Università
degli Studi
della Campania
Luigi Vanvitelli

DOTTORATO DI RICERCA IN "SCIENZE BIOCHIMICHE E BIOTECNOLOGICHE" XXXIV CICLO

SETTORE SCIENTIFICO-DISCIPLINARE BIO/12

Curriculum: Biologia, Biochimica e Biotecnologie Mediche

ANNO ACCADEMICO 2020-2021

Chlorogenic Acid as a naturally occurring molecule with potential anticancer action: its biochemical and biological effects in human osteosarcoma cells

Dottorando: **Dott.ssa Alessia Salzillo**

Docente guida: **Prof. Silvio Naviglio**

Coordinatore: **Prof. Fulvio Della Ragione**

Table of Contents

SUMMARY

Chlorogenic acid (CGA) is a biologically active dietary diphenol derived from the esterification of hydroxyhymamic acids (including caffeic, ferulic and p-cumaric) with quinic acid [1]. It is present in significant amounts in green coffee beans and other plant-based compounds such as herbs, fruits and vegetables [2].

In addition to its antimicrobial, anti-inflammatory and antioxidant properties, several evidence has highlighted a possible antitumor role of CGA in various cancerous models, including leukemia, hepatocarcinoma, lung and breast cancer [3, 4]. Indeed, pharmacological administration of CGA has shown relevant anticancer effects, such as induction of mesenchymal-epithelial transition [5], inhibition of proliferation and migration [6], and enhancement of the sensitivity to conventional antineoplastic drugs [7,8].

Until today, instead, very few evidence has been obtained in osteosarcoma (OS) concerning CGA as antitumor agent [9,10]. Evaluating the biological activity of organic/inorganic PEGsilica hybrid materials containing CGAs, my research group observed a pronounced antiproliferative action of CGA in OS cell model both as a free molecule and as part of these PEG-silica materials [11]. However, the anticancer role of CGA was only marginally investigated in that study. For all the reasons above, the aim of my thesis has been to explore, firstly, the anticancer properties of CGA alone and then in combination with one of the anticancer drugs conventionally used in the treatment of OS, named Doxorubicin (Doxo). In addition to OS, Doxo is currently approved for the treatment of other neoplastic disorders, such as gynecological, breast and lung cancers [12]. Unfortunately, in addition to the resistance mechanisms that tumor cells develop over time, Doxo practice in oncology is severely limited by cardiovascular toxicity that occurs in both cancer patients and survivors [13]. To bypass these problems, in recent years, the chemotherapy combination is receiving more attention, in order to increase the therapeutic index of anti-neoplastic drugs and also to reduce the side effects [14]. Indeed, identifying an innovative

combination chemotherapeutic strategy, in which one novel compound is able to minimize the dosage of chemotherapeutic drugs required to obtain antitumor effects, would definitely advantage cancer patients. At this regard, naturally occurring molecules constitute possible attractive candidates [15,16]. In this context, CGA usage in combination therapy could increase the pharmacological action of Doxo and minimize its side effects, especially those due to cardiotoxicity.

The experimental approach, initially, has foreseen the investigation of the CGA's effects on the cell viability, cell growth, and colony-forming ability in different OS cell lines. Our data showed that CGA exerts significant growth and viability inhibitory effects on U2OS, MG-63 and SAOS-2 with a different sensitivity among them (U2OS and MG-63 the most and the least sensitive, respectively). Furthermore, we demonstrated that CGA exposure affects cell cycle division and causes cell death in U2OS mainly by apoptosis induction, as suggested by the increase of the apoptotic rate and the activity of caspase-3 and PARP. Besides showing promising anticancer features in single therapy administration, CGA also enhances the Doxo mediated outcome. Specifically, although CGA differently affected U2OS and MG-63's behaviors, combination treatment mostly impaired cell viability and growth, as well as colony-forming ability, compared with single agents. Importantly, we observed that CGA oppositely affected OS and cardiac cells when co-administrated with Doxo, enhancing Doxo-induced cytotoxicity in OS cells and ameliorating cell behaviours in H9c2 cardiomyocytes, respectively.

Finally, the research activity focused on the characterization of the molecular mechanisms underlying the antiproliferative and pro-apoptotic activity of the CGA in OS. In this regard, and in line with some of the scientific evidence, significant results highlighted an important involvement of the MAPK pathway, in particular of p44/42 protein [17,18]. In details, we observe that CGA-induced antiproliferative/proapoptotic effects in U2OS cells are accompanied by a strong increase of p44/42 phosphorylation in all times observed. The use of PD98059 compound, to affect the CGA- mediated p44/42 activation, further increased cell growth

inhibition and death, strengthening the hypothesis of a possible pro-survival and antiapoptotic role of p44/42 activation in response to CGA. Given the CGA ability of modulating p44/42 MAPK, we investigated the influence of CGA plus Doxo on this protein. Even in this case, the employment of the PD98059 inhibitor in our experimental conditions promoted combination treatment efficacy.

With this in mind, our findings significantly enforce the evidence of in vitro anticancer activity by CGA alone and in Doxo co-treatment, encouraging the design of future in vivo/clinical studies in order to explore the CGA as “sensitizer” of antitumor drugs for possible use in OS therapy.

SOMMARIO

L'Acido Clorogenico (CGA) è una sostanza organica naturale biologicamente attiva, derivata dall'esterificazione degli acidi idrossiimamici (inclusi caffeico, ferulico e p-cumarico) con acido chinico [1]. È presente in quantità significative nei chicchi di caffè verde e in altri composti vegetali come erbe, frutta e verdura [2]

Oltre alle sue proprietà antimicrobiche, antinfiammatorie e antiossidanti, diversi studi hanno evidenziato un possibile ruolo antitumorale del CGA in diversi modelli cancerosi, tra cui leucemia, epatocarcinoma e cancro al seno [3,4]. Infatti, la somministrazione farmacologica di CGA ha mostrato effetti antitumorali rilevanti, come l'induzione della transizione mesenchimale-epiteliale [5], l'inibizione della proliferazione e della migrazione [6], e anche il potenziamento della sensibilità ai farmaci antineoplastici convenzionali [7,8].

Fino ad oggi, pochissime evidenze sono state ottenute nell'osteosarcoma (OS) riguardanti il CGA come possibile agente antitumorale [9,10]. In studi pregressi, il mio gruppo di ricerca ha valutato l'attività biologica di biomateriali, ibridi organici/inorganici, e in particolare, di materiali PEGsilica contenenti CGA, notando una pronunciata azione antiproliferativa di CGA in modelli cellulari di OS sia come molecola libera che come parte di materiali PEG-silice [11]. Tuttavia, il ruolo antitumorale del CGA è stato marginalmente indagato in quello studio. Per i motivi sopra citati, lo scopo della mia tesi è stato quello di studiare le proprietà antitumorali del CGA da solo, in primo luogo, e poi in combinazione con uno dei farmaci antitumorali convenzionalmente utilizzati nel trattamento della OS, ovvero la doxorubicina (Doxo). Oltre che per la cura dell'osteosarcoma, la doxorubicina è attualmente usata anche per il trattamento di altre neoplasie, come i tumori ginecologici, mammari e polmonari [12].

Sfortunatamente, oltre ai meccanismi di resistenza che le cellule neoplastiche sviluppano nel tempo, l'utilizzo di Doxo in oncologia è fortemente limitato dalla tossicità cardiovascolare che si verifica sia nei pazienti oncologici che nei sopravvissuti al tumore [13]. Per bypassare questi

problemi, negli ultimi anni, la combinazione chemioterapica sta ricevendo maggiore attenzione, al fine di aumentare l'indice terapeutico dei farmaci antineoplastici e di ridurre gli effetti collaterali [14]. In effetti, identificare un'innovativa strategia chemioterapica combinata, in cui un nuovo composto è in grado di ridurre al minimo il dosaggio dei farmaci chemioterapici necessari per ottenere effetti antitumorali, sarebbe sicuramente vantaggiosa per i malati di cancro. A questo proposito, le molecole presenti in natura costituiscono possibili candidati interessanti [15,16]. In questo contesto, l'uso di CGA nella terapia di combinazione potrebbe aumentare l'azione farmacologica di Doxo e minimizzarne gli effetti collaterali, soprattutto quelli dovuti alla cardiotossicità.

L'approccio sperimentale ha previsto, inizialmente, lo studio degli effetti del CGA sulla vitalità cellulare, sulla crescita cellulare e sulla capacità di formazione di colonie in diverse linee cellulari di OS. I nostri esperimenti hanno dimostrato che il CGA esercita significativi effetti inibitori di crescita e vitalità sulle cellule U2OS, MG-63 e SAOS-2 con una sensibilità diversa tra loro (le cellule U2OS e quelle MG-63 le più e le meno sensibili, rispettivamente). Inoltre, i dati ottenuti indicano che l'esposizione al CGA influenza la divisione del ciclo cellulare e causa la morte cellulare nelle cellule U2OS, principalmente per induzione dell'apoptosi, come suggerito dall'aumento del tasso apoptotico e dall'attività di caspase-3 e PARP. In aggiunta, i nostri dati evidenziano l'efficacia del CGA nel migliorare l'azione della Doxo nelle cellule di OS. In particolare, di interesse è l'evidenza che il trattamento combinato con Doxo e CGA ulteriormente inibisce la vitalità e la crescita delle cellule U2OS e MG-63, nonché la capacità di formazione di colonie, rispetto ai singoli agenti. Come prova dell'interazione sinergica farmaco-farmaco, l'analisi CompuSyn ha svelato valori CI inferiori a uno in tutte le condizioni sperimentali testate.

Molto interessante, inoltre, è la nostra evidenza che il CGA influenza in modo opposto le cellule di OS e quelle cardiache quando co-impiegato con Doxo: da un lato il CGA, infatti,

aumenta la tossicità indotta dalla Doxo nelle cellule di OS, dall'altro, invece, riduce quella indotta dalla stessa Doxo nei cardiomiociti H9C2.

Infine, l'attività di ricerca si è rivolta alla caratterizzazione dei meccanismi molecolari alla base dell'attività antiproliferativa e pro-apoptotoca del CGA in OS. A tal proposito, e in linea con alcune delle evidenze scientifiche presenti, i risultati ottenuti nel mio lavoro di tesi suggeriscono un importante coinvolgimento del pathway delle MAPK e in particolare di p44/42 [17,18]. Nel dettaglio, i dati indicano che gli effetti antiproliferativi/proapoptotici indotti dal CGA nelle cellule U2OS sono accompagnati da un forte aumento della fosforilazione di p44/42, in tutti i tempi osservati. L'uso del composto PD98059, inibitore a monte della chinasi p44/42, che contrasta l'attivazione di p44/42 indotta dal CGA, ne aumenta ulteriormente l'azione antiproliferativa e morte cellulare, rafforzando l'ipotesi di un possibile ruolo pro-sopravvivenza e anti-apoptotico dell'attivazione di p44/42 in risposta al CGA. Considerata la capacità del CGA di modulare p44/42 MAPK, abbiamo, inoltre, studiato l'impatto della combinazione CGA/Doxo sulla fosforilazione/attivazione di questa chinasi. Anche in questo caso, l'impiego dell'inibitore PD98059 nelle nostre condizioni sperimentali ha favorito l'efficacia del trattamento combinato.

Sintetizzando, i risultati raccolti in questa tesi rafforzano significativamente l'evidenza dell'attività antitumorale in vitro da parte del solo CGA e nel co-trattamento con Doxo, incoraggiando la progettazione di futuri studi in vivo/clinici al fine di studiare il CGA come "sensibilizzante" di farmaci antitumorali per un possibile uso nella terapia dell'OS.

1. INTRODUCTION

1.1 Chlorogenic Acid (CGA)

1.1.1 Dietary consumption and CGA assimilation

Chlorogenic acids (CGAs) are phenolic compounds derived from the esterification of hydroxycinnamic acids (including caffeic, ferulic, and p-coumaric) with quinic acid [1]. Among the others, 3-caffeoyl quinic acid (3-CQA) is the most abundant and well-characterized isoform in nature, enough to be known with the trade name of CGA [2]. Green coffee beans represent the major source of CGA, even if a relevant amount has been also found in other plant-related products such as herbs, fruits, and vegetables [19]. In fact, CGA is also included in apples, pears, peaches, plums, cherries, tomatoes, and potatoes [20]. In the human body, intact CGA overpasses the gastric tract and is absorbed in intestinal levels both as an entire molecule and as cleavage of quinic and caffeic acid by gastrointestinal microflora [21,22]. Briefly, the metabolic steps are indicated as follows: (I) in humans, part of the CGA (about 33%) from food is absorbed intact, without hydrolysis, in the stomach and/or the upper part of the gastrointestinal tract, and enters into the bloodstream; (II) a small amount of the CGA (around 7%) is absorbed throughout the small intestine involving its hydrolysis into caffeic acid (CA) and quinic acid (QA); (III) colonic microbiota mediates CGA metabolism helping the absorption of metabolites in the colon; (IV) after the intact CGA and its metabolites enter the bloodstream, they are absorbed [23]. Indeed, following the hydrolysis, methylation, glucuronidation and other reactions that happen when the CGA dimer enters the digestive and metabolic systems, CGA's metabolites were observed in blood and urine, respectively, inducing and exhibiting various biological activities [24,25].

Interestingly, CGA is one of the most commercially important herbal ingredients for weight loss dietary supplements in the global market [26]. Its favorable effects on body fat management

have been clearly reported and clinical studies with oral administration of CGA for up to 200 mg/day as a weight loss agent have not shown relevant side effects in patients [27,28].

Image 1: Chlorogenic Acid (CGA)

1.1.2 CGA as a safe molecule: its beneficial effects on heath

The natural compound CGA has been used for centuries in traditional medicine, it is considered to be affordable, and its safety has also been reported in modern medicine [29]. In fact, CGA has been known to exhibit a range of pharmacological activities such as antioxidant, anti-inflammatory, cardiovascular, antimicrobical, hepatoprotective, renoprotective, anti-diabetic and anti-lipidemic [30]. In this context, literature has reported that CGA exerts its anti-inflammatory effects by inhibiting the production of some mediators such as TNF-α, IL-6 and IL-1β [31] and has positive effects on glucose and lipid metabolism regulation by inhibiting the activity of α-glucosidase, altering GIP concentrations, activating the AMPK, upregulating the expression of hepatic PPAR-α, and inhibiting the β-hydroxy-β-methyl glutaric acyl coenzyme A reductase [32,33]. Concerning hypolipidemic effects, numerous are the data obtained in preclinical in vivo models in which the diet intake of CGA decreases serum and hepatic triglyceride and cholesterol levels, and positively impacts on low density lipoprotein oxidation

state [34]. Evidence has proposed that consumption of CGA causes a reduction in LDL-cholesterol and malon dialdehyde (MDA) levels [35]. In obesity, CGA displays a helpful effect through the adjustment of obesity-related hormones and adipokine levels and by upregulating fatty acid oxidation in the liver and downregulating fatty acid and cholesterol biosynthesis [36]. Bodyweight gain and visceral fat mass accumulation are also reverted by CGA administration [37]. Furthermore, CGA may boost the impairment of endothelial function, which develops in diseases, such as hypertension, diabetes, metabolic syndrome, atherosclerosis [38]. In fact, CGA may protect the endothelial function through different mechanisms, allowing the release of vasoactive molecules such as nitric oxide (NO) and thromboxane A2 (TXA2) [39]. Among its beneficial effects, CGA exhibits a positive action on brain disorders [40]. In fact, some studies reported that CGA may defend nervous cells from damage induced by neurotoxic agents, countering autophagy and apoptosis processes [41]. Also in vivo, its injection attenuated the apoptotic rate, mitigating caspase-3, caspase-7, and poly ADP-ribose polymerase expression levels in order to increase the number of TUNEL-positive cells in the cerebral cortex of MCAO (male adult rats via middle cerebral artery occlusion) animals [42]. Moreover, CGA intraperitoneally administered rats decreases the oxidative damage in rat brain cerebellum and reduces lipopolysaccharide (LPS)-induced IL-1β and tumor necrosis factor alpha (TNF-α) release in the substantia nigra supporting its neuroprotective action [43].

In addition, several studies have asserted that CGA could be useful for the conservation of food products, showing antimicrobial properties against several pathogens [23]. In this respect, CGA has activity against a wide range of microorganisms, including bacteria, yeasts, molds, viruses, and amoebas [23,44]. In fact, CGA has shown to erase different Gram-negative (minimum inhibitory concentrations (MICs) ranging from 0.008 to 10 mg/mL) and positive (MICs of 0.02–10 mg/mL) bacteria [23,45].

Moreover, inhibited yeasts by CGA include Candida albicans, Malassezia furfur, Saccharomycses cerevisiae, and Trichosporon beigelii with MICs ranging from 0.04 to 10

mg/mL [23,46]. Taken together, this evidence shows that the CGA is a natural and safe compound and could be used as food additive supplement to replace the synthetic chemical growth promoters (antibiotics) and immune boosters for the prevention and treatment of metabolic syndrome and associated disorders.

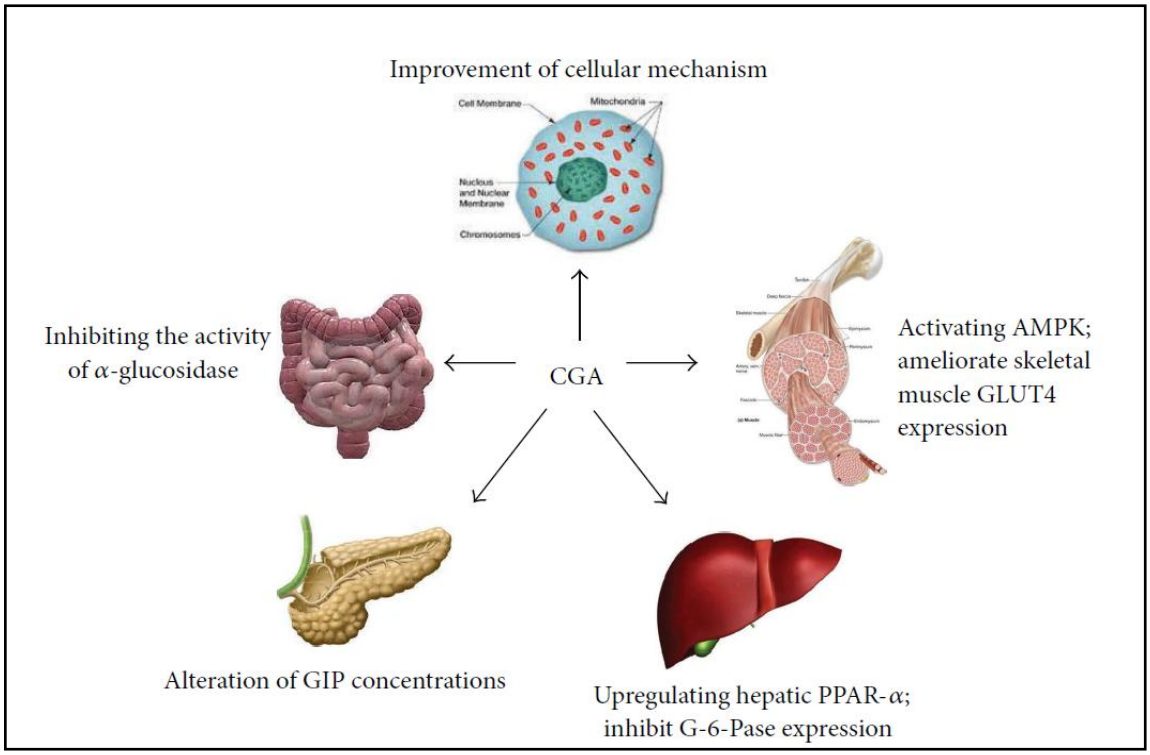

Image 2: CGA regulates glucose and lipid metabolism

1.1.3 Potential anticancer ability of CGA: evidence from literature

In addition to its beneficial properties to health, CGA has been reported to possess antitumor activity in many human cancer models, including leukemia, colon, breast and lung cancer [47,48,49,50]. In this regard, literature has proposed several evidence concerning the anticancer activity of CGA in vivo and in vitro tumor models, some of which are listed below and in *Table 1*. CGA induces inhibitory properties in lung cancer through the reduction of gene expression of stem cell markers including POU5F1, SOX2, NANOG, the down-regulation expression of vascular endothelial growth factor, and the amplification of p53 and PUMA proteins [51]. In addition, CGA significantly decreases the hypoxia-induced HIF-1α protein level and suppresses the transcriptional activity of HIF-1α under hypoxic conditions, leading to a decline in the expression of its downstream target VEGF, in A549 human lung cancer cells [52]. In vivo, CGA prevents the development of new tumors in naïve mice and inhibits growth of lung cancer in tumor bearing mice [53].

Moreover, in hepatocarcinoma cells, CGA is able to prevent cell proliferation and colony formation, induce cell death, hinder invasion and migration by increasing the activity and expression of p53 and p21, and decreasing other markers such as DNA methyltransferase1, ERK1/2 phosphorylation, MMP-2/TIMP 2, MMP-9 expression [58,59].

In addition, CGA provokes apoptosis in K562 leukemia cell line inducing cellular DNA damage and formation of topoisomerase I- and II-DNA complexes [62]. In the same cells, CGA is able to improve Imatinib leukemic effects determining an anti-proliferative effect and triggering apoptosis [63]. Also, in breast cancer, CGA suppresses cell growth and induces apoptosis via p53, Bax, Bcl-2, and caspase-3 signaling pathways [66].

Furthermore, CGA is considered a potential differentiation inducer and it has been widely reported that it increases the expression of key genes associated with differentiation (e.g., KHSRP, p53, and p21) and decreases the expression of genes associated with poor

differentiation (e.g., c-Myc andCD44) in solid tumor cell lines from hepatoma, glioma, breast and colon cancer [69, 70]. In particular, in U373 glioma cells, CGA significantly inhibited the proliferation and the migration, provoking cell cycle arrest in the G2/M phase and apoptosis [71]. Interestingly, in 2018, the first human phase I study of CGA injection was published. In details, a phase I clinical trial of CGA administration was conducted to determine tolerance, safety, pharmacokinetics in patients with recurrent high-grade glioma. This study demonstrates that CGA injection in patients, at the MTD of 5.5 mg/kg, was safe and well tolerated with potential antitumor activity [80]. Overall, this data shows the pharmacological effectiveness of CGA in different tumor cell models, demonstrating its safety and proposing it as a new strategy for cancer treatment.

Table 1: CGA acts on various type of cancer

Cancer Type	Cell line	Application	Reference
Lung Cancer	A549 lung cancer cell	In-vitro	[51,52,55,56]
	Male BALB/c nude mice and A549 cell	In-vivo and in-vitro	[53,54,57]
Liver Cancer	HepG2, Hep3B human hepatocarcinoma cells	In-vitro	[59,60,61]
	HepG2 cell and HepG2 xenograft tissue	In-vitro	[58]
Blood Cancer	Human U937 leukemia cell	In-vitro	[64]
	Human HL-60 leukemia cell	In-vitro	[65]
	Bcr-Abl(+) chronic myeloid leukemia and K562 xenograft nude rats	In-vivo	[62,63]
Breast Cancer	Mouse 4T1 breast cancer cell	In-vivo	[66]
	EMT6, BT-549, and MDA-MB-231 cell and EMT6 xenograft model	In-vivo and in-vitro	[67]
	MCF-7 breast cancer cell	In-vitro	[68]
Brain Cancer	Human glioma cell	In-vitro	[71]
	U87MG and patients derived IV grade glioma Cells	In-vitro	[72,73]
Skin Cancer	Human melanoma (SK-MEL-2) cell	In-vitro	[74]
Kidney Cancer	A498 human kidney cancer cell	In-vitro	[75]
Colon Cancer	HT-29, CT-26 and Caco-2 colon cancer cells	In-vitro	[76,77,78]
	Human HT-29 and HTC-116 colon adenocarcinoma cells	In-vitro	[79]

1.2 Osteosarcoma

1.2.1 Osteosarcoma: from diagnosis to treatment

With more than 3.1 cases per million per year, osteosarcoma (OS) represents, after lymphomas and brain tumors, the third most common cancer in adolescence [81]. Although the main peak of incidence is observed between 10 and 14 years, a second peak is detected after the age of 60, describing a characteristic bimodal distribution [82]. The different occurrence time generally defines the site and body location of the primary tumors that often are lower metaphysis of long bones for OS developed during the adolescence stage, and craniofacial, the terminal portion of the long bones, and axial for OS cases over 60 years [83]. In most patients, the etiology of OS remains obscure. The predilection of OS for the age of the pubertal growth spurt and the site of maximum growth suggests a correlation with rapid bone proliferation. Germinal and somatic mutations of the TP53, alterations of RB1 and overexpression of insulin- like growth factor- I receptor pathway (IGF- RI) represent the most common genetic alterations identified in the primary human OS tumors, suggesting a crucial role of these genes in its pathogenesis [84, 85].

Concerning OS diagnosis, local pain, followed by localized swelling and limitation of joint movement, are the typical signs and symptoms of OS. In rare cases, particularly in patients with osteolytic tumors, a pathological fracture can be the first sign of disease. Although OS can occur in any bone, it is most common in the metaphyses of long bones. The most common primary sites are the distal femur, the proximal tibia, and the proximal humerus, with 50% originating around the knee [86]. About 10% develop in the axial skeleton, most commonly the pelvis. Plain radiography is helpful to describe osseous changes: osteosarcomas can present with osteoblastic, osteolytic or mixed appearance. They often have a soft tissue component in which patchy calcifications resulting from new bone formation or spiculae may be observed. A triangular area of periosteal calcification in the border region of tumor and healthy tissue is known as a Codman

triangle, which is considered typical for osteosarcomas. Magnetic resonance imaging (MRI) is the best modality to assess the soft tissue component, its relationship to surrounding tissues, vessels and nerves, and its intramedullary extension, which is essential for safe definitive surgery. MRI has to include the whole involved bone as well as the neighboring joints, so as to not miss skip lesions, i.e. intramedullary tumor foci without direct contact with the primary lesion. In addition, approximately 15% of patients present radiographic metastases, typically to the lung, but metastases can also develop in bone and rarely in lymph nodes [86, 87].

OS lacks specific tumor markers; however, an elevation of lactate dehydrogenase or, more frequently, alkaline phosphatase levels in serum is found in some patients [88]. The diagnosis of OS must always be verified histologically. Open biopsy is considered the most suitable technique to obtain sufficient material for histological evaluation and additional studies. The hallmark of diagnosis is the proliferation of malignant mesenchymal tumor cells and the production of osteoid and/or bone by these tumor cells, observed by biopsy. The amount of osteoid and/or bone production varies greatly between tumors and within an individual tumor [89].

Neoadjuvant multidrug chemotherapy, composed by cisplatin, doxorubicin, methotrexate (MAP therapy) and ifosfamide, represents the current OS treatment, followed by surgery and postoperative chemotherapy. Multiple chemodrugs regimens can be combined by pairing the above chemotherapy drugs according to different doses and usage sequence. Most hospitals worldwide conduct 2–6 courses of pre-operative chemotherapy for a total of 6–18 weeks [90].

Curative treatment for primary metastatic OS is identical to that of localized disease, with the mandatory addition of surgical removal of all know metastatic foci, usually by exploratory thoracotomy including palpation of the whole lung. Approximately 30% of all patients with primary metastatic osteosarcoma and >40% of those who achieve a complete surgical remission can become long-term survivor. Treatment for relapse, either locally or within the lungs, is primarily surgical. The prognosis is poor, with long-term post relapse survival in <20%.

Complete removal of all metastasis must be attempted, as the disease is otherwise almost universally fatal. In contrast, more than a third of the patients with a second surgical remission survive 5 years or more. Even patients with multiple recurrences may be cured as long as recurrences are resectable, and repeated thoracotomies are often warranted [86, 90].

The role of second-line chemotherapy for recurrent OS is much less well defined than that of surgery, and there is no accepted standard regime. Choice may take into account the prior free interval and disease resectability, and often includes ifosfamide, etoposide and/or carboplatin [86, 89].

Although chemotherapy significantly improves the prognosis of patients with non-metastatic OS, patients are often forced to cease and modify chemotherapy regimens due to drug resistance, toxicity, and/or side effects. In fact, the toxic consequences of chemotherapeutics cannot be ignored, which include incidents such as liver and kidney function damages, bone marrow suppression, neurotoxicity, and gastrointestinal reactions, and continuing research into novel treatment and all types of developed therapeutic forms is required [90,91].

1.2.2 Doxorubicin (Doxo): advantages and side effects

Doxorubicin (Doxo) is an antibiotic derived from the Streptomyces peucetius bacterium. It has widespread use as a chemotherapeutic agent since the 1960s [92]. Currently, Doxo is approved to treat several neoplastic disorders including breast and gynecologic tumors, lymphoma, and lung cancer [93]. Moreover, Doxo-based chemotherapy also constitutes the standard care in OS [94].

The primary mechanism of action of Doxo involves the drug's ability to intercalate within DNA base pairs, causing breakage of DNA strands and inhibition of both DNA and RNA synthesis. In addition, Doxo inhibits the enzyme topoisomerase II, causing DNA damage and induction of cell death. When combined with iron, it also causes free radical-mediated oxidative damage to DNA, further limiting DNA [92]. More recent studies have suggested that Doxo-induced damage and fragmentation caused to DNA by the mechanisms discussed above, or by other mechanisms, may induce apoptosis or other cell biological events, leading to cell death or cell growth arrest. In addition to apoptosis, there is evidence that autophagy, early or accelerated senescence and necrosis, may also be cell biological events key to Doxo's activity [95].

Just as with other cancers, even in OS, the successful rate in first-line is usually followed by a dramatic decline in responsiveness and collateral effects, which impede a further dose intensification [96]. In fact, apart from eliciting resistance mechanisms, cardiovascular toxicity represents, instead, one of the major and potentially lethal Doxo-induced side effects in both cancer patients and survivors, suggesting the existence of such a memory mechanism in cardiac cells [97]. However, despite the beneficial effects in tumor progression, Doxo-mediated cardiovascular diseases are quite frequent in long-term cancer survivors, where they may significantly contribute to heart failure and death [98,99]. To date, several mechanisms have been implicated in the Doxo-induced cardiotoxicity, such as increased oxidative stress and lipid peroxidation, DNA damage, apoptosis and, more recently, autophagy [100]. However, increased reactive oxygen species (ROS) production remains the major mechanism associated with Doxo-

induced oxidative stress, mitochondria injury and cellular apoptosis in cardiac cells [101]. The restricted antioxidant enzymes pool, especially catalases, in cardiac cells are often inadequate to counteract the ROS production amount during Doxo administration [102].

Clinically, the acute cardiac toxicity of Doxo occurs within days of the drug's administration and appears in approximately 11% of patients who receive the drug as myopericarditis, left ventricular dysfunction, or arrhythmias. These conditions, in most cases, are reversible. On the contrary, chronic late cardiac toxicity may also exist after Doxo administration and is the most serious and potentially lethal adverse effect. Its incidence is approximately 1.7% and may also be followed by congestive heart failure. Risk factors for Doxo-induced congestive heart failure include a higher cumulative drug dose, extremes of age, combination chemotherapy with other cardiotoxic drugs, pre-existing left ventricular dysfunction, hypertension, and previous radiation to the mediastinal region. It has been estimated that the 1-year mortality rate of patients with this problem is approximately 50% [103].

1.3 Combination chemotherapy: an overview

Combination therapy was first conceptualized in 1965 by Emil Frei, James F. Holland and Emil J. Freireich who proposed the possibility of the first ever combination therapy for acute leukemia. More in details, Pediatric patients with acute lymphocytic leukemia were treated with the combination of methotrexate, 6-mercaptopurine, vincristine and prednisone (formally known as the POMP regimen) and was proven successful in reducing tumour burden and prolonging remission [104]. From the success of the POMP regimen, cancer combination therapy received more attention and the research became focused on investigating combination therapies that target different pathways to induce a synergistic or additive effect. For example, in a study by Quinn et al., it was demostrated that sabutoclax, a pan-Bcl-2 inhibitor, in combination with minocycline, an antibiotic that has previously displayed anticancer effects, acted synergistically on the intrinsic apoptotic pathway.

In addition, this combination displayed selective toxicity and a reduction in tumour growth in vitro and in vivo on pancreatic ductal adenocarcinoma [105]. Thus, using a combination of compounds that target different pathways, a synergistic or potentiation effect could yield significant anticancer results.

In the last decades, combination therapy has received great attention as a potential successful strategy to overcome resistance and mitigate undesirable toxicity. In fact, the combination of two or more therapeutic treatments to specifically target cancer-inducing or cell-sustaining pathway is becoming a base of cancer therapy [14,106]. The agents used in this treatments work in synergistic or additive manner, and therefore a lower therapeutic dosage of each drug is required 106,107].

In addition, it has been observed that combination chemotherapy, in many cases, prevents the toxic effects on normal cells while simultaneously producing cytotoxic effects on cancer cells. This may occur if one drug in the combination regimen is antagonistic, in terms of cytotoxicity, to another drug in normal cells, essentially protecting normal cells from cytotoxic effects [14].

In this regard, naturally occurring molecules with antitumor activity and with the least toxicity to normal tissues are proposed as possible intriguing candidates to be investigated for their synergistic efficacy in combination with conventional antineoplastic drugs [15,16]. In addition, the concept of chemoprevention is increasingly gaining attention in cancer; particularly, cancer chemoprevention by natural compounds, especially phytochemicals, minerals, and vitamins, has shown promising results against various malignancies in numerous studies both under in vitro and in vivo treatments [108]. In the development of bioactive chemical, natural products from medicinal plants have a rich and long history and represent a very interesting research area for novel therapeutic anticancer strategies [109].

Among the natural compounds from medicinal plants, CGA can be suggested as one of the most interesting molecules to be possibly use in OS therapy.

2. RESULTS

2.1 Impact of CGA on osteosarcoma models

2.1.1 CGA inhibits proliferation of human osteosarcoma cells

To evaluate whether CGA is able of changing human OS cells' behavior, firstly, we determined the impact of CGA on cell viability. To this purpose, U2OS, Saos-2, and MG-63 cells were treated with increasing concentrations of CGA (from 12.5 to 400 μM) for 72 hours and then cell viability was determined by MTT Assay. As reported in Figure 1A, C, and E, no significant changes have been tracked using a concentration of 50 μM, or lower, of CGA, whereas 100 μM produces a reduction of cell viability of ≈20% in all three cell lines tested. Examined the data obtained with 200 μM and 400 μM CGA, the reaction to CGA was different among the three OS cell lines. Particularly, U2OS cells display a decrease in cell viability of 54% and 71% in response to 200 μM and 400 μM, respectively (Figure 1A). Inhibition values of 25% and 56% have been obtained in Saos-2 (Figure 1C), whereas MG-63 cells show a higher strength to survive in presence of CGA, with reduction in cell viability of ≈40% obtained only with the highest dose of CGA (400 μM; Figure 1E). Successively, all cell lines were exposed to 200 μM and 400 μM of CGA for up to 72 hours and cell growth curves were assessed by cell number counting. Figure 1B exhibits that in U2OS, the treatment with 200 μM CGA causes a cell number reduction of 13%, 47%, and 74%, after 24, 48, and 72 hours of treatment, respectively, whereas a robust growth inhibition is clearly visible already after 24 hours in response to 400 μM CGA. Concerning Saos-2 we noted a cell number decrease of 29% at 48 hours and 54% at 72 hours in response to 200 μM CGA, and one of 50% at 24 hours with 400 μM CGA (Figure 1D). Even by cell growth curves, MG-63 cells are confirmed as a model poorly responding to CGA showing a drop in cell number of 15% and 40% respectively at 48 and 72

hours with 200 μM (Figure 1F). This evidence demonstrate a clear role of the CGA as an antiproliferative molecule in human OS cells with different reactivity among U2OS (the most sensitive), Saos-2, and MG-63 (the least sensitive).

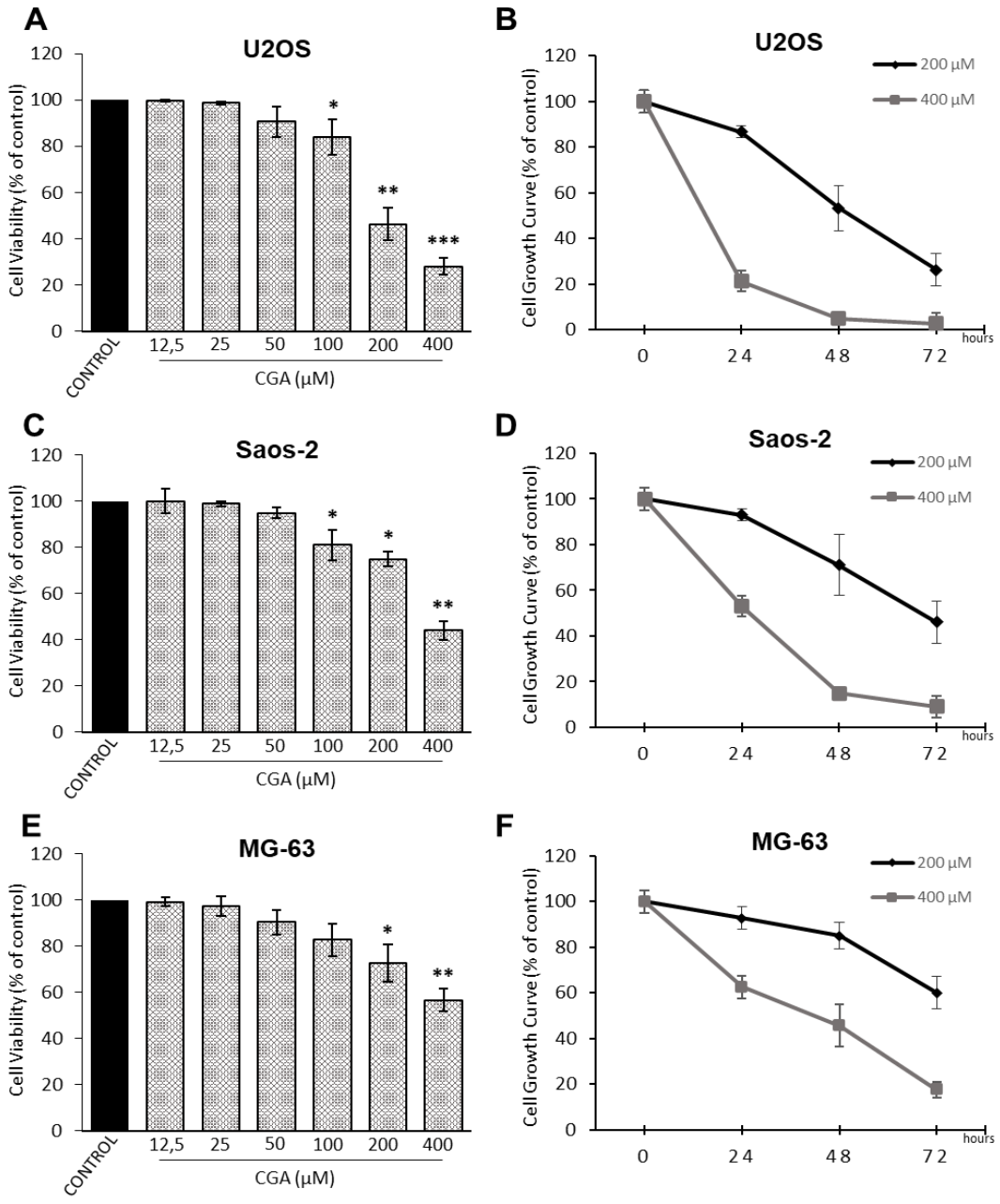

Figure 1. Assessment of CGA effects on cell viability and proliferation in osteosarcoma cell lines.

U2OS (**A**), Saos-2 (**C**), and MG-63 (**E**) were treated, or not (control), for 72 hours with increasing concentrations of CGA, from 12.5 to 400 μM. Therefore, cell viability was estimated by MTT Assay. Growth curves at 24, 48, and 72 hours were reported in response to 200 and 400 μM of CGA in U2OS

(**B**), Saos-2 (**D**), and MG-63 (**F**). Both cell viability and cell growth curves were expressed in % of control. The average and standard deviation (SD) of three independent experiments were reported. *p < .05, **p < .01, ***p < .001 by unpaired t-test.

2.1.2 CGA affects colony forming ability in osteosarcoma cells

In order to assess the consequences of CGA treatment on colony-forming ability in OS cells, initially we selected U2OS and MG-63 cells as the most sensitive and the least sensitive models to CGA, respectively. Figure 2A shows that CGA provokes a substantial reduction in a number of colonies in both cell lines tested. In particular, only a few and very small colonies are visible in U2OS upon just 50 μM CGA, whereas less obvious changes in the number and in size appear in MG-63 cells at 50 and 100 μM CGA. To increase the specificity of the above results, we also examined the impact of CGA to generate colonies in non-tumor mouse embryonic fibroblast NIH3T3 cells. Interestingly, in concomitant experiments, we found that 100 μM of CGA suppresses, partially or totally, colonies formation in U2OS and in MG-63, while no significant differences have been noted in NIH 3T3 cells (Figure 2C). These data indicate that CGA treatment negatively affects long-term proliferative potential in OS cells.

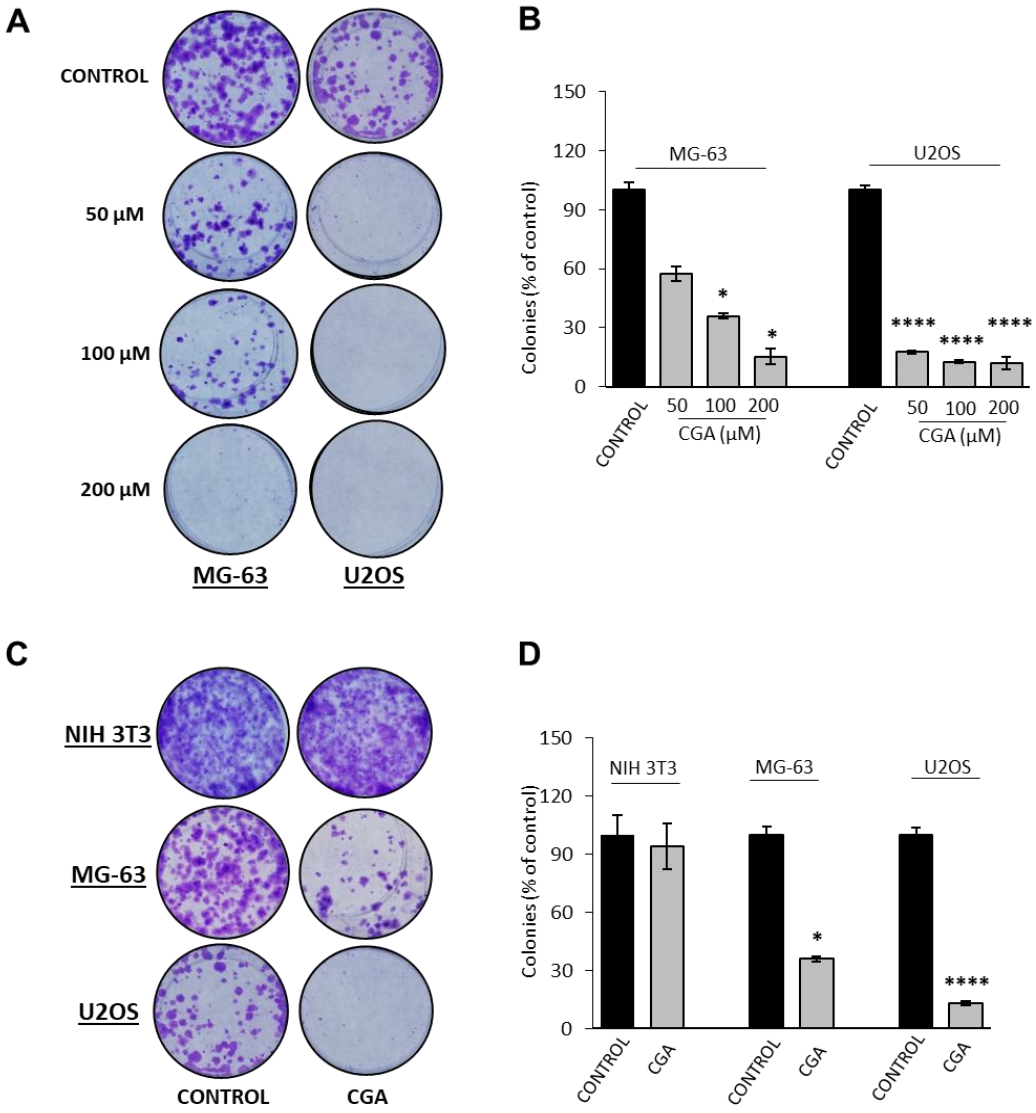

Figure 2. Impact of CGA on colony forming ability in U2OS, MG-63, and NIH3T3 cells.

(**A**) CGA concentration of 50, 100, and 200 µM were used to treat U2OS and MG-63 for 10 days, later colony forming assays were performed. (**C**) Crystal violet staining of U2OS, MG-63 osteosarcoma cells, and NIH 3T3 normal mouse embryonic fibroblasts, obtained in response to 100 µM of CGA, were reported. (**B, D**) Quantification analysis of biological triplicate was reported on the right-hand side of Panel a and c, respectively. A number of colonies were expressed in % of control. Means and SD were indicated. *p < .05, ****p < .0001 by unpaired t-test.

2.1.3 CGA affects cell cycle division and causes cell death in osteosarcoma cells

With the purpose of further studying the CGA mediated antiproliferative effects in OS cells, we performed cell cycle analysis to assess cell phases distribution in response to CGA by flow cytometric analysis of PI- stained cells. Figure 3A shows that no relevant changes have been detected in cell cycle distribution after 24 hours of treatment, whereas at 48 and 72 hours the percentage of cells in S-phase in CGA treated cells is higher than the untreated ones. This data is already clear at 48 hours (+10% comparing CGA vs. control) but becomes more consistent at 72 hours (+15%). In addition, a concomitant reduction in the G0/G1 phase is also seen in response to CGA and a little increase in the G2/M phase is observed at 48 hours. The expression analysis of some relevant proteins implicated in cell cycle regulation indicates an increase chiefly of Cyclin A protein levels, according to the CGA- induced S phase accumulation (Figure 3B). Additionally, we observed a marked increase of the sub-G1 phase as a consequence of CGA. In detail, Figure 3A shows a detectable percentage of sub-G1 equal to 11% after 48 hours of CGA administration, and a large amount at 72 hours (≈27%), suggestive of cell death. To confirm the CGA-induced cell death in U2OS, we also measured PI uptake to distinguish death versus live cells, in which membranes turn out to be permeable. According to sub-G1 data, we also detected an increase in PI uptake (cell death) in response to CGA. To note, parallel testing indicates a comparable percentage number of PI-positive cells and sub-G1population (Figure 3C). To confirm cell death, we assessed the levels of some proteins involved in apoptosis in our specific conditions. To this purpose, U2OS cells were treated for 24, 48, and 72 hours with 200 μM of CGA. Thereafter, cell extracts were prepared and analyzed by western blotting with the aim of evaluating the levels of cleaved caspase-3 (CC3), total poly (ADP-ribose) polymerase (PARP) and its cleaved form (c-PARP). The activation of executioner CC3, the concomitant decrease of PARP and the appearance of its cleavage in U2OS, especially after 48 and 72 hours of treatment, clearly suggest that CGA provokes programmed cell death (Figure 3D). Altogether, the above

data indicate that the CGA-induced antiproliferative actions in U2OS cells are principally depending on apoptotic cell death induction.

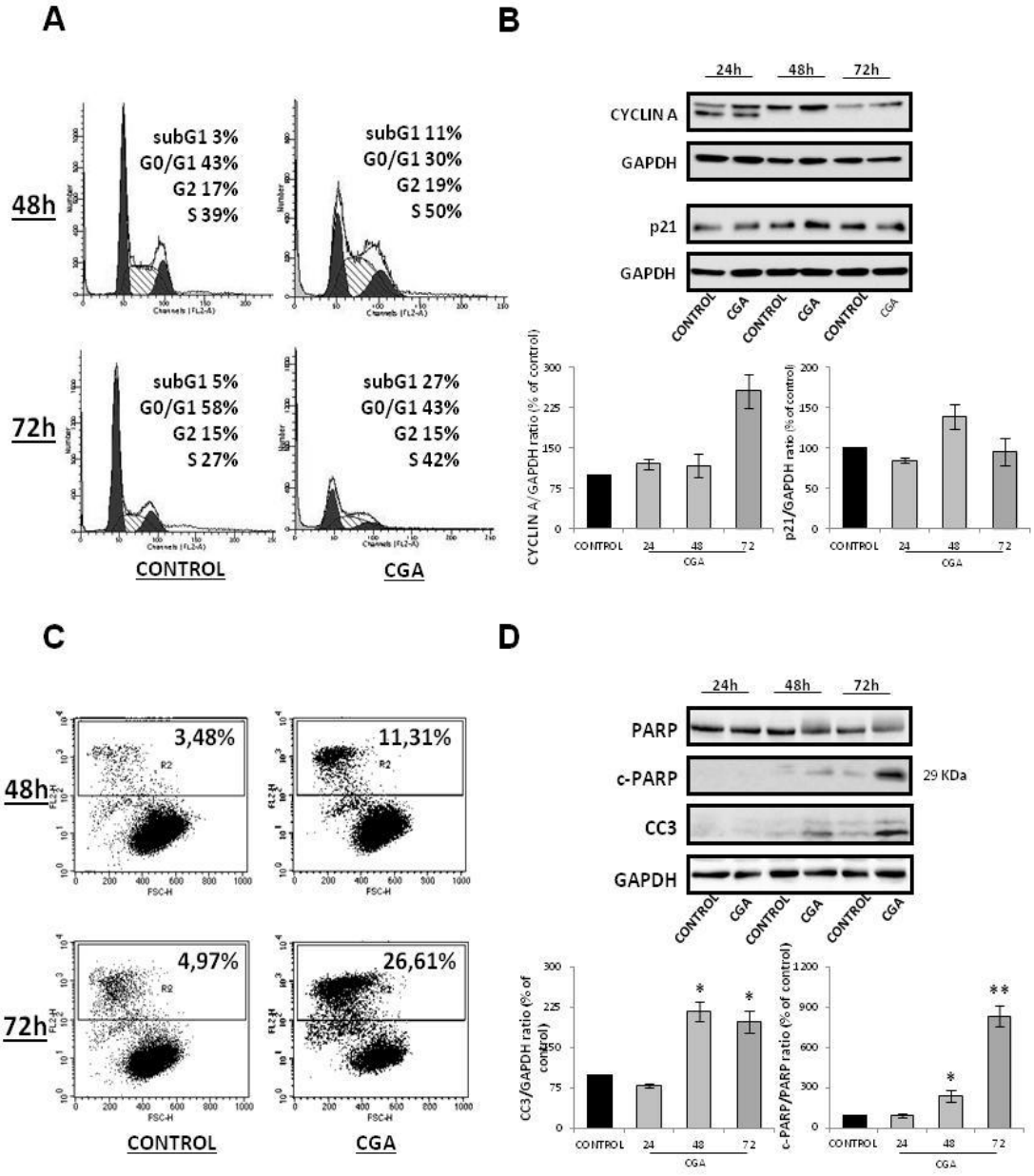

Figure 3. Evaluation of cell cycle distribution and cell death in response to CGA by Flow cytometry and western blotting analyses.

(**A**) U2OS were exposed to 200 µM of CGA for 24, 48, and 72 hour and subG1 and cell phases distribution were evaluated by PI staining. (**C**) Representative cell death experiments obtained in reaction to 200 µM after 48 and 72 hours of treatment were shown. (**B**) Levels of Cyclin A and p21 detected after 24, 48, and 72 hours of exposure to 200 µM of CGA. (**D**) PARP, cleaved PARP, and CC3 were assessed by western blotting analysis. Bands ratio of each CGA time point was indicated in % of its relative control. *p < .05, **p < .01 by unpaired t-test.

2.2 Effects of CGA and Doxo co-treatment on osteosarcoma models

2.2.1 Combination CGA plus Doxo markedly injures cell viability and growth in osteosarcoma cells

Leveraging the above results, we selected three effective concentrations of both CGA (100, 200, and 400 μM) and Doxo (0.05, 0.1 and 0.2 μM), and tested their impact on cell viability with the purpose of assessing potential cooperating effects between these two compounds. In this respect, U2OS and MG-63 were exposed for 48 hours to single and combinatory treatments, mixing CGA and Doxo with a constant dilution ratio. Figure 4A displays a clear dose–response relationship between the CGA concentrations and cell viability inhibition in U2OS, as well as in response to Doxo. However, co-administration of CGA plus Doxo further decreases cellular metabolic activity compared to single treatments, suggesting an additional reduction in viability. Although already obvious at even lower doses, 200 μM CGA plus 0.1 μM Doxo provoked an extra shrinkage of about 27% and 36% versus CGA and Doxo, respectively. Analogously, although CGA was less effective in MG-63 than U2OS cells, all tested combination treatments displayed a greater ability to reduce cell viability even in this other OS model (Figure 4B). In comparison with single agents, co-administration of 200 μM CGA plus 0.1 μM Doxo further affects cell viability in the amounts of 40% and 31% versus CGA and Doxo, individually. In order to define the nature of drug–drug interaction, CompuSyn analysis was applied to calculate combination indexes (CIs). Employing the obtained MTT data, CIs were defined for all tested conditions in both U2OS and MG-63 cells. Whilst a moderate synergism came out at very low concentrations in U2OS, a more consistent outcome was revealed when higher doses, affecting 60% and 90% of cells, were employed (Figure 4C). The MG-63 Fa-CI plot suggests a stronger synergism in every experimental condition assessed, even at very low doses instead (Figure 4D). Subsequently, using 200 μM CGA and 0.1 μM Doxo as effective working dosages, we performed time-course experiments intended to establish the relative impact on cell growth.

Although both compounds were already effective in reducing cell number, combination treatment affected tumor doubling to a greater extent (Figure 4E, F). Compared with Doxo alone, co-treatment further decreased cell growth of approximately 15% after 48 hours in OS cells, and a similar trend was maintained 24 hours later. Regarding CGA instead, a pronounced and different outcome was detected between these two models. Specifically, an additional 39% and 60% of cell number reduction was detected after 48 hours of treatment with both CGA and Doxo in U2OS and MG-63, respectively. Taken together, these findings suggest that combination treatment CGA plus Doxo can synergistically affect cell viability and growth in OS cells.

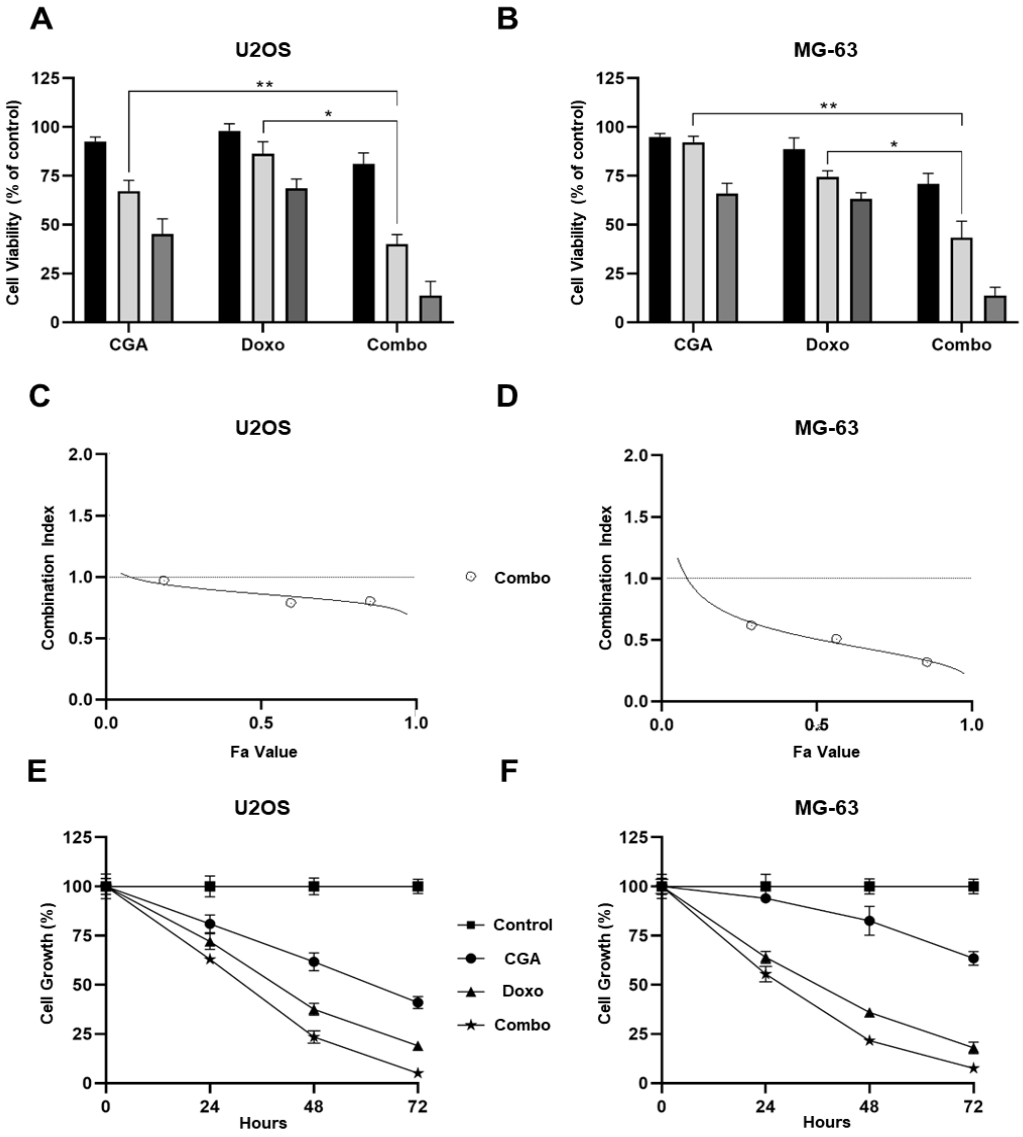

Figure 4. Assessment of CGA, Doxo, and CGA plus Doxo-mediated effects on cell viability and proliferation in OS cells.

100 μM (black bar), 200 μM (light gray), and 400 μM (gray) of CGA and 0.05 μM (black bar), 0.1 μM (light-gray), and 0.2 μM (gray) of Doxo were administrated individually and together in U2OS (**A**) and MG-63 (**B**) for 48 hours. Subsequently, cell viability was estimated by MTT assays and shown as mean ± SD in percentage of control. U2OS (**C**) and MG-63 (**D**) Fa-CI plots were determined employing CompuSyn software analysis. Representative reports are illustrated in figure. Growth curves at 24, 48, and 72 hours were recorded in response to untreated (control), 200 μM CGA, 0.1 μM Doxo, and CGA plus Doxo (combo) in both U2OS (**E**) and MG-63 (**F**). Percentage growth values of three distinct biological replicates, expressed as average _ SD, are displayed. * $p < 0.05$, ** $p < 0.01$ by an unpaired

2.2.2 Co-administration CGA plus Doxo further decreases the chlorogenic ability in osteosarcoma cells

To further characterize the CGA plus Doxo anticancer-mediated action in OS, we studied the consequences of combination treatment on colony-forming ability.

To do this, we added the OS cells with a very low concentration of both CGA and Doxo for 10 days before staining the newly formed colonies with crystal violet. As intentionally designed, the utilize of small doses of both CGA and Doxo had a limited impact on OS colony-forming ability (Figure 5A). Instead, the combination of CGA plus Doxo radically reduces the OS chlorogenic potential, affecting both colony number and size. Quantification analysis further asserted differences among the experimental conditions (Figure 5B). Altogether, these data show that combination CGA plus Doxo induces a longer lasting reduction in chlorogenic potential compared with single-agent treatment.

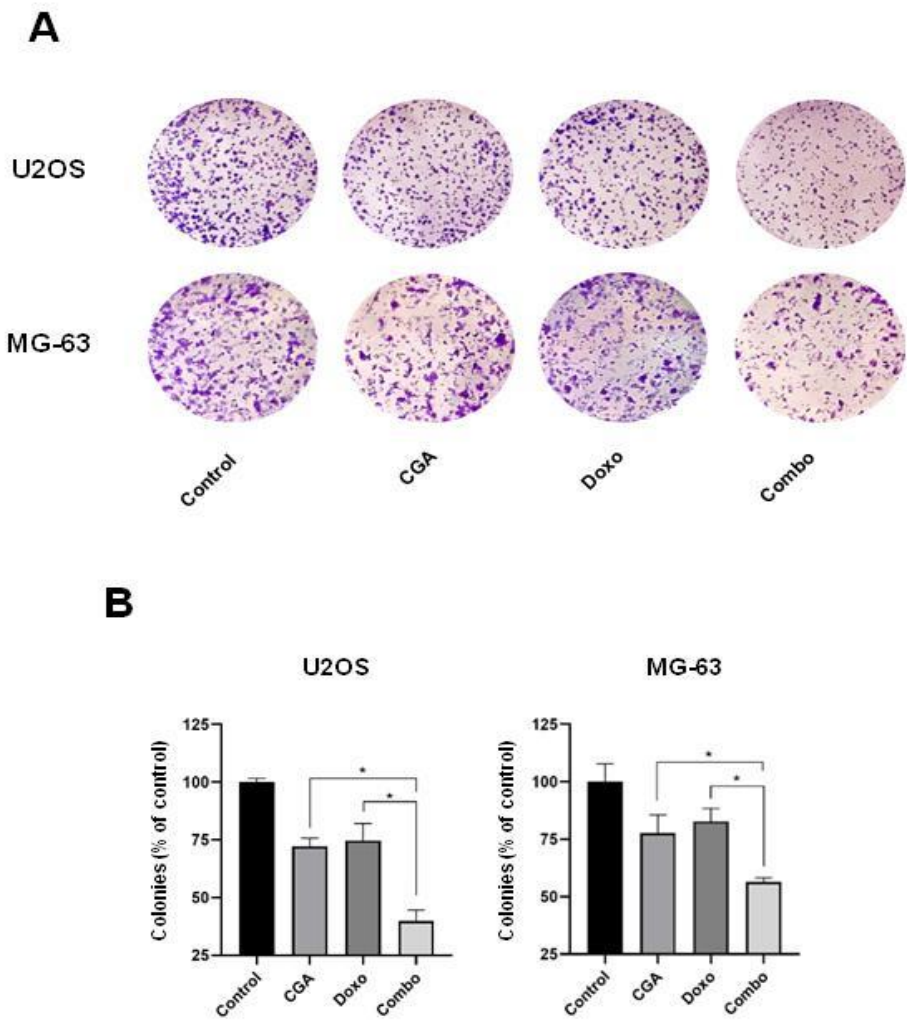

Figure 5. Estimation of CGA, Doxo, and CGA plus Doxo induced colony-forming ability in OS cells.

U2OS and MG-63 were treated and not (control) with 20 μM (U2OS) or 40 μM (MG-63) CGA, 0.005 μM Doxo, and CGA plus Doxo (combo); thereafter, chlorogenic ability was assessed by crystal violet staining. Representative experimental images are displayed in panel (**A**). Quantification analysis from three different experiments has been carried out and reported in panel (**B**). * $p < 0.05$.

2.2.3 CGA improves Doxo-mediated cytotoxic effects in osteosarcoma cells

To address whether the combination treatment CGA plus Doxo could improve single-agent cytotoxicity, we, initially, discriminated living from dead cells using Trypan Blue as the exclusion dye. Figure 6A shows the selective analysis of living cells revealed a 40% and 66% reduction in U2OS when exposed to 200 μM CGA and 0.1 μM Doxo for 48 hours, respectively. About the dead cells, we find out a modest rise in response to CGA (+3%) and a more consistent accumulation with Doxo (+7%). The dead vs. live ratio significantly increased by up to 17% in the presence of CGA plus Doxo, duplicating the Doxo percentage (Figure 6C). MG-63 shown a similar tendency, where CGA plus Doxo co-treatment diminished living cells and enhanced death occurrence compared a single agent, simultaneously (Figure 6B, D). We also performed FACS-based Propidium Iodide (PI) analysis to define the cell death contribution to CGA plus Doxo co-administration. In details, shifting the percentage of PI-positive cells from 8.16% to 14.86%, CGA potentiated Doxo-mediated cytotoxic actions in U2OS (Figure 6E). Analogously, compared with Doxo, a six-point augmentation was detected in MG-63 when CGA and Doxo were administered concurrently (Figure 6F).

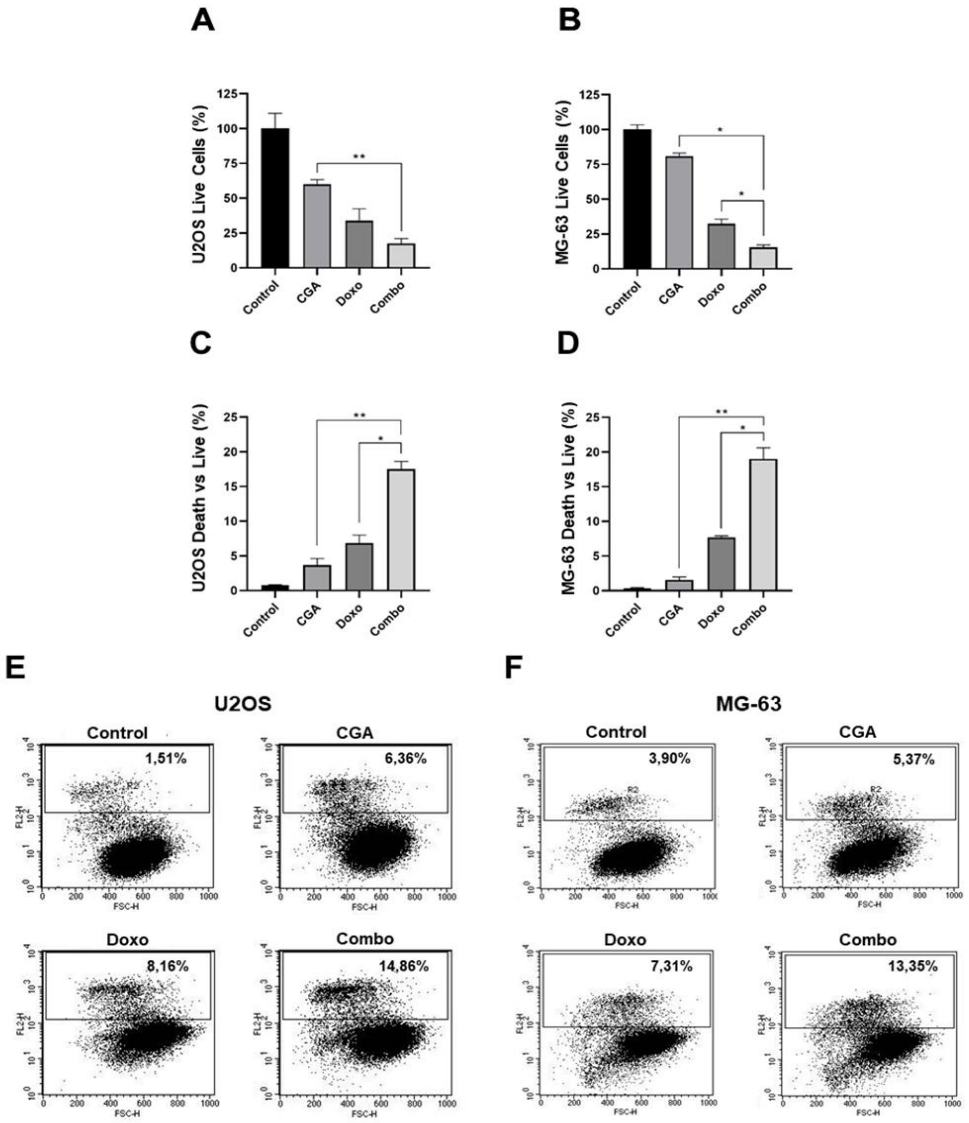

Figure 6. Investigation of CGA, Doxo, and CGA plus Doxo induced cell death in OS cells.

U2OS and MG-63 were treated and not (control) with 200 μM CGA, 0.1 μM Doxo, and CGA plus Doxo (combo) for 48 hours. Subsequently, living and dead cells were discriminated either by Trypan Blue (**A**–**D**) or by PI assay (**E**, **F**). Representative experimental dot-plots are shown in figure. Living cell number, as well as dead vs. live ratio, is expressed in percentage as mean ± SD. * $p < 0.05$, ** $p < 0.01$ by an unpaired two-tailed *t*-test. Experiments shown in the figure were repeated four times.

2.2.4 Combination CGA plus Doxo improves Caspase-3 and PARP Activation in osteosarcoma cells

Depending on the dosage applied, Doxo can destroy tumor cells through multiple death mechanisms, including mitotic catastrophe and programmed cell death [110]. Similarly, in most cancer studies in which CGA exhibits cytotoxic properties, apoptosis constitutes one of the main signaling involved in [5,51,78]. In light of the emerging results, which propose CGA as a potential cell death enhancer in Doxo-mediated effects, we assessed the expression levels of two distinct biochemical apoptosis hallmarks, CC-3 and PARP. Figure 7A, B clearly show a stronger PARP cleavage in reaction to CGA plus Doxo compared with a single agent in both U2OS and MG-63 cells. These results, together with a concomitant intensification in caspase-3 fragmentation (Figure 7C, D), suggest that CGA could improve Doxo-mediated cytotoxicity via apoptosis induction in OS cells.

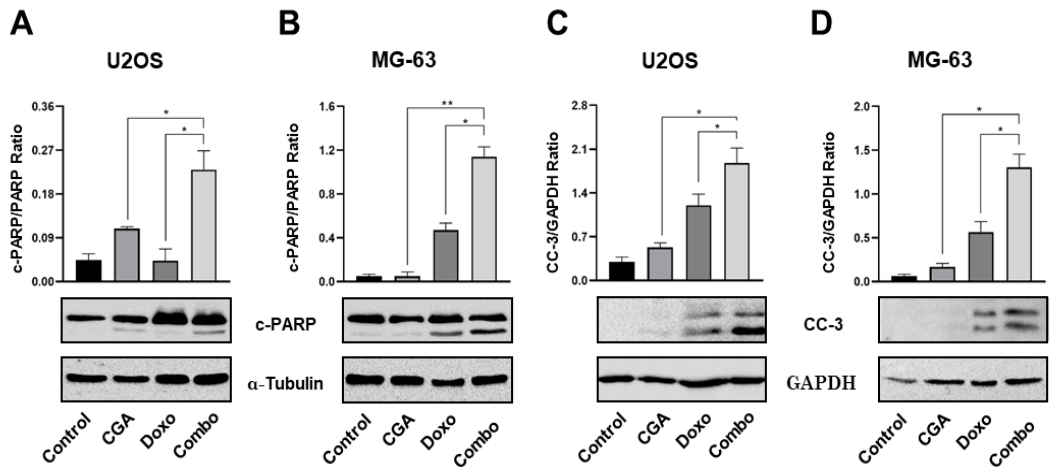

Figure 7. Evaluation of CC-3 and PARP activation in response to CGA, Doxo, and CGA plus Doxo in OS cells.

U2OS and MG-63 were exposed and not (control) to 200 µM of CGA, 0.1 µM of Doxo, and CGA plus Doxo (combo) for 48 hours. Whole extracts were prepared and then samples were analyzed by Western blotting for PARP (**A**, **B**) or CC-3 (**C**, **D**) protein levels. GAPDH and α-Tubulin were employed as internal loading controls. Representative Western blotting films were shown in the figure together with the relative densitometric analysis obtained from at least three biological replicates. * $p < 0.05$, ** $p < 0.01$ by an unpaired t-test.

2.2.5 CGA ameliorates Doxo-mediated cytotoxicity in H9c2 cardiomyocytes

Doxo-mediated cardiotoxicity represents the main limiting factor for its prolonged administration in cancer [96,99]. Therefore, increasing Doxo efficacy should go together with reducing side effects, expressly cardiotoxicity. In order to assess the combination action on Doxo-induced toxicity in cardiac cells, we employed embryonic rat cardiac tissue-derived H9c2 cells as a suitable in vitro system to study the anthracycline-mediated effects [111-113]. More in detail, H9C2 and MG-73 cells were treated using 200 µM CGA and 0.1 µM Doxo alone and in combination for 48 hours. Interestingly, while the combination treatment further suppressed the cell number and improved the percentage of PI-positive cells in MG-63 compared with Doxo alone, this trend was completely reverted in H9c2 cells (Figure 8). Indeed, although Doxo drastically diminished the cell growth rate, combination treatment ameliorated this collateral

effect by approximately 27% (Figure 8A). Moreover, CGA extensively counteracted Doxo-induced cell death, considering that the percentage of PI positive cells moved from around 12.65% to 6.08% when those compounds were administered together (Figure 8B, E).

These data propose the CGA as a non-toxic agent in H9c2 cardiac cells and further suggest this compound as a potential cardioprotective candidate in Doxo-based chemotherapy.

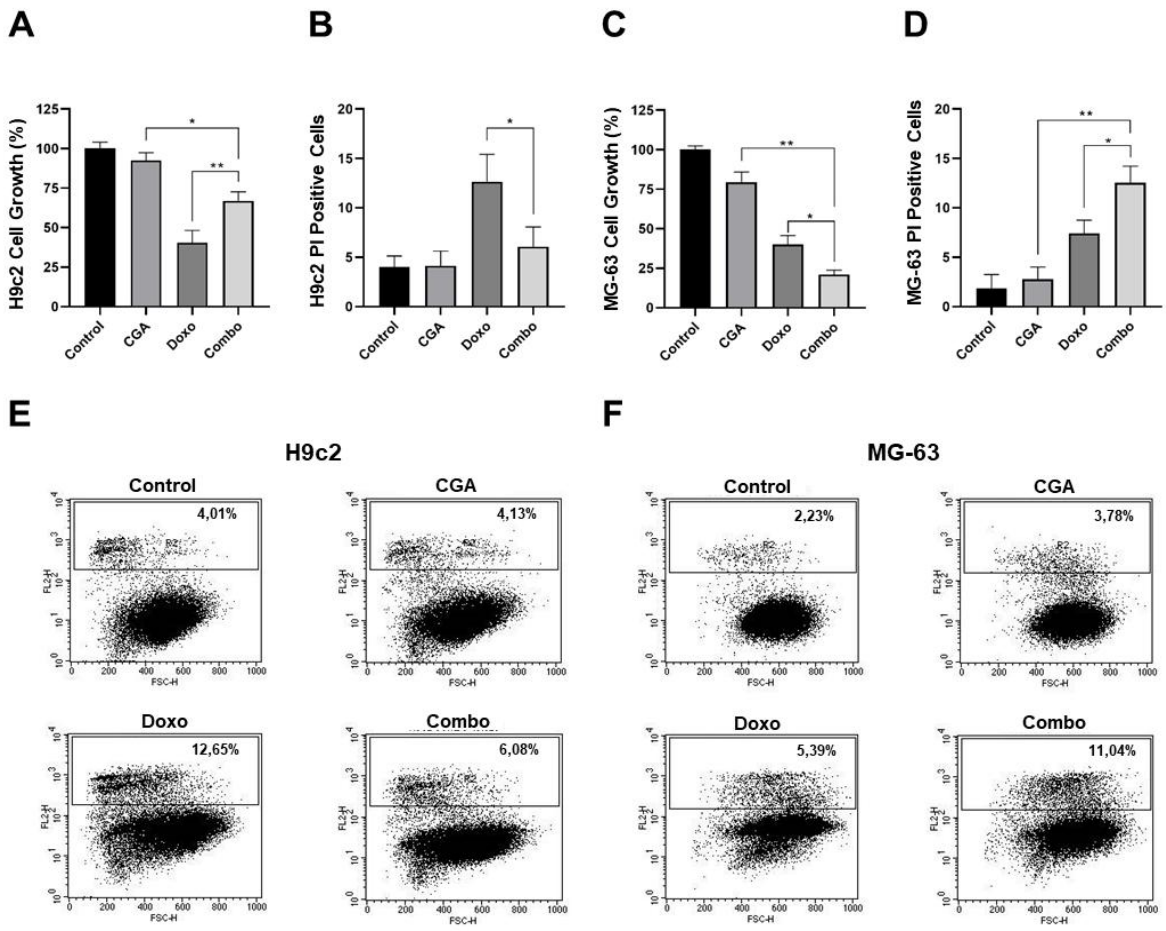

Figure 8. Evaluation of CGA, Doxo, and CGA plus Doxo-mediated effect on cell growth and death in H9c2 and MG-63 cells.

H9c2 and MG-63 were treated and not (control) with 200 μM CGA, 0.1 μM Doxo, and CGA plus Doxo (combo) for 48 hours. Later, the percentage of cell growth (**A**, **C**) as well as cell death (**B**, **D**) was established. Representative dot-plots are shown in (**E**, **F**). * $p < 0.05$, ** $p < 0.01$ by an unpaired two-tailed t-test. Experiments were repeated thrice.

2.3 Involvement of p44/42 signaling pathway in CGA and Doxo action in osteosarcoma models

2.3.1 CGA improves p44/42 activation in osteosarcoma cells

Aberrant expression of the mitogen-activated protein kinase (MAPK) pathway has extensively been reported in several neoplastic disorders [114]. Moreover, higher phosphorylation levels of MAPK are generally associate p44/42 pathway is relevantly involved in many cellular functions, such as cell cycle progression and differentiation, DNA repair, and apoptosis [115]. In addition, there is large evidence indicating the involvement of p44/42 in cancer cell proliferation, including in OS [116-118]. Moreover, recent findings suggest that CGA can modulate p44/42 in tumor and nontumor models [119,120]. With this in mind, to assess the possible engagement of the ERK pathway in the CGA-induced antiproliferative effects in OS cells, we first determined the impact of CGA on p44/42 phosphorylation. To do this, U2OS were treated with 200 μM for a time from 3 to 24 hours, and then total p44/42 and phospho-p44/42 was evaluated by immunoblotting. Impressively, a marked increase of p44/42 phosphorylation is observed in response to CGA starting from 6 hours of treatment, and that becomes stronger at 18 and 24 hours (Figure 9A). No obvious differences in the total amount of p44/42 protein levels in response to CGA occur. A similar activation, substantial after 6 hours and maintained up to 24 hours, has also been observed in MG-63 cells. In addition, we found that the exposure to CGA up to 72 hours keeps risen the ratio between the phosphorylated and total p44/42 protein levels, although such activation was less strong if compared to the early time points (Figure 9B).

Taken together, these findings indicate that CGA treatment results in discrete effects on different signaling pathways, mainly causing profound changes in p44/42 phosphorylation levels, suggesting an involvement of this pathway in CGA-induced antiproliferative action in U2OS cells.

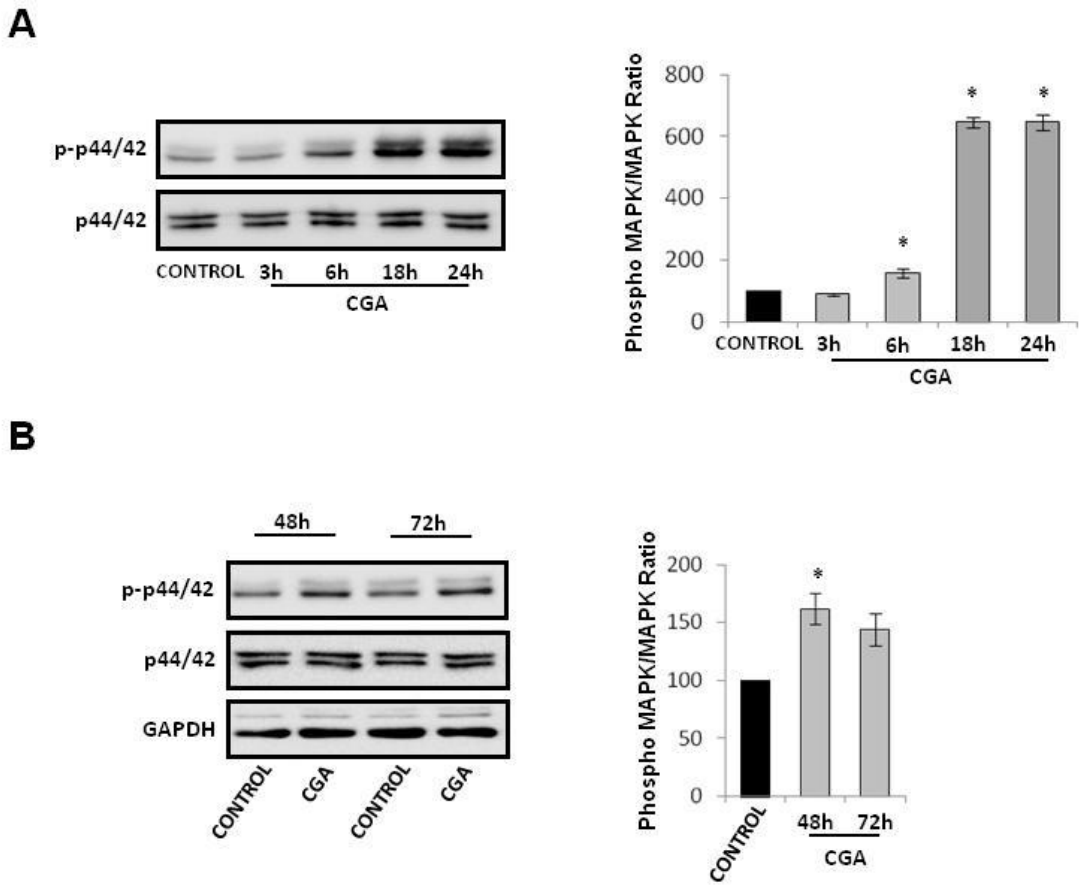

Figure 9. p44/42 signaling pathway involvement in CGA response in U2OS.

Western blotting analyses were performed to evaluate total and phosphorylated amount of p44/42 in response to 200 μM. (**A**) Effects of early time course experiments, from 3 to 24 hours, on total and phosphorylated levels of p44/42. (**B**) Consequence of long-time course experiments, 48 and 72 hours, on p44/42 pathways. Densitometric intensity, of phosphorylated/total amount ratio, was reported to determine their relative activation. Bands ratio of each CGA time point was indicated in % of its relative control. *$p < .05$ by unpaired t-test.

2.3.2 Impairment of p44/42 enhances CGA-mediated cell growth inhibition in osteosarcoma cells

To further investigate the engagement of the p44/42 MAPK pathway in the CGA-induced antiproliferative effects in U2OS cells, we used PD98059 as a potent and selective inhibitor of MEK1 and MEK2, upstream activators of p44/42. In detail, we tested the consequences of CGA, PD98059 and CGA/PD98089 combination on cell proliferation and cell death in U2OS cells (Figure 10). Using 10 μM of PD98059, as an effective and widely concentration used in preclinical in vitro studies, we noted a minimal impact on both parameters analyzed by itself [121,122]. Instead, to assess the possible outcomes of CGA/ PD98059 co-treatment, we tested two distinct CGA concentrations (200 and 400 μM) and two different time points (24 and 48 hours).

Remarkably, we observed that in the all-experimental setting shown in Figure 10, the co-treatment CGA/PD98059 clearly enhances the CGA-mediated antiproliferative effects. In this regard, while the growth inhibition after 24 hours of treatment moved from ≈75%, with 400 μM CGA alone, to ≈90% in the presence of both CGA and PD98089 (Figure 10A), the exposure to the combination 200 μM CGA/PD98059 for a time of 48 hours enhances the inhibition index from ≈47%, CGA alone, to ≈76% (Figure 10B). Comparable results have also been obtained analyzing the PI uptake. Indeed, the combinatory treatment CGA/PD98059 visibly increases the percentage of cell death compared to CGA and PD98059 as single agents (Figure 10C, E). Overall, the above data very likely indicate that perturbation of CGA mediated p44/42 activation further increases cell growth inhibition and cell death, strengthening the sensitivity of U2OS cells to CGA.

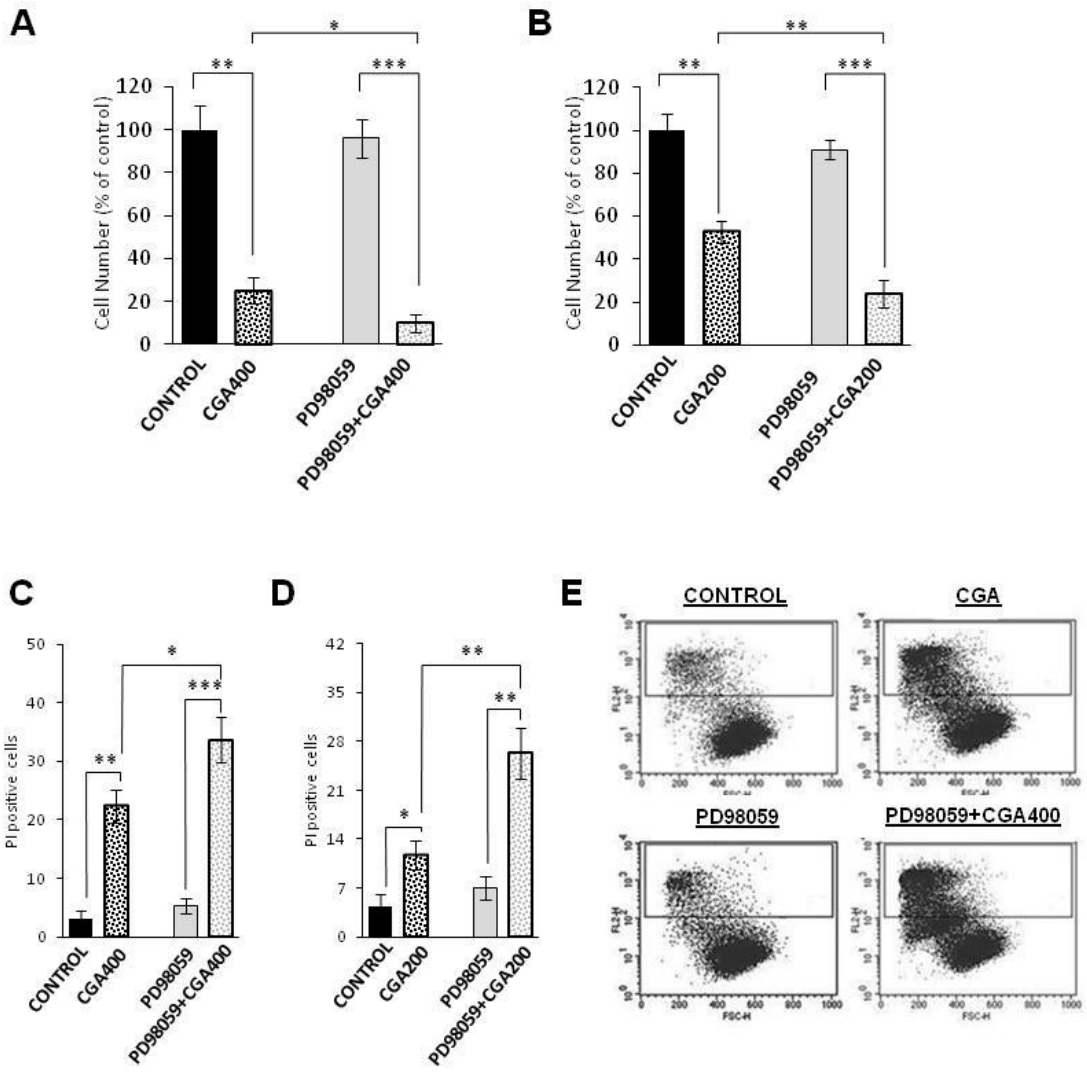

Figure 10. Effects of PD98059 on CGA mediated cell growth inhibition and death in U2OS.

Cells were exposed to 400 μM of CGA and 10 μM of PD98059, alone or in combination, for 24 hours. Thereafter, cell number (**A**) and cell death by PI uptake (**C**) were assessed. Relative cell number (**B**) and percentage of cell death (**D**) were also determined after 48 hours in response to 200 μM of CGA, 10 μM of PD98059, and CGA/ PD98059 cotreatment. (**E**) Representative image of typical cell death experiments reported in Panel c was shown. The cell number was expressed in % of control. The average and SD of three independent experiments were reported. $p < .05$, $^{**}p < .01$, $^{***}p < .001$ by unpaired-test.

2.3.3 p44/42 pathway perturbation promotes CGA-mediated Doxo sensitization in osteosarcoma cells

Starting from this evidence and bearing in mind that also the Doxo administration usually promotes p44/42 MAPK activation either as a cell resistance mechanism or as cell death signaling, we also evaluated the impact of co-treatment CGA/Doxo treatment on p44/42 MAPK signaling. [123,124]. Very interestingly, here we found that CGA plus Doxo downregulated p44/42 MAPK phosphorylation, suggesting a potential involvement in CGA-based combination treatment (Figure 11A, E).

In order to further define the p44/42 MAPK role, we also tested the impact of the selective upstream inhibitor of p44/42 MAPK, namely PD98059, on CGA plus Doxo mediated cell growth effects, firstly in U2OS. As expected, 10 μM of PD98059 was effective in inhibiting p44/42 MAPK phosphorylation (Figure 11B). Nevertheless, although CGA improved Doxo-induced cell growth inhibition, PD98059-mediated p44/42 MAPK impairment enforced this outcome, increasing the inhibition rate by approximately 14% (Figure 11C, D)

Comparable PD98059-mediated results were also obtained in MG-63 cells (Figure 11F). On the whole, these findings propose the p44/42 MAPK pathway as relevantly involved in CGA-mediated Doxo susceptibility.

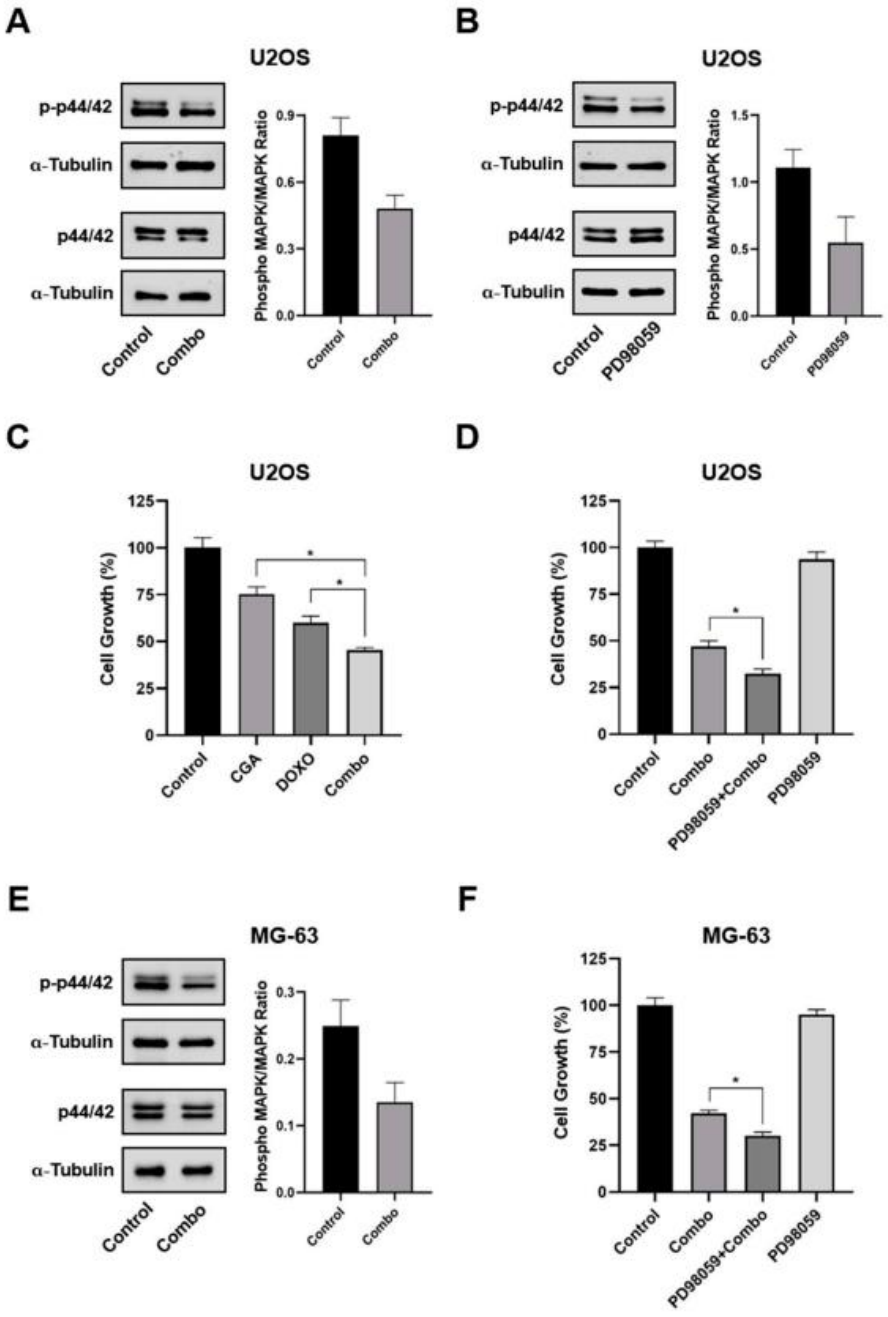

Figure 11. Estimation of MAPK involvement in CGA-mediated Doxo sensitization.

U2OS (**A**) and MG-63 (**E**) were treated and not (control) with 300 μM CGA plus 0.2 μM Doxo (combo) for 24 hours, and then the relative impact on p44/42 MAPK was assessed by Western blotting. (**B**) Analysis of total and phosphorylated p44/42 MAPK levels following 24 hours of exposure to 10 μM PD98059. (**C**) U2OS were treated and not (control) with 300 μM CGA, 0.2 μM Doxo, and CGA plus Doxo (combo) for 24 h, and then the relative cell number in percentage of control was estimated. Combination treatment impact on U2OS (**D**) and MG-63 (**F**) cell growth in an MAPK proficient and hampered background. Three hours pre-treatment with 10 μM of PD98059 preceded and not combo administration. * $p < 0.05$ by an unpaired two-tailed t-test. Experiments showed in the figure are the end-result of three distinct biological replicates.

3. DISCUSSION & CONCLUSION

The high metastatic rate and the lack of effective therapy make OS a very aggressive pediatric and adolescent tumor whose clinical outcome remains extremely poor [81]. Despite the clinical approval of novel chemotherapeutics and formulations, Doxo still remains a cornerstone for OS management and Doxo-based therapy constitutes the widely-used partner in combination therapy [125]. Unfortunately, the limited success rate of Doxo treatment, as well as the relative ease in developing chemoresistance and carditoxity, is crying out for more effective therapeutic approaches in OS [13]. Currently, new efficient therapeutic strategies are required for the cure of OS; in this regard combination chemotherapy, which promises higher efficacy and lower toxicity of clinical anticancer drugs, is consistently being investigated [14]. In this area, naturally occurring molecules and plant-related products that have shown pronounced anticancer effects, are becoming attractive candidates as therapeutic agents in cancer prevention and treatment [15,16].

Among the natural products from medicinal plants, CGA can be considered as a very promising compound for possible use in cancer therapy. In fact, increasing evidence has highlighted the capability of CGA to modulate many cellular functions in the human body and its aptitude to inhibit proliferation in several preclinical cancer models [126].

With the purpose of further addressing the CGA candidacy in OS treatment, herein we shown the potential outcome of CGA alone and its dynamic interaction with Doxo in different OS cells.

Our results reveal that CGA prevents cell viability, cell growth, and colony-forming ability in all OS cell lines tested, with a different sensitivity among them (U2OS and MG-63 the most and the least sensitive, respectively). Notably, our data are fully in agreement with a very recent study in which CGA exerts antiproliferative effects in p53-deficient OS cells Saos-2 and MG-63 [9]. Herein, we confirm and extend such data, providing the first evidence regarding CGA and p53-proficient U2OS cells. The different sensitivity that is emerging from our findings in response to CGA and its possible connection with a specific genetic background, p53- status

first, represents a very intriguing data that need to be addressed more in detail. Moreover, we prove that CGA is able to impact cell cycle phases distribution and induce cell death in U2OS. Increased cell death is accompanied by a rise of the activity of caspase-3 and PARP, suggesting an apoptosis induction due to CGA action.

Concerning the cooperating effects between CGA and Doxo, our results reveal no shortcoming in using these two compounds together; quite to the contrary, their combination could have a greater therapeutic impact compared with single ones. Moreover, as suggested by CompuSyn analysis, potential synergistic action could exist between CGA and Doxo. The cooperative interaction is clearly supported by cell growth and colony results, which show a combination-mediated stronger and deeper outcome in limiting OS tumorigenicity. In addition, their concomitant administration further promoted cell death potentially via apoptosis induction, as suggest by CC-3 and PARP cleavage. Furthermore, we demonstrated that CGA oppositely affected OS and cardiac cells when co-administrated with Doxo, ameliorating cell attitude in H9c2 cardiomyocytes. These results are in agreement with the previusly published study in which CGA is proposed as the best hydroxycinnamic acid for preventing Doxo-induced injuries in Wistar rats' isolated cardiomyocytes [127]. Similar findings have recently been reported in other cardiomyocyte models by Elrazik and coworkers [7].

Signaling pathway examination revealed instead a possible involvement of p44/42 MAPK in the responses elicited by CGA plus Doxo in OS cells. Firstly, we provide evidence that CGA strongly activates p44/42 starting from 6 hours up to 48 hours. Over the last decade, several studies have established that the engagement of p44/42 is absolutely required for cell cycle progression and that, an aberrant pathway activation can generate an uncontrolled proliferation, driving the oncogenic process [116,117]. To underline the importance of this specific pathway in cancer as well as in some other pathological conditions, we must draw attention to its blockage/inhibition that already constitutes a pursuable pharmacological target. In this regard, the use of p44/42 inhibitor, PD98059, further enhances cell growth inhibition and death,

reinforcing the hypothesis of a possible pro- survival and antiapoptotic role of p44/42 activation in reply to CGA. On the other hand, CGA and Doxo co-admistration induces a p44/42 MAPK reduction, but the use of PD98059 enhanced the outcome in OS cells promoting the combination treatment efficacy. If on one side our results further support the MAPK role in Doxo-mediated action, at least in OS cells, on the other, they certainly hint at other molecular mechanisms involved in CGA-mediated Doxo susceptibility.

In conclusion, we firstly provide evidence of potential synergistic effects between CGA and Doxo in constraining OS progression. Apart from supporting the antineoplastic feature, our results recognize an additional CGA therapeutic usage in OS, potentially as a partner in Doxo-based combination therapy.

.

4. MATERIALS & METHODS

4.1 Chemical Reagents

Chlorogenic Acid (C3878; Sigma-Aldrich, St. Louis, MO, USA), Doxorubicin (#5927; Cell Signaling Technology, Danvers, MA, USA), PD98059 (#P215; Sigma-Aldrich), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M2128; Sigma-Aldrich), Propidium Iodide (#P4864; Sigma-Aldrich), Trypan Blue solution (T8154; Sigma-Aldrich), and Crystal Violet (C0775; Sigma-Aldrich).

4.2 Antibodies

p44/42 MAPK (ERK1/2) (#9102) (1:1000, 5% w/v BSA in T-TBS), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101) (1:1000, 5% w/v BSA in T-TBS), PARP (#9542) (1:1000, 5% w/v no-fat dry milk in T-TBS), cleaved caspase-3 (Asp175) (5A1E) (#9664) (1:500, 5% w/v BSA in T-TBS), α -Tubulin (DM1A) (#3873) (1:500, 5% w/v milk in T-TBS), GAPDH (D16H11) (#5174) (1:1000, 5% w/v milk in T-TBS), p-STAT3 (Tyr705) (#9131) (1:500 w/v BSA in T-TBS) and goat anti-rabbit IgG HRP-linked (#7074) (1:2500, 5% w/v milk in T-TBS) were purchased from Cell Signaling Technology. Cyclin A (H-432) (#sc-751) (1:1000, 5% no-fat dry milk in T-TBS), STAT3 (C-20) (#sc-482) (1:500, 5% no-fat dry milk in T-TBS), were acquired from Santa Cruz Biotechnology. p21(EPR362) (#109520; Abcam) (1:1000, 5% w/v BSA in T-TBS).

Goat anti-mouse IgG HRP-linked (1:2500, 5% w/v milk in T-TBS) and goat anti-rabbit IgG HRP-linked (1:2500, 5% w/v milk in T-TBS) were employed as secondary antibodies for immunoblotting.

4.3 Cell Culture and Experimental Procedures

Human osteosarcoma U2OS, Saos-2 and MG-63 cell lines and embryonic rat cardiac tissue derived H9c2 cardiomyoblasts were purchased by the American Type Culture Collection. Cells

were routinely maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin at 37 °C in a 5% CO_2 controlled atmosphere. All the experimental procedures, whose results have already been displayed, consist of three distinct steps, which include seeding, treating, and collecting. Specifically, cells were initially split into an equal number depending on the performed test. After 24 hours, the culture medium was replaced with a fresh one containing CGA and Doxo, both alone and in combination. Times and doses are indicated in the "Results" section, as well as in "Figure Legends". Since CGA and Doxo were reconstituted in ethanol and dimethyl sulfoxide (DMSO), respectively, preliminary tests were carried out with the purpose of addressing the solvent outcome on cell growth and viability. Although no toxic effects were noticed, the same ethanol rate was supplemented in the control group, being that v/v% was usually greater or equal to 0.4%. Apart from colony-forming and cell viability assays, trypsin-mediated cell detachment was applied to collect each treatment condition in every procedure.

4.4 Cell Viability Assessment

Cells were seeded in 96-multiwell plates at the density of 2×10^3 cells per well (U2OS), 1.5×10^3 cells per well (MG-63), or 2.5×10^3 cells per well (Saos-2) and exposed for 72 hours to different CGA and Doxo concentrations as described in "Results" paragraph. Viable cells were determined by the MTT assay. Briefly, 100 µl of MTT solution (5 mg/ml) was added to each well and incubated at 37°C for 3 hr, then media was removed and formazan salts were solubilized using 100 µl of Isopropanol-HCL 0.04N at room temperature on horizontal shaking for 30 min. The optical density (OD) of each sample was determined to measure the absorbance at 570 nm.

4.5 Cell Growth Evaluation and Colorimetric Exclusion

Using a 6-well plate, U2OS, SAOS-2, MG-63, and H9c2 cells were, respectively, seeded at the density of 6×10^4, 7×10^4, 4.5×10^4, and 7.0×10^4 per well and then treated in accordance

with the experimental design. After reaching the designed end-point, cells were harvested before being manually counted by Bürker's chamber, employing an inverted phase contrast microscope. By diluting with Trypan Blue (1:1 Ratio), dead cells, formerly stained in blue, were further discriminated from living cells, unstained. Taking an average of no less than two technical replicates, the cell number of each experimental point was defined.

4.6 Colony Formation Assay

An identical number of U2OS and MG-63 cells, equal to 1.5×10^3 per well, were plated in a 6-well plate and chronically stimulated for 10 days with the indicated doses of CGA, Doxo, and combination treatment. Once past this period, growing media were thrown away and adherent colonies were fixed and stained for three hours using the all-in-one aqueous solution containing 0.1% crystal violet and 7% ethanol. After incubation, distilled water was used to remove a specific and residual dye. Successively, plates were air-dried and acquired by photographic equipment. Quantitative analysis was performed dissolving colonies-bound crystal violet in 10% acetic acid and establishing the OD at 590 nm.

4.7 Evaluation of cell cycle by PI-staining

Flow cytometry analysis was performed to assess cell cycledistribution using FACS-Calibur flow cytometer (BD Biosciences). Cells were fixed in 70% ice-cold ethanol/phosphate-buffered saline (PBS) and incubated overnight at 4°C. The day after cells were spundownat 400×g for 5 min, washed in PBS twice and re-suspended in1ml of PI staining solution (15 μg/ml PI and 20 μg RNaseA in PBS). After 10 min of incubation, at room temperature and protected fromlight, at least 50 K events for each sample were acquired. Lastly, ModiFIT software was used to analyze the percentage of G1, S, and G2/M phases and also for the sub-G1 events. All biological replicates have been performed in triplicate.

4.8 Propidium Iodide Analysis

As a membrane damage marker, PI was employed to further evaluate drug-induced cell death. Physiologically, an intact phospholipid bilayer completely impedes the PI uptake; nevertheless, massive cell impairment transiently permeabilizes the cell membrane, thus allowing the transport of molecules otherwise not permitted, including PI. Taking advantage of this easy principle, dead cells (PI positive) can be easily discriminated by living ones (PI negative). Briefly, U2OS, MG-63, and H9c2 cells were seeded and treated as previously described in Subparagraph 2.1.8. After 48 hours, cells were collected and stained with 0.4 μg/mL of PI in 1× PBS buffer. The percentage of positive cells was later defined using a BD FACS Calibur analyzer (Franklin Lakes, NJ, USA).

4.9 Cell Extracts Preparation

Whole protein extraction was carried out as follows. A number of 3.5×10^5 (U2OS), 4.5×10^5 (Saos-2) and 2.6×10^5 (MG-63) cells were allowed to attach to 100 mm plates for 24 hours. The following day, drugs were supplemented to the growing media in doses and timings as indicated in the respective experimental procedures (see results for more details). Once harvested, cells were spun-down (5 min at 1500 RPM), rinsed with PBS, and spun-down again. Then, adding 3–5 volume of RIPA buffer, supplemented with both Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail, pellets were lysed on ice for 30 min. Finally, samples were centrifuged (15 min at 14,000 RPM), and the supernatant phase was recovered. The protein content of each sample was quantified by Bradford Assay.

4.10 Western Blotting

Detection of specific proteins among the experimental series required a proper sample preparation, in which, for each specimen, the Laemmli buffer 2X (S3401; Sigma-Aldrich) was mixed with an equal protein amount before heat denaturation (5 min at 95 °C). Subsequently, loading a protein range from 15 to 30 μg, electrophoresis was performed in sodium dodecyl

sulfate polyacrylamide gel (SDS-PAGE). After moving proteins to the nitrocellulose membrane, foils were firstly blocked for one hour in no-fat milk (5% w/v) and then incubated overnight at 4 °C with specific primary antibodies. The next morning, HRP secondary antibodies, recognizing the related primary species, were applied to the membrane for 1 hour at room temperature. Finally, chemiluminescence was detected with the Chemi Doc XRS instrument using liteablot chemiluminescent as a substrate. Each incubation step was preceded and followed by three washes in TBS plus 0.05% Tween-20 (T-TBS).

4.11 Statistical Analysis

Mean SD of biological replicates was reported. Analysis of variance (ANOVA) and t-Student' tests were applied with the purpose of discriminating significant differences among the experimental groups. A value of less than 0.05 was recognized as significant. Image J (NIH, Bethesda, MD, USA) was employed to carry out densitometric analyses.

5. REFERENCES

1. Naveed M., Veghar H., Muhammad A., et al. *Chlorogenic acid (CGA): A pharmacological review and call for further research*. Biomed Pharmacother, 2018.
2. Tajik N., Tajik M., Mack I., et al. *The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature*. Eur J Nutr, 2017.
3. Liang N. & Kitts D. Role of Chlorogenic Acids in Controlling Oxidative and Inflammatory Stress Conditions. Nutrients, 2015.
4. Nwafor E.O., Lu P., Zhang Y., et al. *Chlorogenic acid: Potential source of natural drugs for the therapeutics of fibrosis and cancer*. Translational Oncology, 2022.
5. Zeng A., Liang X., Zhu S., et al. *Chlorogenic acid induces apoptosis, inhibits metastasis and improves antitumor immunity in breast cancer via the NF‐κB signaling pathway*. Oncology Reports, 2021.
6. Miao M. & Xiang L. *Pharmacological action and potential targets of chlorogenic acid. Advances in Pharmacology*, 2020.
7. Nesma A., Elrazik A., El-Mesery M., et al. *Chlorogenic acid potentiates antitumor effect of doxorubicin through upregulation of death receptors in solid Ehrlich carcinoma model in mice.* Egyptian Journal of Basic and Applied Sciences, 2019.
8. Refolo M.G., Lippolis C., Carella N., et al. *Chlorogenic acid improves the regorafenib effects in human hepatocellular carcinoma cells.* International journal of Molecular Sciences, 2018.
9. Zhang F., Yin G., Han X., et al. *Chlorogenic acid inhibits osteosarcoma carcinogenesis via suppressing the STAT3/Snail pathway*. Journal of cellular Biochemistry, 2019.
10. Son K.D. & Kim Y.J. *Anticancer activity of drug-loaded calcium phosphate nanocomposites against human osteosarcoma*. Biomaterials research, 2017.
11. Catauro M., Tranquillo E., Salzillo A., et al. *Silica/Polyethylene glycol hybrid materials prepared by a sol-Gel method and containing chlorogenic acid*. Molecules, 2018.
12. Sritharan, S. A comprehensive review on time-tested anticancer drug doxorubicin. Life Sci, 2021.
13. Li D., Yang Y., Wang S., et al. *Role of acetylation in doxorubicin-induced cardiotoxicity*. Redox Biology, 2021.
14. Bayat Mokhtari R., Homayouni T.S., Baluch N., et al. *Combination therapy in combating cancer.* Oncotarget, 2017.
15. Blagosklonny MV. Overcoming limitations of natural anticancer drugs by combining with artificial agents. Trends Pharmacol Sci, 2005.
16. Naviglio S. & Della Ragione F., *Naturally occurring molecules and anticancer combination therapies in the era of personalized medicine and economic crisis.* Curr Pharm Des, 2013.
17. Wei M. *Natural Polyphenol Chlorogenic Acid Protects Against Acetaminophen-Induced Hepatotoxicity by Activating ERK/Nrf2 Antioxidative Pathway.* Toxicol. Sci, 2018.
18. Gao, W. *Acid Attenuates Dextran Sodium Sulfate-Induced Ulcerative Colitis in Mice through MAPK/ERK/JNK Pathway*. BioMed Res. Int, 2019.
19. Maalik A., Bukhari S.M., Zaidi A., et al. *Chlorogenic acid: pharmacologically potent molecule.* Acta Pol Pharm, 2016.
20. Lara M.V., Bonghi C., Famiani F., et al. *Stone Fruit as Biofactories of Phytochemicals With Potential Roles in Human Nutrition and Health.* Front Plant Sci, 2020.

21. Farrell T., Dew T. P., Poquet, L., et al. *Absorption and metabolism of chlorogenic acids in cultured gastric epithelial monolayers.* Drug Metabolism and Disposition, 2011.
22. López-Froilán R., Ramírez-Moreno E., Podio N.S., et al. *In vitro assessment of potential intestinal absorption of some phenolic families and carboxylic acids from commercial instant coffee samples.* Food & Function, 2016.
23. Lu H., Tian Z., Cui Y., et al. *Chlorogenic acid: A comprehensive review of the dietary sources, processing effects, bioavailability, beneficial properties, mechanisms of action, and future directions.* Comprehensive reviews in food science and food safety, 2020.
24. Li J., Qin Y., Yu X., et al. *In vitro simulated digestion and in vivo metabolism of chlorogenic acid dimer from Gynura procumbens (Lour.) Merr.: Enhanced antioxidant activity and different metabolites of blood and urine.* Food Biochem, 2019.
25. Ohnishi R., & Ito H. *Effects of chlorogenic acid and its metabolites on spontaneous locomotor activity in mice.* Biosci Biotechnol Biochem, 2006.
26. Lafay S., Gil-Izquierdo A., Manach C., et al. *Chlorogenic Acid Is Absorbed in Its Intact Form in the Stomach of Rats.* Journal of Nutrition, 2006.
27. Vinson J.A., Chen X., Garver D.D., et al. *Determination of Total Chlorogenic Acids in Commercial Green Coffee Extracts.* J. Med. Food, 2019.
28. Gil M. & Wianowska D. *Chlorogenic Acid: Recent Advances on Its Dual Role as a Food Additive and a Nutraceutical against Metabolic Syndrome*. Molecules, 2017.
29. Moreira E.A., Pilon A.C., Andrade L.E., et al. *New Perspectives on Chlorogenic Acid Accumulation in Harvested Leaf Tissue: Impact on Traditional Medicine Preparations*. ACS Omega, 2018.
30. Upadhyay R. & Mohan Rao L. J. *An outlook on chlorogenic acids occurrence, chemistry, technology, and biological activities.* Critical Reviews in Food Science and Nutrition, 2013.
31. Shi H., Dong L., Jiang J., et al. *Chlorogenic acid reduces liver inflammation and fibrosis through inhibition of toll-like receptor 4 signaling pathway.* Toxicology, 2013.
32. Bagdas D., Gul Z., Meade J.A., et al. *Pharmacologic Overview of Chlorogenic Acid and its Metabolites in Chronic Pain and Inflammation.* Curr Neuropharmacol, 2020.
33. Wan C.W., Wong C.N.Y., Marcus P., et al. *Chlorogenic Acid Exhibits Cholesterol Lowering and Fatty Liver Attenuating Properties by Up-regulating the Gene Expression of PPAR-α in Hypercholesterolemic Rats Induced with a High-Cholesterol Diet.* Phytotheray research, 2013.
34. Meng S., Cao J., Feng Q., et al. *Roles of Chlorogenic Acid on Regulating Glucose and Lipids Metabolism: A Review*. Evid Based Complement Alternat Med, 2013.
35. Tajik N., Tajik M., Mack I., et al. *The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature.* European Journal of Nutrition, 2017.
36. Seon A.S., Jeonb M., Kim M.J., et al. *Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice.* Food and Chemical Toxicology, 2010.
37. Wang Z., Lam K.L., Hu J., et al. *Chlorogenic acid alleviates obesity and modulates gut microbiota in high-fat-fed mice.* Food Science & Nutrition, 2019.
38. Kazuo Yamagata. *Do Coffee Polyphenols Have a Preventive Action on Metabolic Syndrome Associated Endothelial Dysfunctions? An Assessment of the Current Evidence.* Antioxidants (Basel), 2018.

39. Lukitasari M., Rohman M.S., Nugroho D.A., et al. *Cardiovascular protection effect of chlorogenic acid: focus on the molecular mechanism.* F1000Res, 2020.
40. Nabavi S.F., Tejada S., Setzer W.N., et al. *Chlorogenic Acid and Mental Diseases: From Chemistry to Medicine.* Curr Neuropharmacol, 2017.
41. Mikami Y., Kakizawa S., Yamazawa T. *Essential Roles of Natural Products and Gaseous Mediators on Neuronal Cell Death or Survival.* Int J Mol Sci, 2016.
42. Shah M.A., Kang J.B., Park D.J., et al. *Chlorogenic acid alleviates neurobehavioral disorders and brain damage in focal ischemia animal models.* Neurosci Lett, 2021.
43. Socała K., Szopa A., Serefko A., et al. *Neuroprotective Effects of Coffee Bioactive Compounds: A Review.* Int J Mol Sci, 2021.
44. Lou Z., Wang H., Zhu S., et al. *Antibacterial activity and mechanism of action of chlorogenic acid.* J. Food Sci, 2011.
45. Bajko E., Kalinowska M., Borowski P., et al. *5-O-Caffeoylquinic acid: A spectroscopic study and biological screening for antimicrobial activity.* Food Sci. Technol, 2016.
46. Sung W.S. & Lee D.G. *Antifungal action of chlorogenic acid against pathogenic fungi, mediated by membrane disruption.* Pure Appl. Chem, 2010.
47. Rakshit S., Mandal L., Pal B. C., et al. *Involvement of ROS in chlorogenic acidinduced apoptosis of Bcr-Abl+ CML cells.* Biochemical Pharmacology, 2010.
48. Deka S., Gorai S., Manna D., et al. *Evidence of PKC binding and translocation to explain the anticancer mechanism of chlorogenic acid in breast cancer cells.* Current Molecular Medicine, 2017.
49. Hou N., Liu N., Han J., et al. *Chlorogenic acid induces reactive oxygen species generation and inhibits the viability of human colon cancer cells.* Anti-Cancer Drugs, 2017
50. Li W., Liu X., Zhang G., et al. *Mechanism of Chlorogenic Acid in Apoptotic Regulation through Notch1 Pathway in Non-small Cell Lung Carcinoma in Animal Level*. Zhongguo Fei Ai Za Zhi, 2017.
51. Yamagata K., Izawa Y., Onodera D., et al. *Chlorogenic acid regulates apoptosis and stem cell marker-related gene expression in A549 human lung cancer cells.* Molecular and Cellular Biochemis. 2018.Mol Cell Biochem, 2018.
52. Park J.J., Hwang S.J., Park J.H., et al. *Chlorogenic acid inhibits hypoxia-induced angiogenesis via down-regulation of the HIF-1α/AKT pathway*. Cellular Oncology, 2015.
53. Huang S., Wang L.L., Xue N., et al. *Chlorogenic acid effectively treats cancers through induction of cancer cell differentiation.* Theranostics, 2019.
54. Wang L., Du H., Chen P. *Chlorogenic acid inhibits the proliferation of human lung cancer A549 cell lines by targeting annexin A2 in vitro and in vivo*. Biomed. Pharmacother, 2020.
55. Burgos-Moron E., Calderon-Monta J.M., Orta M.L. *The coffee constituent chlorogenic acid induces cellular DNA damage and formation of topoisomerase Iand II-DNA complexes in cells*. J. Agric. Food Chem, 2012.
56. Hongtao L., Xiaoqi G., Junni L., et al. *Chlorogenic acid induced inhibition of non-small cancer cells occurs through regulation of histone deacetylase 6.* Cell. Mol. Biol, 2018.
57. Li W., Liu X., Zhang G., et al. L. *Mechanism of chlorogenic acid in apoptotic regulation through Notch1 pathway in non-small cell lung carcinoma in animal level.* Chin. J. Lung Cancer, 2017.
58. Yuan Yan 1, Na Liu., Hou N., Dong L., Li J., et al. *Chlorogenic acid inhibits hepatocellular carcinoma in vitro and in vivo*. J Nutr Biochem, 2017.

59. Liu Y., Feng Y., Li Y., et al. *Chlorogenic acid decreases malignant characteristics of hepatocellular carcinoma cells by inhibiting DNMT1 expression.* Front. Pharmacol, 2020.
60. Chen X., Yang J.H., Cho S.S., et al. *5-Caffeoylquinic acid ameliorates oxidative stress-mediated cell death via Nrf2 activation in hepatocytes*. Pharm. Biol, 2020.
61. Yan Y., Li J., Han J., et al. *Chlorogenic acid enhances the effects of 5-fluorouracil in human hepatocellular carcinoma cells through the inhibition of extracellular signal-regulated kinases.* Anticancer Drugs, 2015.
62. Burgos-Morón E., Calderón-Montaño J.M., Orta M.L. *The Coffee Constituent Chlorogenic Acid Induces Cellular DNA Damage and Formation of Topoisomerase I– and II–DNA Complexes in Cells.* J Agric Food Chem, 2012.
63. Feriotto G., Tagliati F., Giriolo R., et al. *Caffeic Acid Enhances the Anti-Leukemic Effect of Imatinib on Chronic Myeloid Leukemia Cells and Triggers Apoptosis in Cells Sensitive and Resistant to Imatinib.* Int J Mol Sci, 2021.
64. Yang J.S., Liu C.W., Ma Y.S., et al. *Chlorogenic acid induces apoptotic cell death in U937 leukemia cells through caspase- and mitochondria-dependent pathways.* In Vivo (Brooklyn), 2012.
65. Liu J.Y., Zhou C.Y., Qiu C.H., et al. *Chlorogenic acid induced apoptosis and inhibition of proliferation in human acute promyelocytic leukemia HL‐60 cells.* Mol. Med. Rep, 2013.
66. Changizi Z., Moslehi A., Rohani H., et al. *Chlorogenic acid induces 4T1 breast cancer tumor's apoptosis via p53, Bax, Bcl-2, and caspase-3 signaling pathways in BALB/c mice*. Journal of Biochemical and Molecular Toxicology, 2020.
67. Li Y. & Li X. *Study on the anticancer effect of an astragaloside- and chlorogenic acid-containing herbal medicine (RLT-03) in breast cancer*. Evid. Based Complement. Alternat. Med, 2020.
68. El-Garawani I.M., El-Nabi S.H., El-Shafey S., et al. *Coffea arabica bean extracts and vitamin C: a novel combination unleashes MCF-7 cell death*. Curr. Pharm. Biotechnol, 2021.
69. Ismail T., Donati-Zeppa S., Akhtar S., et al. *Coffee in cancer chemoprevention: an updated review*. Expert Opin Drug Metab Toxicol, 2021.
70. Huang S., Wang L.L., Xue N.N., et al. *Chlorogenic acid effectively treats cancers through induction of cancer cell differentiation.* Theranostics, 2019.
71. Zhou J., Zhang F., Chen J., et al. *Chlorogenic Acid Inhibits Human Glioma U373 Cell Progression via Regulating the SRC/MAPKs Signal Pathway: Based on Network Pharmacology Analysis.* Drug Des Devel Ther, 2021.
72. Skała E., Toma M., Kowalczyk T., et al. *Rhaponticum carthamoides transformed root extract inhibits human glioma cells viability, induces double strand DNA damage, H2A.X phosphorylation, and PARP1 cleavage.* Cytotechnology, 2018.
73. Sitarek T., Kowalczyk S., Santangelo A.J., et al. *The extract of Leonurus sibiricus transgenic roots with AtPAP1 transcriptional factor induces apoptosis via DNA damage and down regulation of selected epigenetic factors in human cancer cells*. Neurochem. Res, 2018.
74. Jin S., Kim K.C., Kim J.S. Anti-melanoma activities and phytochemical compositions of Sorbus commixta fruit extracts. Plants, 2020.
75. Wang X., Liu J., Xie Z., et al. *Chlorogenic acid inhibits proliferation and induces apoptosis in A498 human kidney cancer cells via inactivating PI3K/Akt/mTOR signalling pathway*. J. Pharm. Pharmacol, 2019.

76. Oleaga C., Ciudad C.J., No´V., et al. *Coffee polyphenols change the expression of STAT5B and ATF-2 modifying cyclin D1 levels in cancer cells. Oxid. Med*. Cell Longev, 2012.
77. Kang N.J., Lee K.W., Kim B.H., et al. *Coffee phenolic phytochemicals suppress colon cancer metastasis by targeting MEK and TOPK*. Carcinogenesi, 2011.
78. Sadeghi Ekbatan S., Li X.Q., Ghorbani M., et al. *Chlorogenic acid and its microbial metabolites exert anti-proliferative effects, S-phase cell-cycle arrest and apoptosis in human colon cancer Caco-2 cells.* Int. J. Mol. Sci, 2018.
79. Hou N., Liu N., Han J., et al. *Chlorogenic acid induces reactive oxygen species generation and inhibits the viability of human colon cancer cells.* Anticancer Drugs, 2017.
80. Li W., Ji M., Xue N., et al. *First in human phase I study of chlorogenic acid injection in recurrent high-grade glioma.* Journal of Clinical Oncology, 2018.
81. Whelan J. S. & Davis L. E. *Osteosarcoma, chondrosarcoma, and chordoma.* Journal of Clinical. Oncology, 2018.
82. Luetke A., Meyers P. A., Lewis I., et al. *Osteosarcoma treatment - where do we stand? A state-of-the-art review.* Cancer Treatment Reviews, 2014.
83. Durfee R. A., Mohammed M., Luu, H. H. *Review of osteosarcoma and current management*. Rheumatology and Therapy, 2016.
84. Olivier M., Hollstein M., Hainaut P. *TP53 Mutations in Human Cancers: Origins, Consequences, and Clinical Use.* Cold Spring Harb Perspect Biol, 2010.
85. Li Y.S., Liu Q., He H.B., et al. *The possible role of insulin-like growth factor-1 in osteosarcoma.* Curr Probl Cancer, 2019.
86. Ritter J. & Bielack S.S. *Osteosarcoma. Annals of Oncology*, 2010.
87. Close S.S., Bielack B., Kempf-Bielack G., et al. *Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1.702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols*. J Clin Oncol, 2002.
88. Evola F.R., Costarella L., Pavone V., et al. *Biomarkers of Osteosarcoma, Chondrosarcoma, and Ewing Sarcoma.* Front Pharmacol, 2017.
89. Zhao X., Wu Q., Gong X., et al. *Osteosarcoma: a review of current and future therapeutic approaches.* BioMedical Engineering, 2021.
90. Zhang Y., Yang J., Zhao N., et al. *Progress in the chemotherapeutic treatment of osteosarcoma*.
Oncol Lett, 2018.
91. Carrle D. & Bielack S.S. *Current strategies of chemotherapy in osteosarcoma*. Int Orthop, 2006.
92. Martins-Teixeira M.B. & Carvalho I. *Antitumour Anthracyclines: Progress and Perspectives*. Chem Med Chem, 2020.
93. Sritharan, S. & Sivalingam N. *A comprehensive review on time-tested anticancer drug doxorubicin.* Life Sci, 2021.
94. Yu D., Zhang S., Feng A., et al. *Methotrexate, doxorubicin, and cisplatinum regimen is still the preferred option for osteosarcoma chemotherapy: A meta-analysis and clinical observation*. Medicine, 2019.
95. Meredith A.M. & Dass C.R. *Increasing role of the cancer chemotherapeutic doxorubicin in cellular metabolism*. J Pharm Pharmacol, 2016.
96. Marinello J., Delcuratolo M., Capranico G. *Anthracyclines as Topoisomerase II Poisons: From Early Studies to New Perspectives.* Int. J. Mol. Sci, 2018.

97. Prudowsky Z.D. & Yustein, J.T. *Recent Insights into Therapy Resistance in Osteosarcoma*. Cancers, 2021.
98. Kalyanaraman, B. *Teaching the basics of the mechanism of doxorubicin-induced cardiotoxicity: Have we been barking up the wrong tree?* Redox Biol, 2020.
99. Gegechkori N., Haines L., Lin J.J. *Long-Term and Latent Side Effects of Specific Cancer Types.* Med. Clin. N. Am, 2017.
100. Martins-Teixeira M.B. & Carvalho I. *Antitumour Anthracyclines: Progress and Perspectives.* ChemMedChem, 2020.
101. Cappetta D., De Angelis A., Sapio L., et al. *Oxidative Stress and Cellular Response to Doxorubicin: A Common Factor in the Complex Milieu of Anthracycline Cardiotoxicity.* Oxid Med Cell Longev, 2017.
102. Sirangelo I., Sapio L., Ragone A., et al. *Vanillin Prevents Doxorubicin-Induced Apoptosis and Oxidative Stress in Rat H9c2 Cardiomyocytes*. Nutrients, 2020.
103. Johnson-Arbor K. & Dubey R.. Doxorubicin. StatPearls Publishing, 2021.
104. Frei E 3rd., Karon M., Levin R.H., et al. *The effectiveness of combinations of antileukemic agents in inducing and maintaining remission in children with acute leukemia.* Blood, 1965.
105. Quinn B.A., Dash R., Sarkar S., et al. *Pancreatic Cancer Combination Therapy Using a BH3 Mimetic and a Synthetic Tetracycline.* Cancer Res, 2015.
106. Albain K.S., Nag S.M., Calderillo-Ruiz G., et al. *Gemcitabine plus Paclitaxel versus Paclitaxel monotherapy in patients with metastatic breast cancer and prior anthracycline treatment.* J Clin Oncol, 2008.
107. Mokhtari R.B., Kumar S., Islam S.S., et al. *Combination of carbonic anhydrase inhibitor, acetazolamide, and sulforaphane, reduces the viability and growth of bronchial carcinoid cell lines.* BMC Cancer, 2013.
108. Shanmugam N., Scott J.X., Kumar V., et al. *Multidisciplinary management of hepatoblastoma in children: Experience from a developing country.* Pediatr Blood Cancer, 2017.
109. Greenwell M. & Rahman P.K. *Medicinal Plants: Their Use in Anticancer Treatment.* Int J Pharm Sci Res, 2015.
110. Eom Y.W., Kim M.A., Park S.S., et al. *Two distinct modes of cell death induced by doxorubicin: Apoptosis and cell death through mitotic catastrophe accompanied by senescence-like phenotype*. Oncogene, 2005.
111. Sangweni N.F., Moremane M., Riedel S., et al. *Prophylactic Effect of Pinocembrin Against Doxorubicin-Induced Cardiotoxicity in an In Vitro H9c2 Cell Model*. Front. Pharmacol, 2020.
112. Dallons M., Schepkens C., Dupuis A., et al. *New Insights About Doxorubicin-Induced Toxicity to Cardiomyoblast-Derived H9C2 Cells and Dexrazoxane Cytoprotective Effect: Contribution of In Vitro (1)H-NMR Metabonomics*. Front. Pharmacol, 2020.
113. Hu Y.H., Liu J., Lu J., et al. *sFRP1 protects H9c2 cardiac myoblasts from doxorubicin-induced apoptosis by inhibiting the Wnt/PCP-JNK pathway*. Acta Pharmacol. Sin, 2020.
114. Lee S., Rauch J., Kolch W. *Targeting MAPK Signaling in Cancer: Mechanisms of Drug Resistance and Sensitivity.* Int. J. Mol. Sci, 2020.
115. Roskoski, R. *ERK1/2 MAP kinases: Structure, function, and regulation*. Pharmacological Research, 2012.
116. Chandhanayingyong C., Kim Y., Staples J. R., et al. *MAPK/ERK signaling in osteosarcomas, Ewing sarcomas and chondrosarcomas: Therapeutic implications and future directions.* Sarcoma, 2012.

117. Ilisso C. P., Sapio L., Delle Cave D., et al. *Adenosylmethionine affects ERK1/2 and Stat3 pathways and induces apotosis in osteosarcoma cells*. Journal of Cellular Physiology, 2016.
118. Silva G. & Aboussekhra A. *p16(INK4A) inhibits the prometastatic potentials of osteosarcoma cells through targeting the ERK pathway and TGF-β1*. Molecular Carcinogenesis, 2016.
119. Wei M., Zheng Z., Shi L., et al. *Natural polyphenol chlorogenic acid protects against acetaminophen-induced hepatotoxicity by activating ERK/Nrf2 antioxidative pathway*. Toxicological Sciences, 2018.
120. Yan Y., Li J., Han J., et al. *Chlorogenic acid enhances the effects of 5-fluorouracil in human hepatocellular carcinoma cells through the inhibition of extracellular signal-regulated kinases.* Anti-Cancer Drugs, 2015.
121. Kim S., You D., Jeong Y., rt al. *Berberine down-regulates IL-8 expression through inhibition of the EGFR/MEK/ERK pathway in triple-negative breast cancer cells.* Phytomedicine, 2018.
122. Yao J., Qian C., Shu T., rt al. *Combination treatment of PD98059 and DAPT in gastric cancer through induction of apoptosis and downregulation of WNT/β- catenin*. Cancer Biology & Therapy, 2013.
123. Illiano M., Sapio L., Salzillo A. *Forskolin improves sensitivity to doxorubicin of triple negative breast cancer cells via Protein Kinase A-mediated ERK1/2 inhibition.* Biochem Pharmacol, 2018.
124. Christowitz C., Davis T., Isaacs A., et al. *Mechanisms of doxorubicin-induced drug resistance and drug resistant tumour growth in a murine breast tumour model*. BMC Cancer, 2019.
125. Liu Y., Bushan Raina D., Sebastian S., et al. *Sustained and controlled delivery of doxorubicin from an in-situ setting biphasic hydroxyapatite carrier for local treatment of a highly proliferative human osteosarcoma*. Acta Biomater, 2021.
126. Hayakawa S., Ohishi T., Miyoshi N., et al. *Anti-Cancer Effects of Green Tea Epigallocatchin-3-Gallate and Coffee Chlorogenic Acid.* Molecules, 2020.
127. Chlopcikova S., Psotova J., Miketova P., et al. *Chemoprotective effect of plant phenolics against anthracycline-induced toxicity on rat cardiomyocytes. Part II. caffeic, chlorogenic and rosmarinic acids*. Phytother. Res, 2004.