

CHARACTERIZATION OF THE CYTOTOXIC EFFECTS OF THE COMBINATION OF CISPLATIN AND FLAVANOL (-)-EPICATECHIN ON HUMAN LUNG CANCER CELL LINE A549. AN ISOBOLOGRAPHIC APPROACH

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Background: Among malignancies, lung cancer is a leading cause of death. Platinum-based therapeutic compounds used to treat lung cancer have not been able to increase the survival of patients and such compounds have a high incidence of adverse and toxic effects. It has been proposed that flavonoids such as catechins may significantly reduce the risk of developing cancer, alongside with other health benefits. The *aim* of this work was to determine the effect of (-)-epicatechin, the main flavanol found in cocoa, on the proliferation of the lung non-small cell adenocarcinoma cancer cell line A549, and to determine its effects when added simultaneously with cisplatin. **Materials and Methods:** Concentration-response curves for cisplatin and epicatechin were obtained, inhibitory concentrations calculated and an isobolographic analysis was then performed. **Results:** We found that epicatechin has a concentration-dependent inhibitory effect on proliferation of tumor cells and the isobolographic analysis reveals that the effect of its combination with cisplatin is synergistic. It was also observed that epicatechin promotes cell death by apoptosis. **Conclusions:** Epicatechin might be considered for future studies to explore its possible use as coadjuvant in cisplatin-based treatments.

Key Words: epicatechin, lung cancer cells, cisplatin, isobologram.

Among malignancies, lung cancer has the higher morbidity and mortality worldwide [1]. According to the American Lung Association, 5-year survival rate is just 17.8%, compared to other types of cancers as colon (65.4%), breast (90.5%) and prostate (99.6%) [2]. Only 15% of cases are diagnosed in early stages.

The treatment depends on the type and stage of cancer and includes surgery, chemotherapy and radiotherapy. Adjuvant platinum-based chemotherapy (cisplatin or carboplatin) is the most common choice, however, the response rate is not high. In addition, cisplatin causes nephrotoxicity, gastrointestinal toxicity, myelosuppression, ototoxicity and neurotoxicity. So, the use of cisplatin leads to comorbidities of difficult control, because no agent conferring protection against these toxic effects has been developed [3].

The primary cellular effect of cisplatin is to form adducts or crosslinks in DNA [4]. *In vitro* studies have shown that interaction between cisplatin and DNA may contribute to the generation of superoxide radicals causing toxicity in cancer cells [5]. DNA damage activates DNA-repair mechanisms and apoptosis through activation of the tumor suppressor protein P53. Lung cancer has a high rate of *TP53* mutations (in 46% of adenocarcinomas and

in 81% of squamous cell carcinomas [6]) decreasing response rate [7].

In this context, there is an urgent need to search for therapeutics that may increase efficacy and reduce the adverse effects of the current chemotherapy in lung cancer.

Several epidemiological studies have suggested that a diet rich in fruits and vegetables is associated with reduced risk of cancer [8, 9]. In this regard, cocoa (*Theobroma cacao*) is particularly interesting because it is a fruit whose seeds and derivatives, rich in bioactive compounds with remarkable beneficial effects on metabolism and cardiovascular system, are worldwide consumed [10]. One of such bioactive compounds of cacao is the flavanol (-)-epicatechin (EC). Although many authors refer to it as an inert compound, EC has shown anti-proliferative activity in stomach, prostate and ovary cancer [11, 12]. Regarding lung cancer, it has been noted that in adenocarcinoma cells A549 and PC-9, EC is able to potentiate the growth-inhibitory effect and to increase apoptosis induced by the polyphenol curcumin [13] suggesting that EC may have a potentiating effect in cancer treatment. It is also known that EC reduces nephrotoxicity [14] and ototoxicity [15] induced by cisplatin so it seems to be an excellent option to reduce the toxic effects of conventional chemotherapies.

The aim of this study was to investigate the effect of EC in combination with cisplatin in A549 lung adenocarcinoma cells, assessing whether such combination achieves an additive effect on cytotoxicity.

Submitted: September 7, 2017.

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Abbreviations used: CDDP – cisplatin; EC – (-)-epicatechin; IC – inhibitory concentration.

MATERIALS AND METHODS

Cell lines. Hel-299 cells derived from human embryonic lung tissue and lung adenocarcinoma A549 cells were cultured under standard conditions with 5% CO₂ at 37 °C. Cells were treated with different concentrations of cisplatin [CDDP (cis-Diammineplatinum (ii) dichloride, Sigma)] (1–100 μM) or (-)-epicatechin (EC, Sigma) (0.1–1000 μM) or the combination of both compounds for 48 h. Both compounds were dissolved in DMSO (0.9%).

Cell viability. Cell viability was determined by MTT assay. Briefly, cell were incubated with 0.1 mg/ml MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) during 40 min at 37 °C. Purple formazan formed was solubilized using 0.01 M HCl-isopropanol. The dissolved material was measured spectrophotometrically at 595 nm (BiotekSynergy HT, USA). Each experiment was made in triplicate in independent assays (n = 5). Percent of cell viability was calculated as the ratio of the optical density in experiment to the optical density in control.

Isobolographic analysis. We plotted concentration — death curves for EC and CDDP using GraphPad Prism 5 software, afterwards an isobolographic analysis was provided. Briefly, after the inhibitory concentrations (IC) for each compound were calculated, theoretical values (e.g. IC₃₀, IC₁₅) of combinations in a fixed 1:1 ratio were obtained according to the equation (Eq. (1)).

Equation (1)

$$\frac{CDDP \text{ Theoretical}}{IC_x} + \frac{EC \text{ Theoretical}}{IC_x} = 1$$

If an additive effect exists, ½ EC effective concentration plus ½ CDDP effective concentration must be equal to 1. As an example, in a combination ½ EC (IC₃₀) + ½ CDDP (IC₃₀), if an additive effect exists there will be a 30% inhibition of cell growth.

In order to determine if the theoretical analysis corresponds to real effects in cells, the interaction of EC with CDDP was then experimentally evaluated by the simultaneous administration of ½ EC (IC_x) + ½ CDDP (IC_x) concentrations, where IC_x corresponds to different concentrations of compounds (we always used a 1:1 ratio). The experimental results obtained with the combinations employed were analyzed with equation 2 ascertaining the type of interaction between the two compounds:

Equation (2)

$$\frac{CDDP_{\text{Experimental}}}{IC_{30}} + \frac{EC_{\text{Experimental}}}{IC_{30}} = \text{Result}$$

When the analysis produces a result equal to 1, there is an additive effect. If the result is < 1, there is a synergism or supra-additive effect and, if the result is > 1 the interaction between compounds is antagonistic.

Acridine orange/ethidium bromide staining. The cells cultured on glass cover slips were stained with acridine orange/ethidium bromide

(15 μM/0.002 μM). For image display and acquisition, a fluorescence microscope (Nikon E600 Eclipse) was used. Images of 5 randomly selected fields were taken and the intensity of green and red fluorescence was measured using the ImageJ software version 1.38x (<http://rsb.info.nih.gov/ij>, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). A bright green color was observed in live cells while the cells in apoptosis and dead cells showed a bright red color.

RESULTS

Cell viability. The results indicate that the CDDP inhibits the growth of Hel-299 (Fig. 1, a) and A549 (Fig. 2) cells in a concentration dependent manner. EC does not inhibit proliferation of Hel-299 cells (Fig. 1, b) however, in A549 cells, EC induces a concentration dependent inhibitory effect (Fig. 3). From the aforementioned curves, we calculated IC (IC₃₀) for each compound.

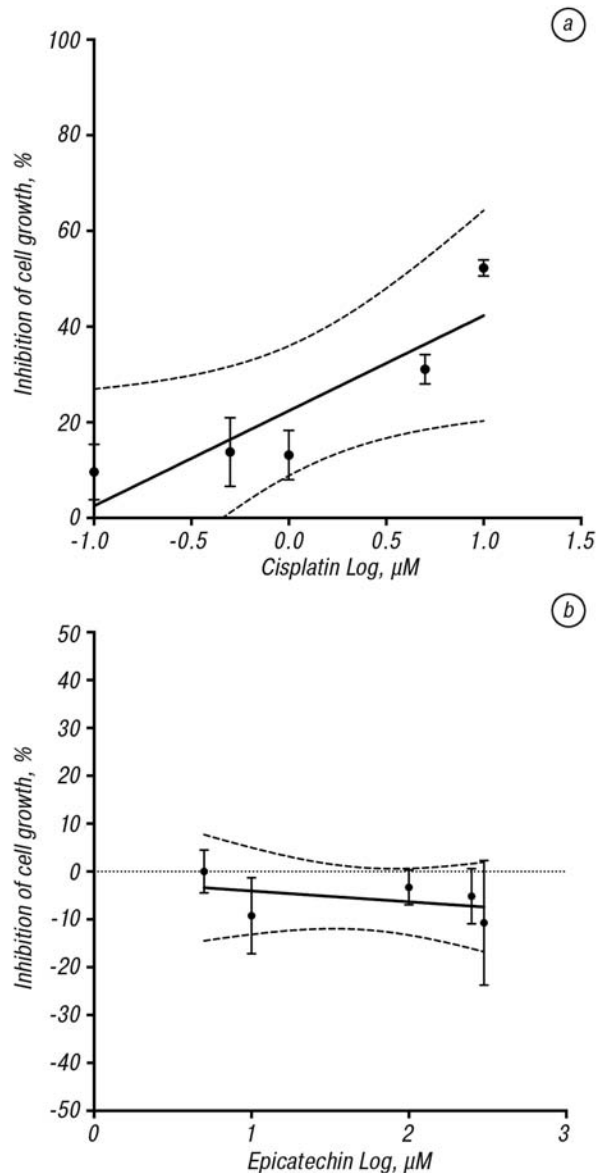


Fig. 1. Growth inhibition of Hel-299 cells: a) by cisplatin; b) by EC. Data are expressed as mean ± SEM. Linear regression curves were obtained using Graphpad Prism software

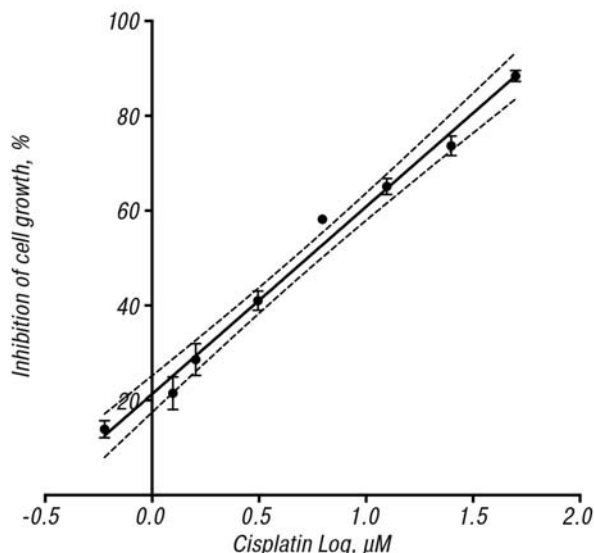


Fig. 2. Growth inhibition of A549 cells by cisplatin. Data are expressed as mean ± SEM. Linear regression curve was obtained using Graphpad Prism software

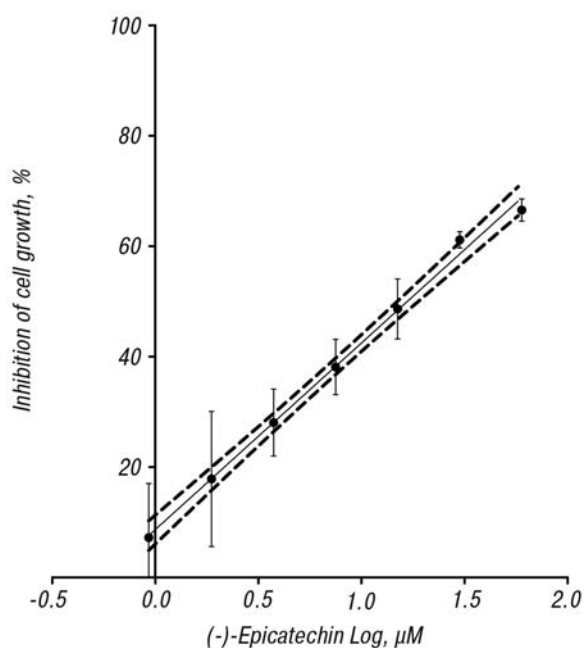


Fig. 3. Growth inhibition of A549 cells by EC Linear regression curve obtained using Graphpad Prism software. Data are expressed as mean ± SEM

Isobolographic analysis. After the IC for each compound were calculated, the theoretical IC₃₀ values of combinations in a fixed 1:1 ratio were obtained according to equation (1).

$$\frac{0.825 \mu\text{M}}{\text{IC}_{30} [1.65 \mu\text{M}]} + \frac{2.135 \mu\text{M}}{\text{IC}_{30} [4.27 \mu\text{M}]} = 1$$

Then, the effects of combinations of one half of IC₃₀ were explored experimentally and the result analyzed using equation (2) and plotted.

$$\frac{0.218 \mu\text{M}}{\text{IC}_{30} [1.65 \mu\text{M}]} + \frac{0.546 \mu\text{M}}{\text{IC}_{30} [4.27 \mu\text{M}]} = 0.26$$

We found that the combination of both compounds induces growth-inhibitory effect superior to that expected in case of the additivity (Fig. 4), the

isobolographic analysis results in an effect < 1 (0.26) when plotted below the line of additivity (Fig. 5). The effect is highly significant ($p = 0.0001$) indicating the synergistic antiproliferative effect of the combination of both compounds in A549 cells.

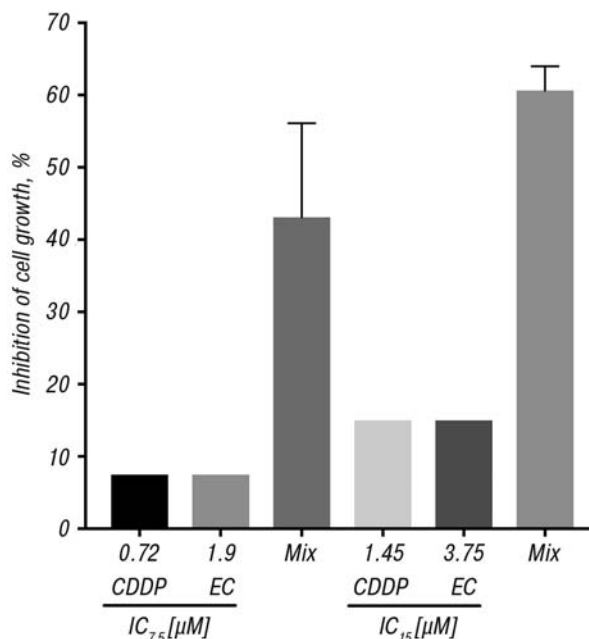


Fig. 4. Individual effects of IC_{7.5} and IC₁₅ of cisplatin and EC and the increased effect when both compounds are combined. Data are expressed as mean ± SEM

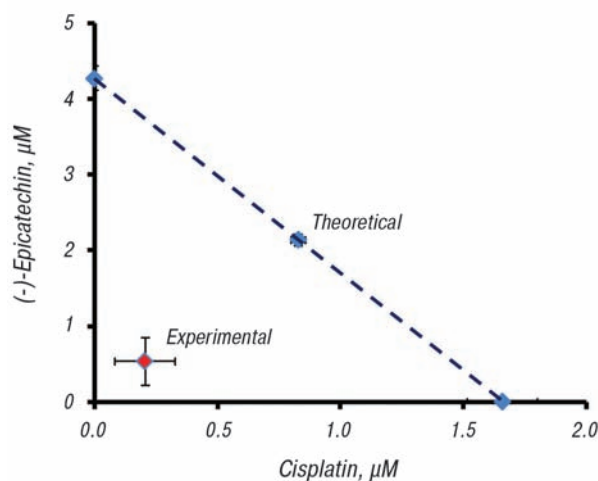


Fig. 5. Graphic representation of isobologram of growth-inhibiting effect of cisplatin and EC combined. Theoretical dotted line represents interaction if effects were additive. Experimentally obtained values show a synergistic interaction

Cell death. Results showed that the cell damage produced by the treatments with CDDP and/or EC is apoptotic. Upon staining with with acridine orange/ethidium bromide we analyzed and counted viable (green fluorescence) and dying (orange fluorescence) cells. Late apoptotic cells with condensed and fragmented nuclei stained by ethidium bromide are seen in specimens treated with CDDP, EC, or both suggesting the apoptotic mode of death (Fig. 6). Moreover, combination of both compounds increased the apoptotic percentage of A549 cells compared to that of CDDP or EC used as single agents (see Fig. 6).

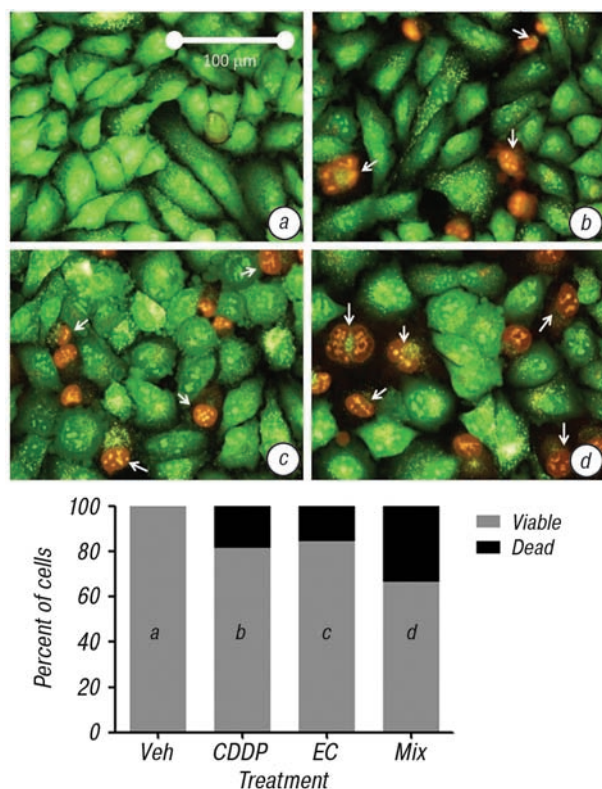


Fig. 6. Identification of cell death after 48 h treatment with compounds alone or in combination (acridine orange and ethidium bromide staining). Viable cells (green) and cells dying (orange) are shown (arrows indicate cells in apoptosis). Graphic representation of fraction of cells in apoptosis

DISCUSSION

EC belongs to a class of polyphenols, the flavanols. It is commonly found in natural products like cacao and cacao products such as dark chocolate, and also in green tea. EC-induced effects seem to be specific since structurally related isomers (catechin, (-)-epigallocatechin gallate (EGCG), etc) [16, 17] do not induce similar effects or even act as antagonists of EC effects [16].

Antiproliferative activity of EC has been shown in stomach [11], prostate and ovary [12] cancer and adenocarcinoma cells A549 and PC-9 (lung cancer). It inhibits the proliferation of Hodgkin's lymphoma cells and Jurkat T cells, effect attributed to its ability to inhibit the binding of NF- κ B to DNA [18]. Interestingly, EC causes DNA damage and apoptosis in acute myeloid leukemia cells in rats [19].

The interest in studying EC effects in lung cancer cell is based on reported EC inhibitory effects on proliferation of cancer but not normal cells [11, 12]. Our results have shown that EC induced a concentration dependent inhibition of proliferation of lung cancer cell A549, without inhibition of proliferation of normal lung cells (Hel 299).

One possible mechanism of EC action is the inhibition of the Na⁺/H⁺ exchanger, in this way cell plasma membrane fluidity and cytosolic pH are disturbed, thus interfering with cell proliferation [20]. Other mechanisms proposed are acting through Erk and/or other signaling pathways leading to an activation

of mitochondrial oxidative phosphorylation, which interferes with Warburg metabolism decreasing the non-oxidative breakdown of glucose and increasing the mitochondrial dependent oxidative breakdown of pyruvate. Interestingly, we and others had been shown that EC induce mitochondrial biogenesis *in vitro* and *in vivo* [21–23]. Through these processes EC could interfere with cancer signaling, thus rendering the cells more susceptible to apoptosis, an effect that could be utilized to sensitize cancer cells to chemotherapy.

EC-induced effects are not limited to inhibition of proliferation, they extend to protective effects decreasing toxic effects of chemotherapeutic drugs in healthy tissues. It has been reported that EC inhibits mitochondrial and renal damage induced by cisplatin administration in mice [14].

On the other hand, it is well known that CDDP forms adducts or crosslinks in DNA, inhibiting replication [4], contributing to the generation of superoxide radicals and causing toxicity in cancer cells [5]. DNA damage in turn activates DNA-repair mechanisms and apoptosis through activation of the tumor suppressor protein P53. Although there are several described ways to develop resistance to cisplatin treatment, mutations in the *TP53* gene (encoding the P53 protein) have an important role in most cancer types and lung cancer has a high rate of specific mutation of *TP53*, so their response to treatment is poor [7].

With all these data in mind, the purpose of the present work was to analyze possible additive anti-proliferative effects of the combination of CDDP and EC. We used a pharmacological approach based on the analysis of the curves of concentration dependent inhibition of proliferation for CDDP and EC, from which inhibitory concentrations were calculated. We use an isobolographic analysis in order to precise the effects of the combination of CDDP with EC in A549 cells [24]. In the present study we demonstrate that co-treatment of A549 cells with CDDP plus EC (in a fixed 1:1 ratio) induced a significant synergistic effect. Moreover, we have shown the increase in apoptotic cell fraction upon the combined treatment as compared to CDDP or EC administered alone. We suggest that even if EC and CDDP act in different ways, the convergent pathways lead to the inhibition of cell growth and the increase in apoptosis.

In addition, the synergism observed between CDDP and EC contributes to the knowledge of the importance of diet rich in flavonoids in cancer prevention and improved response to treatment [25]. The findings reported here warrant the implementation of trials using EC as adjuvant in the treatment of non-small cell lung carcinoma increasing the cytotoxic effect of cisplatin on malignant cells, protecting normal cells and perhaps reducing secondary effects. We believe that the synergistic effect can set the base for more complex studies including clinical trials in search for the decrease of cisplatin-induced secondary or toxic effects. Finally, EC may be expected to use as co-

adjuvant of traditional chemotherapy helping to reduce dosing or at least reducing secondary effects.

Sources of funding CONACYT postdoctoral fellowship #290805-IPN to NN., CONACYT Masters fellowship to OV. This work was supported by an IPN grant and CONACYT grant # 253769 to GC.

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