were added, plates were incubated for 10 hours at 37 ºC, and optical densities were measured at 540 nm on a multiwell plate spectrophotometer reader. Cell viability was expressed as percentage in relation to controls. All data were averaged from at least three independent experiments and were expressed as means ± standard error of the means (SEM).

Measurement of glycolytic rates

Glycolysis rates were assessed by measuring concentrations of glucose (initial product of glycolysis) and lactate (final product of glycolysis) in supernatants of A549 lung cancer cells and MRC-5 lung non-malignant cells. Briefly, 4 x 10^5 cells were allowed to grow in 24-well plates for 8 h. After

medium removal, cells were washed with PBS and 300 µL of fresh medium were added to each well. Afterwards, cells were allowed to grow for 8 h, and glucose and lactate concentrations were determined in cell supernatants by using the Accutrend® Plus analyzer together with Accutrend glucose strips and BM-Lactate Strips (Roche Diagnostics). After calibrating the instrument with glucose and lactate calibration strips, test strips were used to determine glucose and lactate levels via colorimetric-oxidase mediator reactions according to the manufacturer's instructions [25]. Results are shown as means ± standard error of the means (SEM) of three independent experiments.

Inhibition of glycolysis

Glycolysis inhibition was assessed by measuring concentrations of glucose and lactate in control and treated cells. Briefly, 4 x 10_5 cells were allowed to grow in 24-well plates for 8 h. After medium removal, cells were washed with PBS and 300 µL of fresh medium were added to each well. Afterwards, drugs were added and, after an incubation period 8 h, glucose and lactate concentrations were determined in cell supernatants as described in the previous section. Results are expressed as percentage of lactate production and percentage of glucose consumption in relation to untreated cells, and are shown as means ± standard error of the means (SEM) of three independent experiments.

Statistical analysis

All data were averaged from at least three independent experiments and were expressed as means ± standard error of the means (SEM). For statistical analysis we used the t-test (paired, two-tailed). A P-value >0.05 is not considered statistically significant and is not represented by any symbol. A P-value <0.05 is considered to correspond with statistical significance and is indicated with an asterisk (*), a P-value <0.01 is indicated with a double asterisk (**), and a P-value <0.001 is indicated with a triple asterisk (***).

Results and Discussion

Most patients with metastatic lung cancers die because the drugs used for the treatment of their disease have a limited capacity to selectively kill lung cancer cells. The initial aim of this work was the evaluation of the selective anticancer activity of several compounds that have shown anticancer potential (and that have entered clinical trials), with the hope of identifying potential new treatments for these patients. We initially selected the natural products ascorbic acid (vitamin C), resveratrol and curcumin (Illustration 1), and their possible selective cytotoxic activity was evaluated by using the human A549 lung cancer cell line and the human embryo lung fibroblastic MRC-5 cell line. These malignant and non-malignant cells were exposed for 48 h to these three natural products and to the commonly used anticancer agents etoposide, cisplatin and oxaliplatin; then cell viability was estimated with the MTT assay. Results, represented in Illustration 2, show that vitamin C and the red wine constituent resveratrol induced a statistically significant selective cytotoxicity towards the cancer cell line. It is worth noting that the selectivity cytotoxicity shown by ascorbic acid at a concentration of 1 mM was higher than that observed for any concentration of the three tested anticancer agents. We also prepared and evaluated the selective cytotoxicity of a lyophilized red wine extract and of a hydroalcoholic extract from commercial red grapes. Illustration 3 shows that the alcohol free-lyophilized red wine extract, but not the hydroalcoholic extract form red grapes, had certain selectivity towards the lung cancer cell line. We cannot conclude, however, that resveratrol is responsible for this activity, as red wine contains other polyphenols that may play a role in its selective cytotoxicity [26].

The curry polyphenol curcumin, which have entered clinical trials for the treatment of several cancers, did not show selective cytotoxicity towards the lung cancer cell line (Illustration 2). Although this dietary constituent has anticancer potential [13;14], it is the authors' opinion that its chemotherapeutic potential may have been overdiscussed in the last years [15;27].

Our next goal was to evaluate the selective anticancer activity of the glycolysis inhibitors 2-deoxyglucose, dichloroacetate and 3-bromopyruvate. Evidence suggests that cancer cells keep sustained glycolytic rates despite the presence of an adequate oxygen supply (Warburg Effect), and that these high glycolytic rates play a key role in their survival. The inhibition of glycolysis has become in recent years an attractive strategy to selectively kill cancer cells [28-32]. The glycolytic inhibitors 2-deoxyglucose, dichloroacetate and 3-bromopyruvate have shown promising anticancer effects in preclinical models [18-24] and 2-deoxyglucose and dichloroacetate have already entered clinical trials for the treatment of specific cancers (http://clinicaltrials.gov/). Results represented in Illustration 4 show, however, that none of these three glycolytic inhibitors displayed selective cytotoxicity against the cancer cell line. In fact, non-malignant lung fibroblasts were somewhat more sensitive to their cytotoxic activity than lung cancer cells.

With the aim of understanding these unexpected results, we considered the possibility that the cancer cell line did not have increased glycolytic rates in relation to the non-malignant cell line. We also speculated with the possibility that these glycolysis inhibitors were not inhibiting glycolysis in these cells. Results represented in Illustration 5 A show, however, that the A549 lung cancer cells consumed more glucose and produced more lactate than the MRC5 non-malignant lung cells. Since glucose and lactate respectively are the initial and final products of glycolysis, our data indicate that the cancer cell line have higher glycolytic rates than the non-malignant cell line. Results represented in Illustration 5 B show that dichloroacetate inhibited glucose consumption and lactate production in both cell lines. Therefore, the lack of selective cytotoxicity displayed by the glycolytic inhibitors (Illustration 4) cannot be explained by similar glycolytic rates in both cell lines or by lack of inhibition of glycolysis in these cell lines. Perhaps these drugs may induce cytotoxicity against these cells through glycolysis-independent mechanisms at lower concentrations than those required to inhibit glycolysis.

Cancer cells are known to produce high levels of hydrogen peroxide constitutively [33;34]. We have recently discussed that the activation of glycolysis in cancer cells may play a key role in the detoxification of hydrogen peroxide by increasing the levels of the hydrogen peroxide scavenger pyruvate and by regenerating NADPH [32]. We also hypothesized that the combination of glycolytic inhibitors with pro-oxidant agents might be therapeutically useful [32]. In a recent paper, Vuyyuri et al. reported that combinations of vitamin C with the glycolysis inhibitor 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3-PO) synergistically enhanced cell death in lung cancer cells but not in non-malignant cells [6]. These data support our hypothesis [32], as vitamin C is known to induce cancer cell death through generation of hydrogen peroxide [3]. To further support this hypothesis, we evaluated whether the combination of the glycolytic inhibitors 2-deoxyglucose, dichloroacetate and 3-bromopyruvate with hydrogen peroxide and the hydrogen peroxide-generating agent pyrogallol [35] resulted in an increase in their cytotoxicity or selectivity against the cancer cell line. Our results revealed, however, that the combination of these glycolytic inhibitors with the pro-oxidant agents pyrogallol and hydrogen peroxide did not result in a significant increase in their cytotoxicity or selectivity against the cancer cell line (Illustration 6).

In conclusion, this report shows that vitamin C and the red wine polyphenol resveratrol induce selective cytotoxicity towards lung cancer cells. Because these natural products have already entered clinical trials for the treatment of specific cancers, these data support their possible advancement into clinical trials for the treatment of metastatic lung cancers. Although the dietary agent curcumin and the glycolysis inhibitors 2-deoxyglucose and dichloroacetate are in clinical trials for the treatment of specific cancers, our data suggest that these agents have a limited potential (at least as single agents) for the treatment of patients with lung cancer.

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University of Seville.

Competing Interests

The authors declare that they do not have conflicts of interest.

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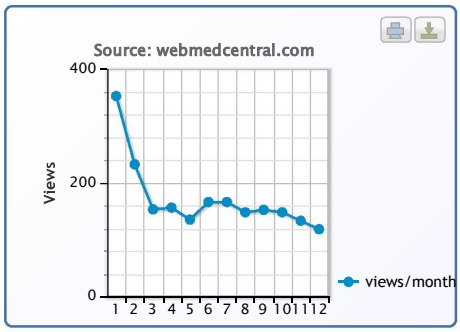

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