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Pharmacological Induction of Heme Oxygenase-1 Reduces KB Cell Viability: Role of Carbon Monoxide

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Abstract

Heme oxygenase-1 (Hmox1) catalyzes the rate-limiting step in heme degradation, releasing iron, carbon monoxide (CO), and biliverdin. The aim of the present study was to investigate Hmox1 as a possible mechanism underlying propolis cytotoxic effects in KB cells. Cells were cultured for 24, 48 and 72 hours and treated with propolis or $SnCl_2$, known inducers of Hmox1 protein expression and activity. Propolis and $SnCl_2$ treatments decreased cell viability and induced Hmox1 expression. Furthermore, propolis increased LDH release and decreased dramatically reactive oxygen species (ROS) formation. Toxic effects of both propolis and $SnCl_2$ were reversed by tin-mesoporphirin (SnMP), a Hmox activity inhibitor. No significant effect was observed on p21 expression following propolis treatment. By contrast, $SnCl_2$ decreased ROS formation and increased p21 expression but did not affect LDH release. These results were further confirmed by the use of CO releasing molecule (tricarbonyldichlororuthenium dimer (II)) (CORM-II) treatment (10-40 μM). Our results suggest that propolis mediates KB cell cytotoxicity, in part by Hmox1 induction, and that KB cells are very sensitive to Hmox1 derived CO, a property that may be relevant for oral squamous cell carcinoma therapy.

Keywords: Squamous cell carcinoma; Hmox1 protein; Heme degradation; Lymphosarcoma

Introduction

Squamous cell carcinoma (SCC) is the most frequent malignant tumor of the oral cavity with poor clinical outcome. Over 197,000 deaths occur per year worldwide, of which 74% are in developing countries [1]. Among many risk factors, tobacco and alcohol are prevalent in the development of oral carcinogenesis, being involved in >75% of oral cancers in the USA, France and Italy [2,3]. Important advances have been made during the last decade in molecular understanding of oral cancer and its application for early and sensitive diagnosis, effective treatment and improved prognosis. In previous studies the potential of using heme oxygenase-1 (Hmox1) and p21, a well-known inhibitor of cellular proliferation, as markers for clinico-pathological features was reported [4,5]. Furthermore, a relation between malignant behavior and alteration of Hmox has been demonstrated. Elevated Hmox activity was found in renal adenocarcinoma, compared with juxtatumor or normal renal tissues and this elevation was attributed solely to Hmox1 gene expression [6]. In addition, increased expression of Hmox1 was detected in lymphosarcoma [7], benign prostatic hyperplesia and prostate cancer and hepatoma [8,9]. In human gliomas, Hmox1 may be a useful marker for macrophage infiltration as well as neovascularization [10]. In this regard, Abraham et al. showed that over-expression of Hmox1 gene potentiates pancreatic cancer aggressiveness, by increasing tumor growth, angiogenesis and metastasis and that inhibition of Hmox system may be of useful benefit for the future treatment of the disease [11]. However, the precise molecular signals by which Hmox1 regulates cellular proliferation in SCC have not been investigated so far.

Hmox isoforms catalyze the conversion of heme to carbon monoxide (CO) and biliverdin, with a concurrent release of iron, which can drive the synthesis of ferritin for iron sequestration [12]. To date, two Hmox isoforms have been shown to be catalytically active in heme degradation, and each is encoded by a different gene [13,14] . Hmox-2 is constitutively expressed in blood vessels, endothelium, testis and most other tissues and its levels are relatively unaffected by factors inducing Hmox1 [15]. Hmox1 is expressed under basal conditions and its expression and activity can be induced by oxidative stress-causing agents, heavy metals and polyphenolic compounds such as rosolic acid, caffeic acid phenethyl ester (CAPE) [16-20]. This is an active compound of propolis, a natural honeybee product exhibiting a spectrum of biological activities, including anti-microbial, anti-

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inflammatory, anti-oxidant and anti-tumoral actions [21-23]. Most of these properties have been attributed, in part, to CAPE anti-oxidant activity [22,24,25], which is primarily due to the phenolic hydroxyl groups being able to furnish hydrogen atoms in scavenging reactive oxygen species (ROS). It has been suggested that ROS may play a key role in signal transduction and activation of specific genes promoting cancer cell proliferation [26]. As such, scavenging ROS with phenolic phytochemicals should inhibit these cellular processes and thus cancer cell proliferation. However, we and others [18,27] suggested a potential novel aspect in the mode of action of phenolic phytochemicals; that is, the ultimate stimulation of Hmox1 pathway is likely to account for the established and powerful antioxidant/anti-inflammatory properties of these polyphenols. Our recent studies [28] evidenced that KB cells are more sensitive to the Chilean propolis ethanolic extract, containing a high concentration of CAPE and exhibiting interesting antioxidant activity, when compared to others tumor cell lines such as Caco-2 and DU-145. In addition, it has been reported that other oral tumor cell lines are more sensitive to CAPE treatment when compared to non tumoral cell lines [29].

Therefore, the present study was designed to evaluate the effect of Chilean propolis on the SCC Hmox system and how this may impact on ROS formation and molecular mechanism leading to cellular proliferation mechanisms.

Materials and Methods

Materials

Cell culture medium and sera were obtained from Life Technologies Ltd. (Milano, Italy). Monoclonal Hmox1 and Hmox-2 antibodies were from Stressgen Biotechnologies (Victoria, BC, Canada). Secondary horseradish peroxidase-conjugated anti-mouse antibody and p21 monoclonal antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ECL (enhanced chemiluminescence) system for developing immunoblots and nitrocellulose membranes was purchased from Amersham (Milano, Italy). Tricarbonyldichlororuthenium was purchased from Sigm-Aldrich (Milan, Italy). All other chemicals were purchased from Merck (Frankfurt, Germany).

Propolis sample

Propolis ethanolic extract was provided by NATURANDES-CHILE. Propolis sample was collected at San Vicente de Tagua-Tagua. One kg of propolis sample was mixed with 5 liters of 60% ethanol and stirred for 24 h at 20°C. After stirring and filtering under vacuum, the filtrate was evaporated to dryness in a Rotavapor. The dry starting material was 1000 g of propolis. The extraction yield was 450 g (45%). The extract was previously standardized [28] and HPLC analysis showed that it had the following composition: galangin 0.43%; hydroxycinnamic acids (caffeic acid 3.85%; p-cumaric acid 0.02%, ferulic acid 0.04%), CAPE 22.30%.

Cell culture and treatments

KB cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and were maintained in RPMI supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained at 37°C under humidified 5% carbon dioxide to allow cell attachment. Cells were then harvested by trypsinization and differently treated with 80 µg/ml concentration of Chilean propolis ethanolic extract in the presence or absence of 10 µM $SnCl_2$ and 15 µM tin-mesoporphirin (SnMP), an inducer and inhibitor of Hmox activity respectively. Even though the ethanolic extract of propolis was dissolved in ethanol, at the treatment stage the final ethanol concentration was never higher than 0.05%. Under these conditions, ethanol was not toxic and did not alter the parameters tested. In order to evaluate the role of CO in this system, we used tricarbonyldichlororuthenium (II) dimer (CORM-II), a well-known and characterized CO releasing molecule (26), at different concentrations (10-40 µM) and time exposures (24, 48 and 72 h). Inactive form of the compound (negative control) was also used in some experiments and it was prepared as follows: CORM-2 was 'inactivated' (iCORM-2) by adding the compound to DMSO and leaving it for 18 h at 37℃ in a 5% CO_2 humidified atmosphere to liberate CO. The iCORM-2 solution was finally bubbled with nitrogen to remove the residual CO present in the solution. This preparation was referred as control in all experiments with this compound. At the end of the treatment cells were scraped, washed with cold phosphate buffered saline (PBS) and immediately processed. The concentration of proteins in the cellular lysate was determined according to the method of Bradford [30].

MTT assay

Cells were set up 6×10^3 cells per well of a 96 multiwell flat-bottomed 200 µl microplate. Cells were then incubated at 37°C in a humidified 5% CO_2/95% air mixture. At the end of treatment time, 20 µl of 0.5% MTT 3(4,5-dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide in PBS were added to each microwell. After one hour of incubation with the reagent, the supernatant was removed and replaced with 100 µl of DMSO. The optical density of each well sample was measured with a microplate spectrophotometer reader (Digital and Analog Systems, Rome, Italy) at 550 nm.

Lactic dehydrogenase (LDH) release

Lactic dehydrogenase (LDH) activity was spectrophotometrically measured in the culture medium and in the cellular lysates at 340 nm by analyzing NADH reduction during the pyruvate-lactate transformation. Cells were lysed with 50 mM Tris-HCl + 20 mM EDTA pH 7.4 + 0.5% sodium dodecyl sulfate (SDS), further disrupted by sonication and centrifuged at 13,000g for 15 min. The assay mixture (1 ml final volume) for the enzymatic analysis contained: 33 µl of sample (5-10 µg of protein) in 48 mM PBS pH 7.5 plus 1 mM pyruvate and 0.2 mM NADH. The percentage of LDH released was calculated as percentage of the total amount, considered as the sum of the enzymatic activity present in the cellular lysate and that in the culture medium. A Hitachi U-2000 spectrophotometer (Hitachi, Tokyo, Japan) was used.

Western blotting

Cell lysate was collected for Western blot analysis and protein levels were visualized by immunoblotting with antibodies against Hmox1, Hmox-2 or p21 as previously described [31]. Briefly, 30 µg of lysate supernatant were separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were incubated overnight with 5% milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20 (TBST) buffer at 4°C. After washing with TBST, the membranes were incubated with a 1:1000 dilution of anti-Hmox1, anti-Hmox-2 or p21 antibody for 1 hour at room temperature with constant shaking. The filters were then washed and subsequently

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probed with horseradish peroxidase-conjugated anti-mouse IgG (Amersham) for Hmox1 and p21 at a dilution of 1:2000, or horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) for Hmox-2 at a dilution of 1:5000. The used Hmox1 antibody recognizes the full length (32 Kda) form of the protein which possesses the complete enzymatic activity. Actin was also used for normalization. Chemiluminescence detection was performed using an ECL detection kit according to the manufacturer's instructions.

ROS determination

ROS determination was performed by using a fluorescent probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA), as previously described [32]. DCFH-DA diffuses through the cell membrane, it is enzymatically hydrolyzed by intracellular esterases and oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. One hundred microliters of 100

M DCFH-DA, dissolved in 100% methanol was added to the cellular medium where the acetate group is not hydrolysed [32], and the cells were incubated at 37°C for 30 min. After incubation, KB cells were lysated and centrifuged at 10,000 g for 10 min. The fluorescence (corresponding to the radical species-oxidized 2', 7'-dichlorofluorescein, DCF) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan): excitation 488 nm, emission 525 nm. The total protein content was evaluated for each sample, so the results are reported as Fluorescence Intensity/mg protein and compared to relative control.

Heme oxygenase activity assay

Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH (0.8 mm), glucose 6-phosphate (2 mm), glucose-6-phosphate dehydrogenase (0.2 units), 3 mg of rat liver cytosol prepared from a 105,000 × g supernatant fraction as a source of biliverdin reductase, potassium phosphate buffer (PBS, 100 mm, pH 7.4), MgCl2 (0.2 mm), and hemin (20 μm). The reaction was conducted at 37°C in the dark for 1 h and terminated by the addition of 1 ml of chloroform, and the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (ε=40 mm−1 cm−1). Heme oxygenase activity was expressed as picomoles of bilirubin/mg of cell protein/h.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Bonferroni's t test was performed in order to estimate significant differences among groups. Each value represents the mean ± SD of three separate experiments performed in duplicate and differences between groups were considered to be significant at $p<0.005$.

Results

Effects of Chilean propolis on KB cells viability

The effects of Chilean propolis extract on KB cell viability following treatment with different concentrations and time exposures are shown in Figure 1A. Treatment of cell cultures for 24, 48 and 72 h with propolis (80 μg/ml) containing high concentration of CAPE, a well-known inducer of Hmox1 expression and activity (35), resulted in a time-dependent decrease in viability ($p<0.001$). Similar results were obtained by using $SnCl_2$ (10 μM), also a well-known inducer of Hmox1 protein expression and activity [19]. In addition, the combination of both propolis and $SnCl_2$ showed a decreased viability when compared to propolis or $SnCl_2$ alone ($p<0.001$). Interestingly, the addition of SnMP, a potent inhibitor of Hmox1 activity (6), significantly increased viability in propolis or propolis plus $SnCl_2$ treated cultures ($p<0.001$). The exposure of cell culture to SnMP alone did not show any significant changes in cell viability when compared to control. We also tested the effects of CORM-II at different concentrations and time exposures, observing a dramatic dose and time dependent decrease in viability, which suggests that KB cells are particularly sensitive to CO when compared to non tumoral cell types [10] (Figure 1B). Concentrations of both propolis and $SnCl_2$, not toxic for normal cell types, derive from our preliminary experiments (unpublished results) where they showed maximal biological effect.

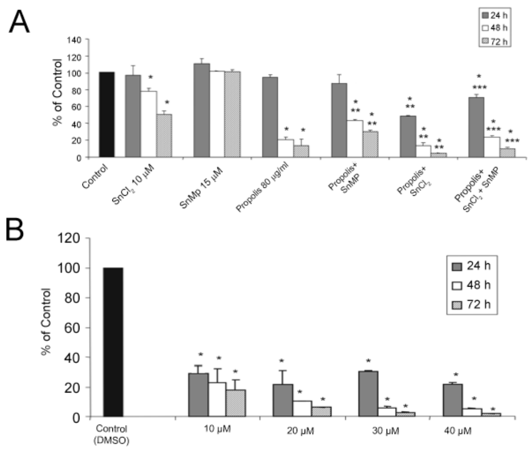

Figure 1. Viability of KB cells treated at different time exposures (24, 48 and 72 h). (A) Cells were treated with Chilean propolis ethanolic extract (80 μg/ml) in the presence or absence of 10 μM $SnCl_2$ or 15 μM SnMP. (B) Cells were treated with different concentrations (5-50 μM) of tricarbonyldichlororuthenium (II) dimer (CORM-II). Each value represents the mean ± SD of three separate experiments performed in duplicate. (*$p<0.001$ vs. Control; **$p<0.001$ vs. Propolis; ***$p<0.001$ vs. Propolis + $SnCl_2$).

LDH release determination

LDH release was also measured to evaluate the presence of cell necrosis as a result of cell disruption subsequent to membrane rupture (Figure 2). Under our experimental conditions, treatment of cell cultures with propolis resulted in a significant increase in LDH release ($p<0.001$) at 72 h. Surprisingly, $SnCl_2$ treatment did not result in a significant release of LDH, thus indicating that a different mechanism, such as cell cycle arrest, may occur in $SnCl_2$ mediated cytotoxicity. Furthermore, propolis plus $SnCl_2$ treated cultures showed a significant increase in LDH release when compared to propolis or $SnCl_2$ alone and this effect was abolished by the addition of SnMP, thus suggesting that induction of Hmox activity renders KB cells more susceptible to propolis mediated cell necrosis.

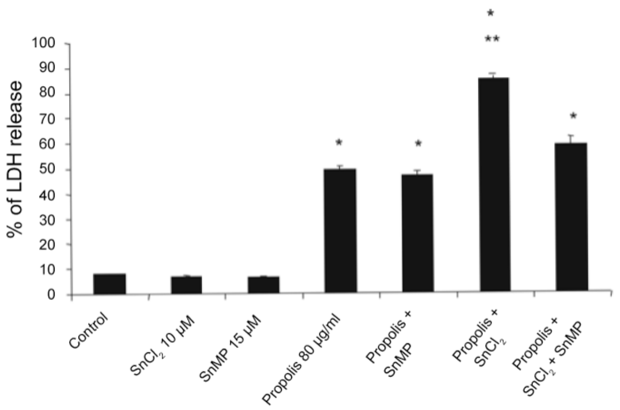

Figure 2. LDH released in KB cells untreated and treated for 72 h with Chilean propolis ethanolic extract (80 µg/ml) in the presence or absence of 10 µM $SnCl_2$ or 15 µM SnMP. Each value represents the mean ± SD of three separate experiments performed in duplicate. (* p<0.001 vs. Control; ** p<0.001 vs. Propolis).

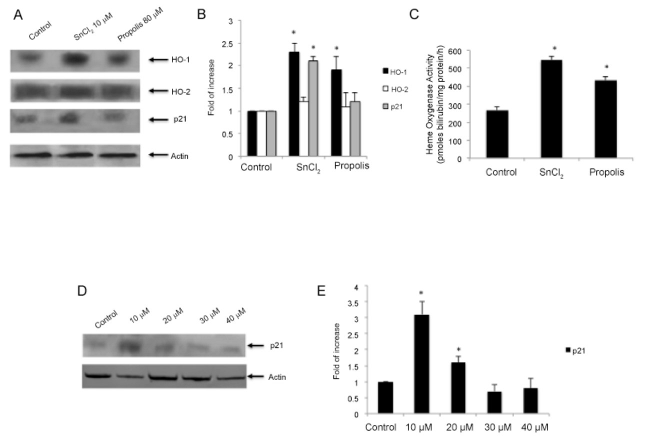

Figure 3. (A) Western Blot analysis of Hmox1, Hmox-2 and p21 following treatments for 72 h with Chilean propolis ethanolic extract (80 µg/ml) and 10 µM $SnCl_2$. (B) Densitometric analysis of western Blot following actin normalization. (C) Hmox activity following different pharmacological treatment. (D) p21 protein expression following treatments with tricarbonyldichlororuthenium (II) dimer (CORM-II) at different concentrations (5-50 µM). (E) Blots shown are representative of Western blot analysis from three separate experiments (*p<0.001 vs control).

Western Blot analysis

Cells were examined for the levels of Hmox1 and Hmox-2 proteins by Western blot analysis. The results of three representative experiments are reported in Figure 3A. Cells showed basal levels of Hmox1 protein and a significant increase after treatment with propolis and $SnCl_2$ as compared to untreated cells (Figure 3A and 3B). No significant effects were observed on Hmox-2 protein levels after pharmacological treatments (Figure 3A and 3B). SnMP, a transcriptional activator of Hmox1 gene and inhibitor of Hmox activity, did not change Hmox1 protein expression in propolis or $SnCl_2$ treated cultures (data not shown) thus confirming our previous studies [1,16,17].

In order to further elucidate the molecular mechanism leading to decreased cell viability, we also examined the expression of p21, a well-known inhibitor of cell cycle progression. The addition of $SnCl_2$ to the culture medium resulted in a significant increase of p21 protein expression (Figure 3A, 3B and 3C). By contrast, propolis was not able to induce p21 protein, thus suggesting that propolis mediated cell number decrease may be related to necrotic cell death. In order to establish a link between Hmox1 derived CO and p21 expression in KB cells, we determined the expression of p21 following CORM-II treatment at different concentrations (Figure 3D and 3E). This set of experiments showed a marked increase of p21 expression following treatment with 10 and 20 µM concentrations whereas higher concentrations did not show any significant effects, suggesting that low CO levels regulate KB cell proliferation via p21 upregulation, but higher concentrations are toxic. Of note is the fact that the same concentrations of CORM-II did not show any significant toxicity on other not tumoral cells such as endothelial cells, and astroglial cells (data not shown) and cardiomyocytes [33], thus further suggesting that KB cells are particularly sensitive to CO.

ROS determination

ROS were determined using a fluorescent probe DCFH-DA. The probe diffuses into the cells, intracellular esterases hydrolyze the acetate groups, and the resulting 2',7'-dichlorofluorescin (DCFH) then reacts with intracellular oxidants resulting in the observed fluorescence. The intensity of fluorescence is proportional to the levels of intracellular oxidant species. As shown in Figure 4, the addition of propolis or $SnCl_2$ for 72 h resulted in a significant decrease in ROS formation when compared to control. This effect was reversed, in part, by the addition of SnMP, thus suggesting that the phenolic components of propolis per se play a key role in the anti-oxidant properties of propolis.

Discussion

There is increasing evidence for an association between a high consumption of fruit and vegetables and reduced risk of oral cancer, suggesting that natural products offer a protective effect against oral cancer [34,35]. In addition many substances derived from dietary or medicinal plants are known to be effective and versatile chemopreventive and antitumoral agents in a number of experimental models of carcinogenesis [36]. In this regard, Li et al. [37] showed that curcumin, a natural Hmox1 inducer, present in turmeric and curry and possessing antioxidant properties, appeared to have an inhibitory effect on the progression from dysplasia to SCC.

We describe, in the present study, the effects of pharmacological induction of Hmox1 using Chilean propolis and $SnCl_2$ in KB cells, and how this may impact on KB cell cytotoxicity and proliferation. We demonstrated that Chilean propolis and $SnCl_2$ showed a significant increase in Hmox1 protein expression which was followed by a decrease in cell viability and this effect was reversed by the addition of SnMP, thus suggesting that Hmox1 may play an important role in both propolis and $SnCl_2$ toxicity. These results are consistent with our previous results showing that KB cells are particularly sensitive to propolis [28]. The possible involvement of the Hmox system was further suggested by the use of CORM-II which showed that, CO, one of the Hmox products, is toxic for KB cells in a dose- and time-dependent manner. We also propose that propolis has different mechanisms of toxicity in KB cells. In fact, we found that this compound caused a significant decrease in cell number as a result of cell necrosis as measured by LDH release. By contrast, $SnCl_2$ showed a significant decrease in cell number unaccompanied by a concomitant cell membrane breakdown. In this case, decreased cell number may be related to the increased expression of Hmox1 which leads to increased CO cellular levels, thus upregulating p21 protein expression. This hypothesis is supported by our experiments with CORM-II, which showed that increased CO levels result in a significant increase in p21 protein expression. These results are in apparent contrast with our recent work showing that the same concentrations of CO releasing molecule resulted in a significant increase of endothelial cell proliferation and angiogenesis, as measured by capillary formation; however high concentrations of CO releasing molecule resulted toxic for endothelial cells and inhibited angiogenesis [38]. These data obtained on different cell types suggest that CO regulates cell proliferation in a cell-specific, dose- and time-dependent manner. In addition, these results are consistent with our previous work [39] demonstrating that Hmox1 regulates proliferation in a cell-specific manner by differentially regulating p21 protein expression; in fact, pharmacological Hmox1 induction increased endothelial cell proliferation, but inhibited smooth muscle cell proliferation. Furthermore, we and others demonstrated that the same CORM-II concentrations showed no significant cytotoxic effects in other cell types such as astrocytes, endothelial cells, smooth muscle cells and cardiomyocyte [33,38,40].

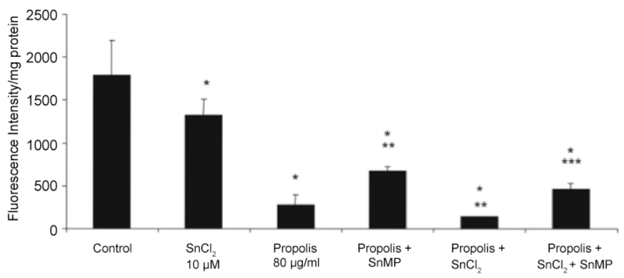

Figure 4. Intracellular ROS formation in KB cells untreated and treated with Chilean propolis ethanolic extract (80 µg/ml) for 72 hours in the presence or absence of 10 µM $SnCl_2$ or 15 µM SnMP. Each value represents the mean ± SD of three separate experiments performed in duplicate. (*p<0.001 vs. Control; **p<0.001 vs. Propolis; ***p<0.001 vs. Propolis + $SnCl_2$).

The idea of the involvement of different mechanisms in propolis toxicity, besides Hmox1 induction, is supported also from our ROS formation data showing that propolis, because of its anti-oxidant extract phenolic components activity (galangin, caffeic acid, p-cumaric acid, ferulic acid and CAPE) [22], resulted in a dramatic reduction in the formation of ROS, a mechanism involved in cancer cell proliferation [41]. This hypothesis is supported also by previous studies showing that phenolic phytochemicals may scavenge the constitutively high amounts of ROS in cancer cells, thereby blocking MAPK signaling, activation of NFkB and AP-1, and ultimately the expression of responsive genes that stimulate cancer cell proliferation [26].

The addition of SnMP, significantly attenuated the anti-oxidant effects of propolis, even though ROS remained significantly low when compared to control, thus confirming that the anti-oxidant properties of this compound are mediated in part by Hmox1 induction and also by the phenolic structure of propolis components. These data are also confirmed by our observations showing that $SnCl_2$ caused a significant decrease in ROS formation eventhough propolis was a more potent ROS scavenger.

Taken all together, our data indicates that KB cells seem to be particularly vulnerable to Hmox1 induction which may represent a mechanism by which these cells regulate their proliferation and cell cycle progression, thus suggesting that the Hmox system may be the Achille's heel of KB cells. In fact, pharmacological induction of Hmox1 is associated with decreased cell proliferation following p21 upregulation and increased cytotoxicity. These effects seem to be mediated by Hmox derived CO as suggested by the results following CORM-II treatment. Furthermore, other minor sources of CO include the auto-oxidation [42] of phytochemical phenols which may account, in part, for propolis mediated cytotoxicity. These in vitro results seem to be consistent with recent clinical findings showing that increased Hmox1 expression was associated with reduced lymph node metastasis in patients affected by oral SCC [5]. These results together with our recent data on angiogenesis strongly suggest that CO may represent an excellent strategy for controlling cancer growth.

In conclusion, our studies demonstrate that Chilean propolis, due to its phenolic components, and $SnCl_2$ not only have cytotoxic and antiproliferative effects in KB cells, but also utilize Hmox1 in exerting their antitumoral effects, thus providing a new and powerful strategy for oral SCC treatments.

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