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NATURAL AND NATURALLY-DERIVED
COMPOUNDS AS NEW
CHEMOPREVENTIVE AGENTS

Dr. SILVIA DI GIACOMO

Doctoral Coordinator: *Supervisor:*
Prof. VINCENZO CUOMO   **Prof.ssa GABRIELA MAZZANTI**

Doctoral Committee
Prof.ssa MARIA LUISA BARBACCIA
Prof. ROBERTO CICCOCIOPPO
Prof.ssa LUIGIA TRABACE

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ABSTRACT

Chemoprevention is an approach based on the use of natural or synthetic compounds to inhibit, suppress or reverse the development and progression of cancer, by blocking the DNA-damage induction or by arresting or reversing its progression. In order to overcome the cancer disease, the identification of chemopreventive compounds became of particular interest. Among them, antimutagens prevent the mutagen-induced DNA-injury or promote the repair and/or the reversion of damage. In addition to antimutagenicity, some agents also act as chemosensitizers, by increasing the effectiveness of cancer chemotherapy and radiotherapy, when used in combination with chemotherapeutical agents. This approach is very interesting to prevent the development of multidrug resistance (MDR), which makes cancer cells not-sensitive to a broad range of drugs.

In this context, present study was aimed at evaluating the potential chemopreventive properties of some natural and naturally-derived compounds, particularly the sesquiterpenes β-caryophyllene (CRY) and β-caryophyllene oxide (CRYO), and the aldehyde α-hexylcinnamal (HCA). The antimutagenic activity (pre-incubation method) was evaluated by the reverse bacterial mutation assay (Ames test), on *Salmonella typhimurium* TA1535, TA1538, TA98, TA98NR, TA98 1,8-DNP and TA100, and *Escherichia coli* WP2*uvr*A and WP2*uvr*A/R strains, both in absence and presence of the S9-metabolic activation system. As mutagens, 2-nitrofluorene (2NF), sodium azide (SA), methyl methanesulfonate (MMS), 2-aminoanthracene (2AA), benzo[a]pyrene (BaP), 4-nitroquinoline N-oxide (4NQO), 1-nitropyrene (1NP), 1,8-dinitropyrene (1,8-DNP) and a sample of condensed smoke (CSC) from standard 3R4F cigarette were used. In addition to antimutagenicity studies, the potential chemosensitizing properties of CRY, CRYO and HCA and their ability to interfere with ABC-transporter function were evaluated, in Caco-2, CEM/ADR5000 and CCRF/CEM human cancer cells. For each compound, low concentrations (IC_{10} and IC_{20}) were assayed in order to verify their potential additive, synergistic or antagonistic effects with the anticancer doxorubicin. The nature and the extent of the interaction were evaluated by the combination index (CI) and the isobologram analysis, respectively; conversely, the potential enhancement of drug effectiveness was quantified by cytotoxicity enhancement ratio (RR). The interaction between test compounds and ABC-transporters was studied by the rhodamine 123 assay.

HCA exhibited an antimutagenic activity against different nitro-compounds (2NF and 1NP in all experimental protocols and 1,8-DNP in the post-treatment) and in various experimental protocols, suggesting the involvement of both desmutagenic and bioantimutagenic mechanisms.

The sesquiterpenes CRY and CRYO resulted able to inhibit the mutagenicity of CSC, although with different potency and specificity: CRYO was the most potent compound, acting at concentrations about ten-times lower than CRY. The antimutagenicity was highlighted in different strains and in all experimental protocols, suggesting the overlapping of various protective mechanisms; the inhibition of CSC-induced oxidative stress seems to be likely and deserves further investigations.

In human cancer cells, the substances produced cytotoxic effects at high concentrations both in resistant and in sensitive cell lines (200 µM $<IC_{50}<$ 1000 µM): HCA was the most effective substance, especially in the sensitive CCRF-CEM cells (IC_{50} = 212.95 µM). All the compounds synergistically acted with doxorubicin, although HCA was the most potent: IC_{20} HCA increased the doxorubicin cytotoxicity of about six, seven and fourthy-seven folds, in Caco-2, CEM/ADR5000, and CCRF-CEM, respectively. In addition, a remarkable inhibition of ABC-trasporter was produced by HCA in the cancer cells tested: the effect was higher that that of the standard inhibitior verapamil. Also CRY and CRY oxide inhibited the ABC transporters but with lower potency than verapamil.

The antimutagenic and chemosensitizing properties of β-caryophyllene, β-caryophyllene oxide and the α-hexylcinnamaldehyde deserves attention and represent a starting point to better evaluate their potential applications in the field of chemoprevention.

GENERAL BACKGROUND

GENERAL BACKGROUND

CHEMOPREVENTION

According to Sporn et al. (1976), the term of chemoprevention indicates the use of natural or synthetic compounds to inhibit, suppress or reverse the development and progression of cancer. Cancer is the second leading cause of death worldwide after heart disease and chemotherapy is the major treatment, when cancer is well established within the patient. Currently there is greater need for more effective and less toxic therapeutic and/or preventive strategies. In this context, natural products are becoming an important research area for identifying novel bioactive molecules.

Phytochemicals and dietary compounds have been used in the cancer treatment throughout history due to their safety, low toxicity, and general availability (Pratheeshkumar et al., 2012). Hippocrates said *"Let food be thy medicine and medicine be thy food"*, while Galen of Pegamon (129–199 A.D.) was said to have prescribed various foods and vegetables for the cancer treatment (Karpozilos and Pavlidis, 2004). Also in the Ayurvedic literature the use of natural products to cure "minor neoplasms" (Granthi) and "major neoplasms" (Arbuda) was reported (Balachandran et al., 2005). Likewise, Traditional Chinese Medicine (TCM) and Mayan civilization applied to natural products to treat or prevent several disease among which cancer (Kunow, 2003; Xutian et al., 2009).

A consistent relationship between fruit and vegetable consumption and cancer risk reduction has been found (González-Vallinas et al., 2013). This beneficial effects have been attributed to different bioactive compounds (Rafter, 2002), for instance isothiocyanates from cruciferous vegetables, polyphenols from green and black tea, and flavonoids from soybeans have been identified (Surh, 2003). A variety of naturally-occurring dietary compounds have been shown to possess significant chemopreventive properties and many experimental attempts have been made to underline their mechanisms of action (Surh, 2003). Recently, also the modulation of the signaling pathways, transcription factors and genes expression by chemicals or chemical combination has been evaluated as new opportunities for future design chemopreventive agents (Kwon et al., 2007; Metha et al., 2010).

Natural products and phytochemicals can act both as chemopreventive as well as chemotherapeutic agents. Until relatively recent times, new potential chemotherapeutic agents have been discovered, for example, alkaloids from vinca (vinblastin, vincristine and vindesine),

epipodophyllotoxins (etoposide and teniposides), taxanes (paclitaxel and docetaxel), and camtothecins (camptothecin and irinotecan).

The mechanisms of chemopreventive agents towards mutagens and carcinogens may depend on several variables, such as doses, route of penetration into the organism, sequence of intake, chemical interactions, biotransformations in the organism (De Flora and Ferguson, 2005). Neverthless, taking into account that mutagenesis and carcinogenesis evolve through a cascade and a network of events (Figure 1), it appears very difficult to establish, when the role of a preventive agent ends during the progression of cancer and when the role of a therapeutic agent begins. For instance, the inhibition of adduct-formation to either nuclear DNA or mitochondrial DNA is a biomarker, which reflects the occurrence of protective mechanisms preceding the binding of electrophilic molecules to DNA (De Flora et al., 1996).

A chemopreventive agents may be useful for individuals at high risk of developing cancer, such as patients whose colon polyps have been removed or individuals who may be at a higher risk of developing cancer due to family history. On the other hand, for cancer patients in whom the goal is to kill the cancer cells, chemotherapeutic agents are routinely used.
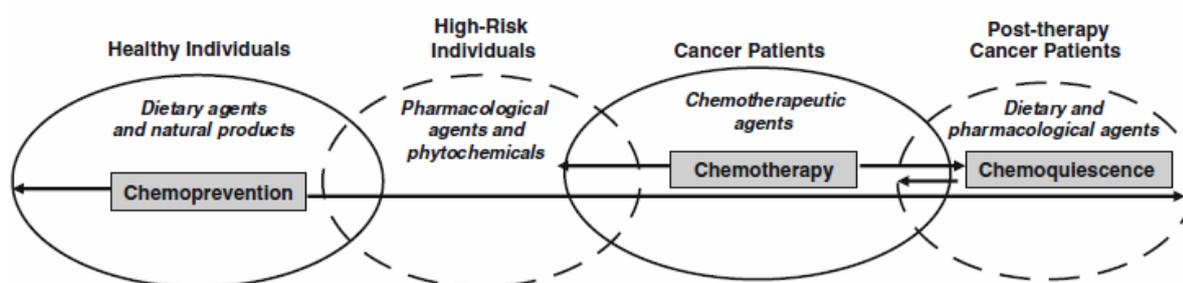

Figure 1. A schematic diagram to show selective responsiveness of healthy population as well as cancer patients to chemopreventive agents. Accordingly, chemopreventive agents (CPA) can be useful to all populations; for cancer patients, it is feasible that CPA can be used in combination with chemotherapeutic agents. For post-therapy patients, dietary modification along with pharmacological intervention should be considered for suppressing or inhibiting the recurrence (Mehta et al., 2010).

It is important to point out that in the case of the cancer patient, chemopreventive agents may be used in combination with chemotherapeutic agents in hopes of providing additive or synergistic effects. Moreover, dietary recommendations (including a variety of fruits, vegetables and whole grains) may provide an additional protection and may be a necessary component in the post-therapy of cancer patients, when recurrence of the disease is an undesired consequence. This kind of protection is termed chemoquiescence as it allows to prevent second primary tumors.

A good chemopreventive agent should be able to interfere with one or more phases of the carcinogenesis process (Figure 2): initiation (days), promotion (several years), and progression (1–5 years) (Russo, 2007). Initiation is irreversible and includes the initial damage by chemical or physical carcinogenic agents directly at DNA level. Promotion, which involves epigenetic mechanisms, is generally a slow and reversible process leading to accumulation of pre-malignant cells abnormally dividing. Finally, progression is the irreversible step, and leads to the final stage of carcinogenesis with tumor growth and acquisition of invasiveness and metastatic potential (Russo, 2007). The passage from pre-malignant to malignant cell requires the activation of proto-oncogenes and/or the inactivation of tumor suppressor genes (Hanahan, 2000). Both categories of genes, when mutated, cause alterations in key cellular processes linked to cell growth and proliferation.

It is well established that mutations in somatic cells play a key role in cancer initiation and other stages of the carcinogenesis process. Antimutagens are agents able to protect the cells by inhibit the induction of DNA-damage or by favouring its repair. These substances can act by extracellular mechanisms, such as inhibition of the mutagen-uptake, complexation, dilution and/or deactivation of mutagens, block of secondary oxidative stress and affection of activation and detoxification systems; the mutagen-inhibition by some physical, chemical or enzymatically catalysed interactions with the antimutagen can also occur in the intracellular compartment (De Flora, 1998). The agents which prevent the mutagen-induced DNA-damage both at extra- or intracellular level, are defined as desmutagenic agents (Shamon and Pezzato, 1994). Conversely, compounds that interfere with fixation and progression of DNA-damage in intracellular compartment, by stimulating the DNA repair and/or reversion systems are bioantimutagens (Shamon and Pezzato, 1994). It is also clear that certain mechanisms are strictly interconnected or partially overlapping.

Carcinogenesis can be activated by various environmental carcinogens (such as cigarette smoke, industrial emissions, gasoline vapors), inflammatory agents (such as tumor necrosis factor, or TNF, and H_2O_2) and tumor promoters (such as phorbol esters and okadaic acid) (Dorai and Aggarwal, 2004).
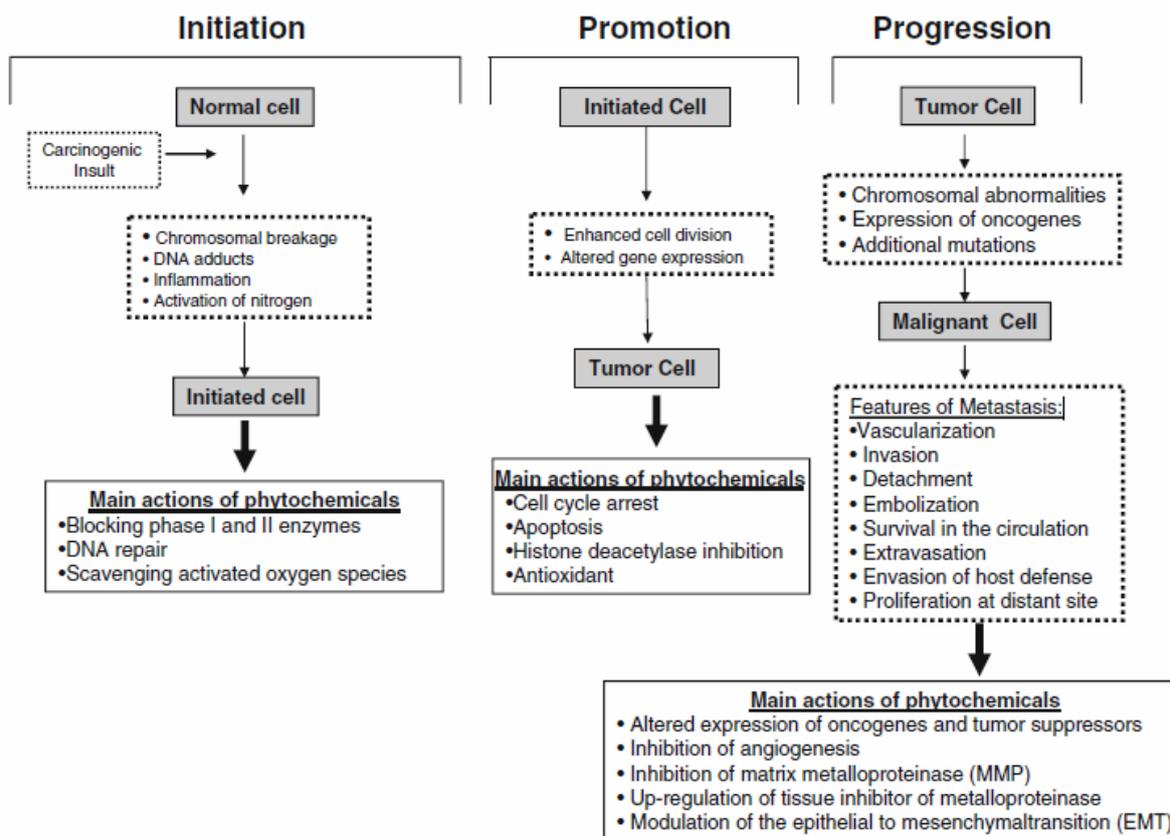
Figure 2. Process of carcinogenesis: initiation, promotion and progression. Progression is shown here to include the growth of malignant tumors, invasion and metastasis. In this diagram for each of the stages, various major actions of phytochemicals involving signaling pathways are summarized. Mehta et al. 2010

In the initial stage of carcinogenesis, carcinogens can induce one or more simple mutations, including transitions, or small deletions in genes which control the process: phase I enzymes (including the cytochrome P450 system) can convert pro-carcinogens into the DNA-reactive metabolites, which form covalent adducts with individual nucleic acids (DNA or RNA). Conversely, phase II enzymes (e.g., glucoronidases, sulfotransferases) play a role in the detoxification of the activated carcinogens. Reactive oxygen species (ROS) can also released as end-products of the breakdown of xenobiotics, so inducing additional DNA-damage. When DNA-damage is not repaired, mutations in critical genes, such as tumor suppressors or oncogenes, can be produced.

Chemopreventive agents with antioxidant properties, such as vitamin C, genistein, and compounds originating from cruciferous vegetables have been shown able to prevent the oxidative DNA-damage (Guilford and Pezzuto, 2008).

The promotion of carcinogenesis is characterized by deregulation of the signaling pathways which normally control cell proliferation and apoptosis; particularly, mutation of the genes responsible for the cell cycle control result in the continued proliferation of transformed cells in

spite of the normal cells. In this context, apoptosis of transformed cells must be expected (Khan et al., 2007). Two pathways, intrinsic (mitochondrial) and extrinsic (death receptor), are involved in apoptosis. Many chemopreventive agents have been shown to regulate the intrinsic pathways of apoptosis (Murillo et al., 2004). The caspases represent a cysteine protease family, which possess a pivotal role in the apoptosis control. Bcl-2 family members are crucial to control the mitochondrial-mediated apoptosis, by halting the mitochondrial membrane disruption and the release of cytochrome c and other pro-apoptotic factors. As well survivin play a key role in regulation of apoptosis in cancer cells. So, anti-promotional agents can target a variety of signaling pathways, including transcription factors, mitogen-activated protein kinases, hormone receptors, cell cycle check-point markers, rate-limiting enzymes and tumor suppressor genes. Finally, the progression is characterized by genetic alterations within the karyotype of the cells brought about by accumulation of mutated genes and resulting in chromosomal abnormalities; invasion, angiogenesis, and metastatic growth constitute the stage of progression. Anti-angiogenic compounds block the development of new blood vessels, which is a crucial step for malignant cell nutrition; as a result, the size of the tumor and metastasis may be reduced. Polyunsaturated fatty acids, EGCG, resveratrol, curcumin, and genistein have been shown to inhibit this process. Also deguelin has been reported to exert anti-angiogenic effects against human hepatocellular carcinoma (Lee et al., 2008).

Recent reports showed that cancer chemopreventive phytochemicals, when used in combination with chemotherapeutical agents, can act as chemosensitizers, so increasing the effectiveness of cancer chemotherapy and radiotherapy. In fact, the use of chemotherapy to treat cancer ever results in the development of broad resistance to a wide variety of drugs with different chemical structures and mechanisms of action. This form of resistance is mediated primarily by classical ATP-driven drug efflux pumps such as the P-glycoproteins and the MRP family of proteins. The earlier agents characterized as transporter blocking, such as verapamil and PSC-833, have shown to produce significant toxic effects. Recent reports on the reversal multidrug resistance (MDR) by chemopreventive compounds, such as curcumin and genistein, have provided encouraging results. The chemosensitization can occur at various levels, by directly competing with the ATP binding site of the MDR or MRP drug efflux pumps, by saturating the pumps and increase the amount of the chemotherapeutic drug within the cell, or by interfering with the pump functioning, so impairing the efflux of anticancer compounds. For instance, curcumin is able to inhibit the pump, and to interfere with MRP transporter function (requiring a steady supply of reduced glutathione), so increasing the intracellular concentrations of chemotherapeutic drugs, such as vinblastine or vincristine (Harbottle et al., 2001). Conversely,

genistein and green tea components (EGCG) act as efflux substrate, competing with the MDR or MRP substrates, saturating the pump and prevent the efflux of chemotherapeutics.

On the basis of these evidences, chemopreventive agents can be used not just to prevent but also in the treatment of cancer. Because of their pharmacological safety, the combination of chemopreventive agents with anticancer drugs enhance the effectiveness of chemotherapy at lower doses, so minimizing toxicity and overcoming the multidrug resistance phenomenon. Again, some chemopreventive agents are also able to suppress multiple pathways involved in cancer development, so representing a potential new source of anticancer compounds.

MULTIDRUG RESISTANCE

Chemotherapy is the treatment of choice for patients diagnosed in the late stage of locally advanced and metastatic cancers, and it is used for primary treatment, adjuvant therapy and palliation. In these patients, administering a drug dosage, which maximizes the efficacy and minimizes the toxicity of treatment is the main challenge. Unfortunately, the tumor does not respond to the therapeutic agents in a significant number of patients. This clinical obstacle is due to known and yet-to-be determined mechanisms of resistance to chemotherapy (MDR), in which the cells become resistant to a variety of structurally and mechanistically unrelated drugs in addition to the drug initially administered (Gillet and Gottesman, 2010). Taking into account that cancer cells are genetically heterogeneous because of the mutate phenotype, the involvement of more than one mechanism of multidrug resistance can be expected: this phenomenon has been called multifactorial multidrug resistance (Gottesman et al., 2002). Resistance can be developed against a wide range of anticancer drugs, especially natural product agents (e.g., anthracyclines, epipodophyllotoxins, taxanes and vinca alkaloids): although their few structural and functional similarities, they are large, hydrophobic molecules and may enter the cell by passive diffusion (Thomas, 2003).

Two general classes of anticancer drug resistance have been identified: 1) the anticancer drug release to tumor cells is impaired; 2) genetic and epigenetic alterations affect drug sensitivity in the cancer cell (Gottesman, 2002).

The main mechanisms that cause drug resistance involve:

➢ the increased activity of the efflux pump (i.e. ATP-dependent transporters) enhances the anticancer agent elimination (classical multidrug resistance): this mechanism affects vinblastine and vincristine, doxorubicin and daunorubicin, and paclitaxel;

➢ the decreased uptake might fail to accumulate the drug without evidence of its increased efflux (es. water-soluble drugs that 'piggyback' on transporters, carriers that are used to bring nutrients into the cell, or agents that enter by means of endocytosis): methotrexate, nucleotide analogues, such as 5-fluorouracil and 8-azaguanine, and cisplatin are examples;

➢ drugs inactivation by induction of detoxifying proteins (such as cytochrome P450 mixed-function oxidases); a synchronize induction of the multidrug transporter P-glycoprotein (Pgp) and cytochrome P450 3A has been observed (Schuetz et al., 1996): this type of multidrug resistance can be induced after exposure to any drug;

➢ activation of the mechanisms that repair drug-induced DNA damage;

➢ alteration or modification of the drug targets;

➢ disruptions in apoptotic signaling pathways: for instance before a malignant transformation (e.g. tumors with mutant or nonfunctional p53; decreased ceramide levels), the cells acquire changes in apoptotic pathways, which can prevent the initiation apoptosis. The main mechanisms that cause drug resistance are showed in Figure 3.
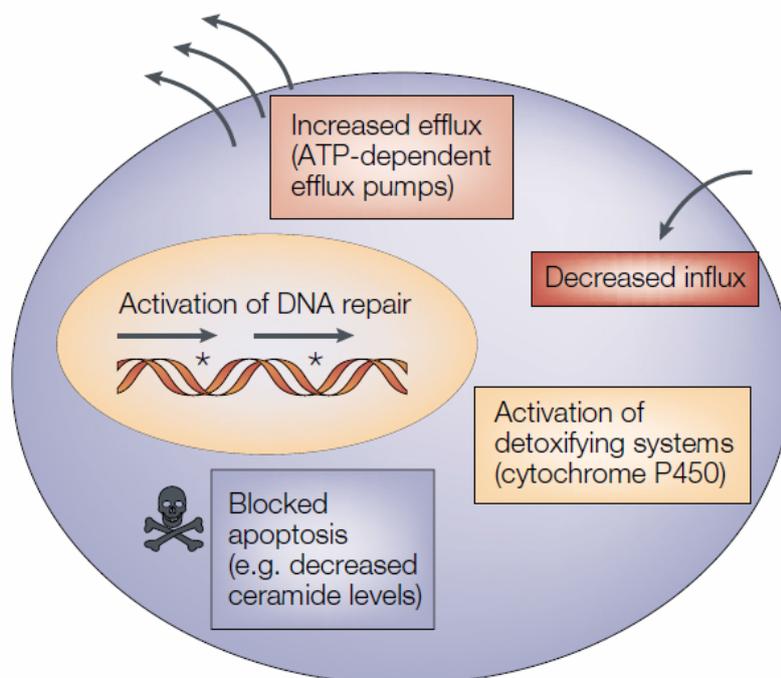

Figure 3. Cellular factors that cause drug resistance (Gottesman et al., 2002)

Alternatively, impaired drug delivery can result from poor absorption of orally administered drugs, increased drug metabolism or increased excretion, resulting in lower levels of drug in the blood and reduced diffusion into the tumor mass. Furthermore, environmental factors, such as the extracellular matrix or tumor geometry, might be involved in drug resistance.

Although a variety of mechanisms are responsible for MDR in cancer cells, the overexpression of ATP-binding cassette (ABC) transporters seems to be of particular importance. Therefore, in order to combat MDR and sensitize resistant cancer cells, a significant effort has been directed recently toward the develop of ABC-transporter inhibitors (Yan et al., 2013).

ATP-BINDING CASSETTE (ABC) TRANSPORTERS

ATP-binding cassette (ABC) transporters are carrier protein superfamily, whose designation was based on the highly conserved ATP-binding cassette (Higgins, 1992). So far, 48 human ABC genes have been identified and divided into seven distinct subfamilies (ABCA–ABCG), on the basis of their sequence homology and domain organization (Dean et al., 2001). MDR1 P-glycoprotein (ABCB1), MRP1 (multidrug resistance protein, ABCC1), and BCRP1 (breast cancer resistance protein, ABCG2, MXR, ABCP) are the most extensively studied transporters at moment.

These carriers are involved the active transport of phospholipids, peptides, steroids, polysaccharides, amino acids, nucleotides, organic anions, drugs, toxicants, food components, and their conjugates with glutathione, glucuronate, or sulfate (Hoffmann and Kroemer, 2004).

The clinical significance of the multidrug resistance pumps P-glycoprotein, and MRP has been initially restricted to the resistance to anticancer drugs by enhancing cellular export of classical cytostatics. Afterwards, it has been described that three members of the ABCC family, MRP4 (ABCC4), MRP5 (ABCC5), and MRP7 (ABCC7), transport cyclic nucleotides thereby having a potentially modulating effect on signal transduction. However, these cyclic nucleotide transporters are involved in cellular export of structurally related anticancer drugs (e.g., methotrexate, adriamycin) and antiviral compounds (e.g., ganciclovir, adefovir) and contribute to drug resistance (Chan et al., 2004; Kruh and Belinsky, 2003).

It should be noted that interactions between cross-stimulatory or cross-inhibitory compounds appear very complex. ABC transporters can transport at the same time multiple different substrates, and the co-transport results in a modification of the transport efficacy. Many transporters have an overlapping substrate specifity or distinct drug. The transport binding sites influences the molecular interactions (Hoffmann and Kroemer, 2004).

From a structural point of view, ABC proteins are composed of about 1300 amino acids and two structurally similar fractions, each of which contains two parts: a transmembrane domaine (TMD) that is arranged into six α-helices, and a nucleotide-binding domain (NBD), (Figure 4). An additional N-terminal extracellular extension consisting of five putative transmembrane segments are present in the MRP 1–3 transporters. NBD represent the binding sites for ATP, whose hydrolysis release the energy to move the substrates across the membrane. Two sequence motifs located in each NBD, designated ''Walker A'' and ''Walker B,'' are critical for ATPase function (Walker et al., 1982). The Walker A motif is involved in the binding β-phosphate of ATP, while the Walker B motif binds magnesium (Sharom et al., 1999). The exact mechanism by which the energy derived from ATP hydrolysis is transduced into drug

transport is not known. All ABC-transporters possess to extracelluar N-glycosylation branches. Moreover, frequently a single gene encodes for the four domains, as is the case for P-gp which has a TMD-NBD-TMD-NBD structure (Gottesman et al. 1996; Higgins, 2001).
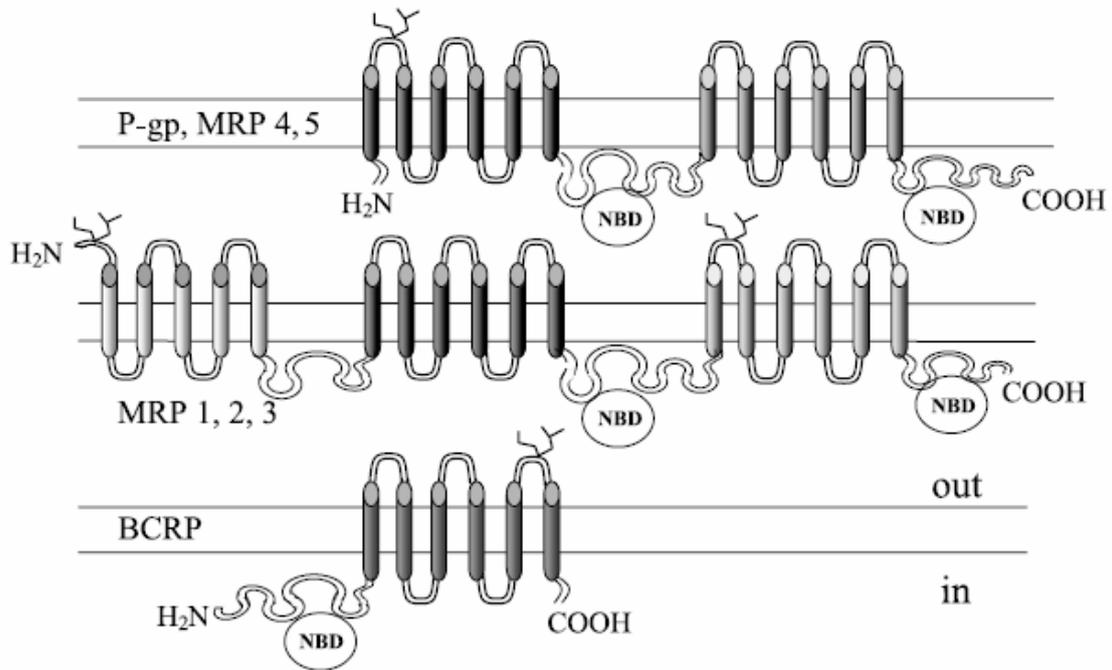

Figure 4. Structures of some ABC-transporters based on the amino acid sequence homology and the assumed secondary structure. 1) P-glycoprotein, MRP4 and MRP5: two transmembrane domains (TMD), each containing six transmembrane α-helices, and two nucleotide binding domains (NBD); glycosylation of P-gp and MRP at first extracellular loop, and at first loop of second extracellular domain, respectively. 2) MRP1-3: additional aminoterminal extension containing five transmembrane segments, glycosylated near N-terminus at extracellular loop. 3) BCRP: six transmembrane helices and one NBD; likely N-glycosylated at third extracellular loop; NBD located on aminoterminal loop (Hoffmann and Kroemer 2004).

MDR1 P-glycoprotein (ABCB1 or P-gp)

It is a single polypeptide (170 kDa) consisting of 1280 residues that are structured in two repeats of 610 amino acids, joined by a linker region (Jones and George, 2000). Each duplicate is formed of a NH2-terminal hydrophobic domain with six transmembrane α-helices, separated by three hydrophilic loops (TMD); the hydrophilic intracelluar domain (NBD) contains the ATP binding site (Figure 5). The transporter is a cylinder of about 10 nm in diameter with one half of the molecule in the lipid bilayer and the rest above and below the membrane (Rosenberg et al., 1997). The central pore, of approximately 5 nm in diameter, is delimited by a hexagonal TMD-array (Dong et al., 1998). The drug binding domain is located near the transmembrane domains. For a functional molecule both ATP sites are necessary, but sites for substrate recognition and ATP binding are different (Dey et al., 1998).

A wide range of structurally unrelated hydrophobic compounds (mainly neutral or basic organic agents), including numerous drugs, can be extruded by P-gp transporter. Molecules characterized by hydrophobicity, planar aromatic rings, and tertiary amino groups are preferentially transported. Among the other structural elements, the presence of two or three electron donor groups with a fixed spatial separation increase the affinity to the P-gp binding sites. Moreover, a high percentage of hydrogen bonding amino acids of P-gp in the transmembrane sequences is relevant for the substrate interaction (Seelig, 1998; Seelig et al., 2000).

P-glycoprotein can be activated by protein kinase C (PKC)-mediated phosphorylation; conversely, PKC inhibitors reduce the phosphorylation, leading to drug accumulation. PKC inhibitors can also directly interact with P-gp protein and inhibit drug transport (Castro et al., 1999; Conseil et al., 2001).
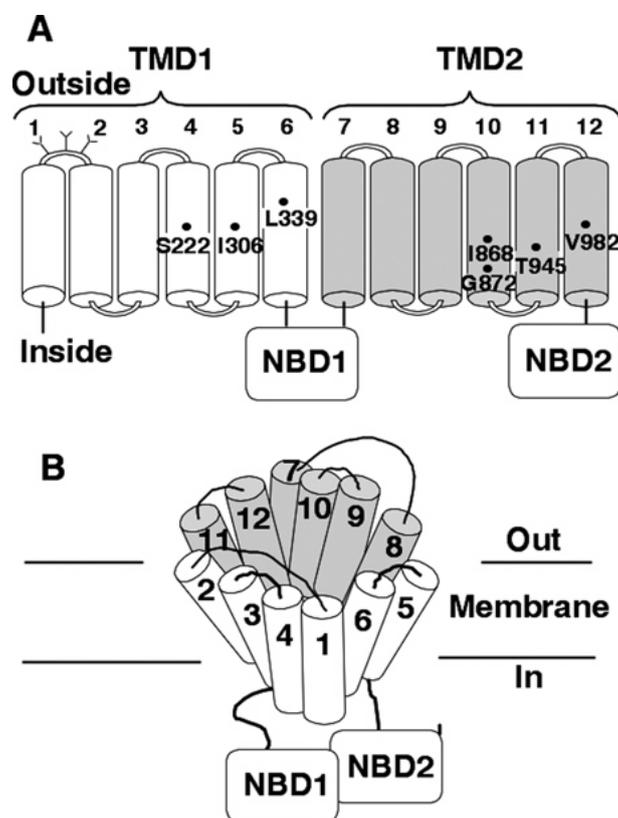
**Figure 5. Schematic P-gp-models.** (A) White and grey cylinders represent TM segments in TMD1 and TMD2, respectively. Branched lines and rounded rectangles indicate the glycosylation sites and NBD domains. Residues in the TM segments are included in the drug-binding pocket. (B) Model of TM-segments organization based on cross-linking and cysteine scanning mutagenesis studies (Loo and Clarke, 2005).

This transporter is localized in the plasma membrane, on the apical or luminal surface of epithelial cells, including the blood brain barrier, the choroid plexus, the brush border membrane of intestinal cells, the biliary cannicular membrane of hepatocytes, and the luminal membrane in proximal tubules of the kidney (van Helvoort et al., 1996). Considering its localization and the vectorial outward transport of the molecules, its physiological function seems to be the cell protection against various toxicants. Protection can be realized by elimination of the toxins in the intestine or blood-tissue barriers or by active excretion in the liver, intestine, or kidney. The P-gp, in the mucosal epithelium of the intestine, contributes to the direct excretion of compounds into the intestinal lumen and determines therefore the reduced uptake of orally administered agents. Many organs such as liver express several ABC transporters with overlapping substrate specificity, thereby allowing elimination and hence protection of hepatic tissue (Müller, 2000). In the epithelial cells of the proximal tubules of the kidneys, P-gp plays both a direct excretory role for drugs and limits the reuptake of xenobiotics (Krishna and Mayer, 2000). Furthermore, P-gp has also modulating role for the penetration of drugs through the blood-brain barrier. In fact, the efflux transporters restrict the uptake of drugs by active elimination from the brain. P-gp seems also active in releasing of active neurotransmitters as β-endorphins, opiods, or glutamate directly from the brain into the blood thereby modulating the activity of the peripheral nervous system (King et al., 2001; Liu and Liu, 2001). P-gp is present in placental barrier, on the maternal side of the brush-border membrane of the trophoblasts (Usigome et al., 2000). A proposed physiological role of P-glycoprotein is also the intracellular transport of lipid and cholesterol (Johnstone et al., 2000). Functional P-gp might play a fundamental role also in regulating apoptosis induced by a range of chemotherapeutic drugs, fas cross-linking, binding of TNF-α to the cell surface receptor, or UV irradiation (Johnstone et al., 1999, 2000; Ruefli et al., 2000).

P-glycoprotein mediated multi-drug resistance is reversed by different compounds, including calcium channel blockers, calmodulin inhibitors, phenothiazines, steroid hormones, opiate antagonists, or herbal remedies (e.g. curcumine, flavonoids, terpenoids) (Wink, 2007). Some compounds, among them tamoxifen, valspodar (PSC 833), biricodar (VX 710), tariquidar (XR 9576) have been clinically evaluated in different oncologic patients, without confirmation of the reversal effects obtained in preclinical studies (Hoffmann and Kroemer, 2004). MDR-modulators also exhibited toxic side effects and poor pharmacokinetics.

Multidrug Resistance Associated Protein (MRP, ABCC)

The second most extensively studied transporter is the multidrug resistance protein family MRP (Borst et al., 2000; Schinkel and Jonker, 2003). Seven different proteins have been identified in cells and tissues (from MRP1 to MRP7) and are involved in drug resistance. Two new members have been identified, MRP8 and 9, but the role in the xenobiotic transport remains to be elucidated (Tammur et al., 2001). The MRP1-5 are organic anion pumps, but they differ in substrate specificity, tissue distribution, and intracellular location. MRP1, 2, 3 and 6 have shown the highest sequence homology (Borst and Elferink, 2002).

In particular, MRP2, also called canalicular multispecific anion transporter (cMoat), is structurally similar to P-gp, but, in addition, it contains a TMD0 domain with five putative transmembrane segments, an extracellular N-terminus, and a long internal loop L0 to the core domain (Figure 4). It is mainly expressed in liver canaliculi on the apical site, where it is responsible for the excretion of endogenous metabolites as well as many exogenous compounds. Also renal proximal tubules, gut enterocytes, syncytiotrophoblast cells of the placenta, and brain capillaries have shown lower levels of this transporter (Hoffmann and Kroemer, 2004). A MRP2-co-expression with relevant phase II enzymes is assumed: so glutathione-S-transferases and UDP-glucuronosyltransferases catalyze the conjugation with glutathione and glucuronic acid (phase II metabolism), then MRP2 mediates their export (phase III metabolism). An overexpression of MRP2 in pathophysiological situations, especially cholestasis, and various hepatic, colorectal, mesothelial, lung, ovarian, or leukemic cancer, has been also highlighted (Zollner et al., 2001, 2003; Hoffmann and Kroemer, 2004).

MRP2 possess high affinity for several metabolites formed in phase-II reactions, such as GSH-conjugates, sulfates, and glucuronide (Lee et al., 2004; Rea et al., 1998). It also transports neutral or basic drugs as vinca alkaloids, etoposide, and anthracylines as P-gp. A GSH co-transport with anticancer drugs is required (Evers et al., 2000; van Aubel et al., 1999). Metal excretion is also GSH dependent, and arsenic and methyl arsenic glutathione were formed and effluxed by MRP2. Likewise, the biliary elimination of zinc, copper, and manganese, and the renal elimination of organic anions have been shown (Kala et al., 2000; Liu et al., 2001; Trauner and Boyer, 2003).

Distribution of MRP2 and P-gp tissue is extensively overlapped: it appears to be conceivable that these two transporters have overlapping features in terms of their pharmacological and toxicological functions (Kruh et al., 2001). MRP2 also confers resistance to a variety of natural products as well as to campthothecins, methotrexate, and cisplatin (Suzuki and Sugiyama, 2002). Reversal agents for MRP2-induced chemoresistance have not been as readily identified. The pyridine analog PAK-104P, is able to inhibit both P-gp and MRP *in vitro* models (Chen et al.,

1999). Likewise, the farnesyl protein transferase inhibitor SCH-66336 (Ionafarnib) showed to inhibit the function of both MRP1 and MRP2. A synergistic effect between cisplatin and 5-fluorouracil mediated by MRP-inhibition has been highlighted (Wang and Johnson, 2003). Also curcumin inhibited the MRP1- and MRP2-mediated transport in cells (Wortelboer et al., 2003).

Breast Cancer Resistance Protein BCRP (ABCG, MXR, ABCP)

BCRP/ABCG2, consists of 655 amino acids and possesses six transmembrane helices and one ATP binding site (Figure 4). It is a halftransporter, requiring to at least homodimerize in order to function (Nakanishi et al., 2003). Biochemical analysis using gel-filtration chromatography suggests that BCRP exists as a homotetramer that may act only to regulate the level of functional homodimerized BCRP transporters (Xu et al., 2004). BCRP has been characterized as an important part of self-defense systems in the organisms, being an efflux transporter for xenobiotics and unwanted toxic compounds. It is present in normal tissues, such as placental syncytiotrophoblasts, hepatocytes, and intestinal mucosal cells (Doyle and Ross, 2003); in addition it is located on the luminal surface of microvessel endothelium, in brain microvasculature (Cooray et al., 2002). This tissue distribution reflects the major role of BCRP in protecting cells from potentially toxic xenobiotics, including dietary mutagens and carcinogens. Numerous natural or physiological BCRP-substrates, among which polycyclic aromatic hydrocarbons, micotoxins, hormon steroid (i.e. 17β-estradiol and dihydrotestosterone) and folic polyglutamate conjugates have been identified and have been well reviewed elsewhere (Volk and Schneider, 2003; Chen et al., 2003; Doyle and Ross, 2003; Ebert et al., 2005; van Herwaarden et al., 2006; Polgar et al., 2008; Nakanishi and Ross, 2012).

BCRP/ABCG2 confers resistance to a limited range of anticancer agents respect to P-glycoprotein and MRP1/2, particularly methotrexate (its polyglutamylated metabolite), anthracyclines, mitoxantrone, and topoisomerase I inhibitors (such as camptothecin) (Hooijberg et al., 2003, 2004; Ifergan et al., 2004; Stark et al., 2003). Conversely, it does not confer resistance to vinca alkaloids, epipodophyllotoxins, paclitaxel, or cisplatin (Allen and Schinkel, 2002).

Development of ABC-transporter inhibitor

The inhibition of ABC-transporter function (or the expression) with potent and low toxic inhibitors (or modulators) is still considered the easiest approach to restore drug sensitivity in MDR cancer cells. The use of a chemosensitizing agent can restore drug sensitivity in MDR cancer cells by direct blocking drug efflux, by improving drug penetration and distribution, and increasing drug accumulation (Wu et al., 2011).

In the past two decades substantial efforts have been carried out to develop potent modulators of ABC drug. Unfortunately, there is still a lack of irrefutable evidence and clinical trial data demonstrate that this approach can improve bioavailability or delivery, or restore drug sensitivity in MDR cancer patients (Szakacs et al., 2006). The difficult to find an ideal inhibitor is often associated with the adverse interactions between modulator and anticancer drug. Furthermore, the variability in the expression levels and polymorphisms of ABC transporters among individuals makes clinical trials related to MDR cancer exceptionally challenging (Polgar and Bates, 2005). Four major categories of ABC transporter-inhibitors have been identified: the *First Generation Inhibitors* included drugs or chemicals with known biological activities (such as channel blockers, immunosuppressants and even cardiovascular drugs); the *Second Generation Inhibitors* and *Third Generation Inhibitors* were specifically designed and synthesized based on structural information obtained from the *First Generation Inhibitors* (Shukla and Ambudkar, 2008). For these inhibitors, the ABC-transporter inhibition was not their primary targets, so unspecific and unfavorable interactions with other molecules could be expected. Also, higher concentrations of these drugs were required to obtain the inhibitory effect, so causing undesirable toxicity (e.g. verapamil) (Shukla and Ambudkar, 2008). *Fourth Generation Inhibitors* are represented by modulating compounds of natural origin. A great variety of materials, such as plants, fungi and even marine organisms, can be use to find new natural compounds. Furthermore, natural extracts are often low in toxicity and are well tolerated in the human body. For that reason, new candidates from natural sources with strong modulating effect on the function and/or the ABC transporter-expression have been screened. When a lead compound is identified, quantitative structure-activity relationship (QSAR) studies and an optimization process are carried out (Raub, 2006). For example, the fungal toxin fumitremorgin C (FTC) is an ABCG2 inhibitor discovered from natural sources, but has unfavorable neurotoxicity (Rabindran et al., 2000). Therefore, the optimization of this molecule into a more potent, specific and less-toxic analog Ko143 has been made (Allen et al., 2002). Over the years, a large number of natural product modulators have been discovered, such as curcumin, flavonoid, terpenoids, etc. (Wu et al., 2011).

BASIC CONCEPT OF GENETIC TOXICOLOGY

Genetic toxicology is a branch of the toxicology field that assesses the toxic effects of damage to deoxyribonucleic acid (DNA). Genetic information, encoded in DNA, is maintained, replicated and transmitted to successive generations with high fidelity. Damage to DNA can occur through normal biologic process or as the result of interaction of DNA, both directly or indirectly, with chemical, physical or biological agents (Brusick, 1980). Genetic toxicology therefore involves the study of DNA single- and double-strand breaks, damage to DNA, mutations in DNA, and recombinational events in DNA mediated by exogenous agents in bacteria, yeast, plant cells, and mammalian cells. In plant and mammalian cells, genetic toxicology also encompasses micronucleus formation, chromosomal aberrations, chromosomal aneuploidy, and morphological and neoplastic transformation (Landolph, 2005). In addition, genetic toxicology allows investigators to determine genetic damage and mutations induced by chemical carcinogens and ultraviolet (UV) and ionizing radiations in lower animals and humans (Landolph, 2005).

The importance of mutations and chromosomal alterations for human health is evident from their roles in genetic disorders and cancer (Table 1).

Table 1. Examples of Human Genetic Disorders (Teaf and Middendorf, 2000).

Chromosome Abnormalities
Cri-du-chat syndrome (partial deletion of chromosome 5)
Down's syndrome (triplication of chromosome 21)
Klinefelter's syndrome (XXY sex chromosome constitution; 47 chromosomes)
Turner's syndrome (X0 sex chromosome constitution; 45 chromosomes)
Dominant Mutations
Chondrodystrophy
Hepatic porphyria
Huntington's chorea
Retinoblastoma
Recessive Mutations
Albinism
Cystic fibrosis
Diabetes mellitus
Fanconi's syndrome
Hemophilia
Xeroderma pigmentosum
Complex Inherited Traits
Anencephaly
Club foot
Spina bifida
Other congenital defects

A critical link exists between DNA mutation, chromosomal rearrangements (genomic instability) and cancer development. The genomic instability can manifest itself as small changes at the nucleotide level or as gross chromosomal alterations. Moreover, mutations in the genes that encode DNA damage response proteins are responsible for a variety of genomic instability syndromes and these disorders often result in a heightened predisposition to cancer (Martin et al., 2010). Oncogenes and tumor suppressor genes have a central role in cancer. Oncogenes can originate from proto-oncogenes, usually involved in normal cellular growth and development, by point mutations or chromosomal alterations, (Bishop, 1991; Barrett, 1993; Rabbitts, 1994), and can stimulate the transformation of normal cells into cancer cells (Bishop, 1991). Tumor suppressor genes usually control the cellular proliferation; inactivation or deletion of these genes, due to mutational events, free the cells from their inhibitory influence (Hanahan and Weinberg, 2000) and have been associated with various cancers, including those of the eye, kidney, colon, brain, breast, lung, and bladder (Fearon and Vogelstein, 1990; Marshall, 1991). Combinations of activating mutations in proto-oncogenes and inactivating mutations in tumor suppressor genes, in somatic (non germline) or germ line cells play a key role in carcinogenesis (Landolph, 2005).
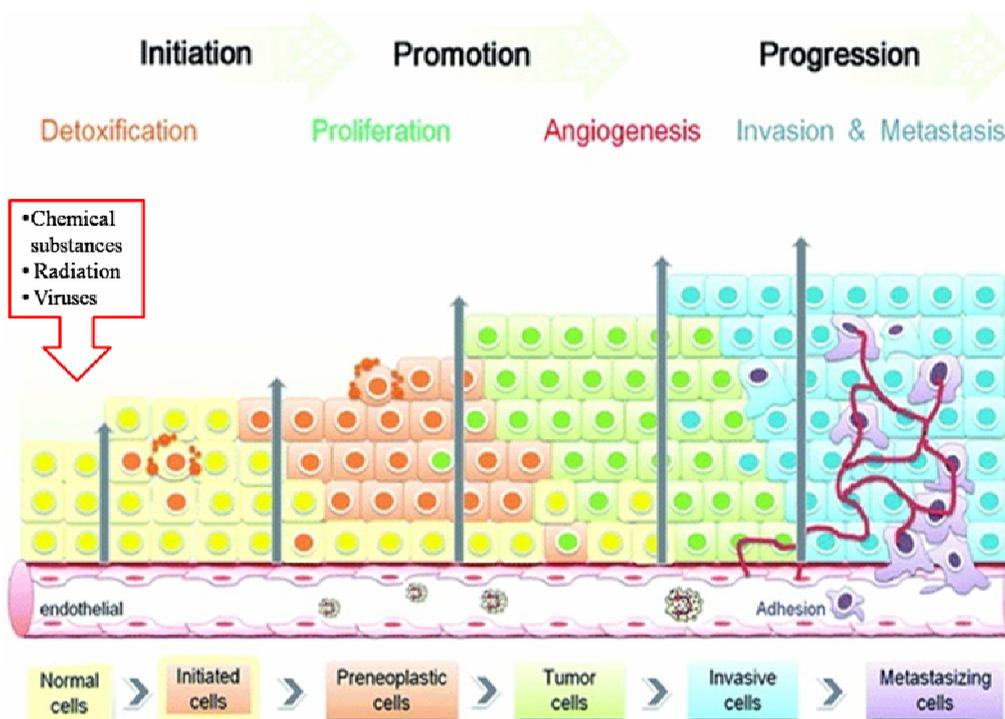

Figure 6. Multistage carcinogenesis process. The mutagenesis hypothesis of cancer formation postulates that the cause of most cancers is mutagenesis. Mutations accumulate during cell proliferation and when mutations alter critical genes, carcinogenesis is initiated. Interference with the normal mechanisms guarding genomic stability would consecutively accelerate genome destabilization, leading to tumor progression and metastasis (modified from Pan et al. 2011).

High association between positive responses in genetic toxicity tests and rodent and human carcinogenicity is showed (McCann et al., 1975a; Purchase et al., 1978). However, genetic toxicity is not a measure of carcinogenicity, but it is often used as a surrogate in the evaluation of cancer risk because the tests measure an initiating or intermediate event in tumorigenesis (Fearon and Vogelstein, 1990).

Damage to the genome can occur both in germ cells and in somatic cells. The injure in germ cells affect the reproductive performance or result in genetic diseases in future generations. Mutations in germ cells (sperm or egg cells), can lead to a predisposition to cancer, such as the Li-Fraumeni syndrome, and still other mutations can be lethal and result in nonviable offspring. Mutation in the somatic cells are involved in the initiation and progression of cancer (Zeiger, 2001).

Today, genetic toxicity assays are used routinely as an initial toxicological screening in chemical and drug development.

Mutagenesis

Mutagenesis results from changes in the sequence of DNA bases; these alterations are transmitted to the RNA (synthesized according to the instructions carried by the DNA) and then to proteins, which conduct chemical (enzymatic) reactions in the cell, or serve as structural materials, giving a cell its shape (Landolph, 2005).

Mutations can have beneficial effects, deleterious effects or no consequences in organisms. The sickle mutation in the hemoglobin gene represents an example of positive mutation in the organism and is thought to give humans in Africa an ability to survive malaria better. In fact, the resulting mutated haemoglobin aggregates in the red blood cells, leading them to assume a sickle shape that makes difficult for the malarial parasite to enter and infect red blood cells.

Many mutations are neutral and have no significant effects on the organism at all. However, certain types of mutations can have harmful consequences in organisms; a deleterious mutation in humans destroys the activity of adenosine deaminase enzyme, leading to a deficient immune system and a consequent inability to fight disease (Landolph, 2005).

Different mutational events can occur, such as frameshift mutations and basepair substitutions at gene level, while aberrations, micronuclei induction and sister chromatid exchange (SCE) at chromosomal level.

Mutation in which one or two nucleotides are inserted or deleted into a DNA sequence, shifting the coding frame out of its original alignment, are defined frameshift mutation (Figure 7). As a result, the original amino acids in the encoded protein are changed, and the code is shifted out of

register and away from the site of this type of mutation. Hence, the site of the mutation produces a new or "scrambled" protein, which can have an altered structure, and if the protein is an enzyme, the enzymatic activity may be decreased or abolished (Landolph, 2005).

In a deletion mutation, one or more bases are removed from the DNA, while in an addition mutation, also called insertion mutation, one or more bases are added; deletion or addition mutations are called large or small if many or only a few bases are involved (Figure 7).

A transition mutations one in which, during replication, one base (purine or pyrimidine) is replaced by another (purine or pyrimidine respectively). Conversely, a transversion mutation occurs when a purine base is substituted for a pyrimidine base (guanine for thymidine) or a pyrimidine base for a purine base (cytosine for adenine) (Figure 7). These types of mutations are also called base substitution mutations (Landolph, 2005).

Figure 7 shows a schematic representation of the point mutations described above.
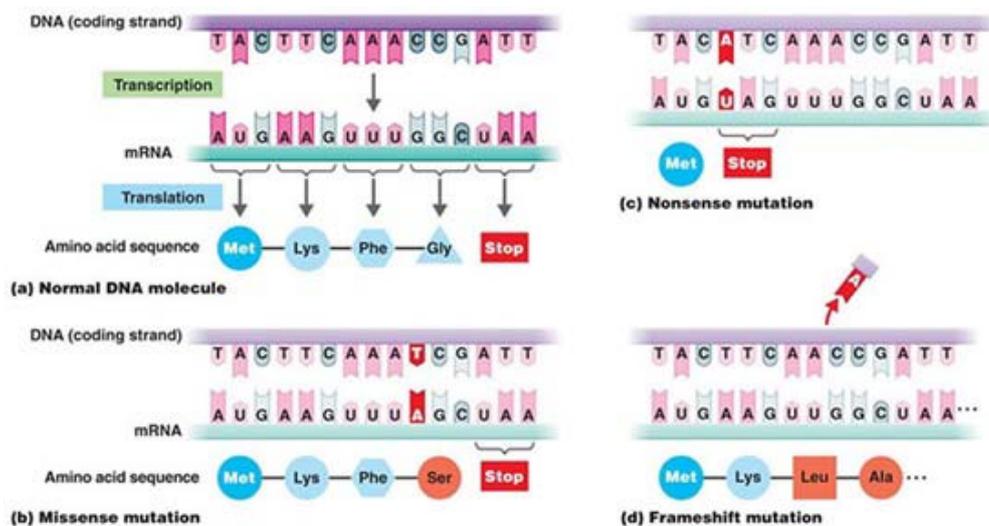
Figure 7. Schematic representation of point mutations.

Also mutation can occur at chromosomal level in mammalian cells. It is defined as macrolesion because may be visualized by microscopy and it can be chromosomal aberration, which may be chromatid gap or break, symmetrical exchange (exchange of corresponding segments between arms of a chromosome), asymmetric interchange between chromosomes and micronuclei (MN) induction. During cell division can occur an incomplete separation of replicated chromosomes. This kind of macrolesion is characterized by the abnormal chromosome numbers that results in the daughter cells and may be recognized as a change in the number of haploid chromosome sets (ploidy changes) or in the gain or loss of single chromosomes (aneuploidy).

Chromosome aberration is another type of macrolesion. It is caused by damage to chromosome structure (clastogenic effect), which showed an abnormal morphology. The lesion may be repaired when an initial lesion induces a break in the chromosomal backbone. However, the lesion can not be repaired when result in a permanent break, misrepaired or joined with another chromosome to cause a translocation of genetic material (Teaf and Middendorf, 2000).

SCE are produced during the S phase and are presumed to be a consequence of errors in the replication process (Preston and Hoffmann, 2001). Finally, MN are small nuclear structures containing one or more whole chromosomes, or pieces of chromosomes, as results of aneuploidogenic or clastogenic mutational events, respectively.

Point mutations can result in altered products of gene expression, but chromosomal aberrations or alterations of chromosome numbers in germ cells can have disastrous consequences (embryonic death, teratogenesis, retarded development, behavioral disorders and infertility) (Teaf and Middendorf, 2000).

The importance of a mutation depends on where it occurs in a gene coding for a protein. It do not have a significant effect on the structure of the cell or on the enzymatic activity of a protein if it occurs in a site that does not significantly change the shape of a protein used to maintain the structural integrity of a cell, or in a site that does not affect the structure of an enzyme. Conversely, the mutation can have severe negative consequences for the survival of the cell if the mutation occurs in a part of the protein that significantly changes its structure or decreases its enzymatic activity (Landolph, 2005).

However, it is important to take into account that all cells possess the capability to repair DNA breaks, point mutations, and nucleotide repeats. The cell can undergo apoptosis (programmed cell death) if the damage is extensive, (Evan and Littlewood, 1998); but the cells can also repair the damage, by repair processes that returns the DNA to its undamaged state (error-free repair) or to an improved but still altered state (error-prone repair), if the damage is less severe. The basic principles underlying most repair processes are damage recognition, removal of damage (except for strand breaks or cleavage of pyrimidine dimers), repair DNA synthesis, and ligation. Figure 8 shows the mechanisms involved in DNA repair.
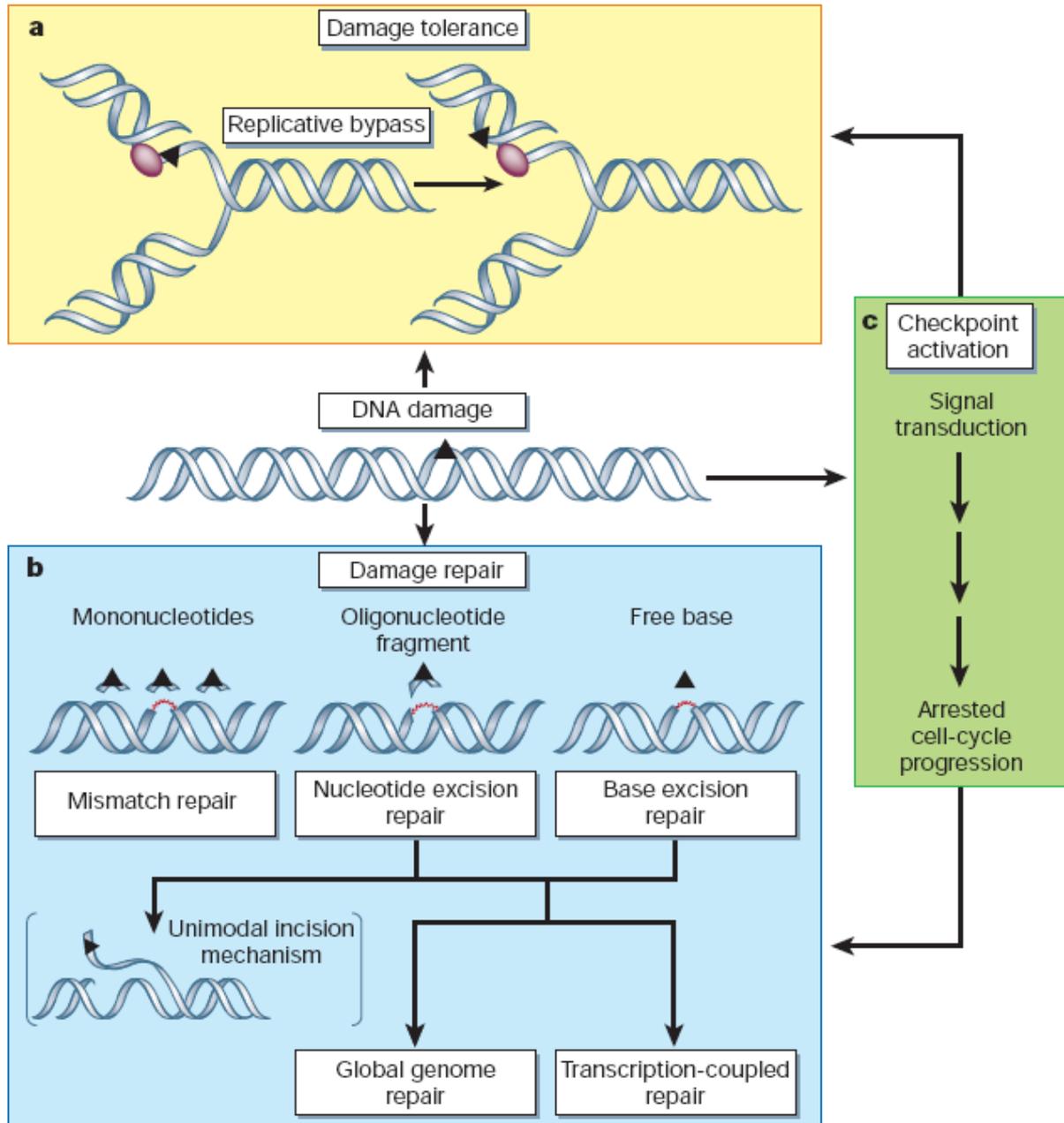
Figure 8. Responses to DNA damage. DNA damage (black triangle) results in either repair or tolerance. a. In damage tolerance, damaged sites are recognized by the replication machinery before they can be repaired, resulting in an arrest that can be relieved by replicative bypass (translesion DNA synthesis); it persists in the genome but its potential for interfering with DNA replication and transcription is somehow mitigated. b. A damaged base is excised as a single free base (base excision repair, BER) or as an oligonucleotide fragment (nucleotide excision repair, NER); mispaired bases are excised as single nucleotides during mismatch repair. c. The cell has a network of complex signalling pathways that arrest the cell cycle and may ultimately lead to programmed cell death (Friedberg 2003).

The major pathway by which DNA base damages are repaired is the Base Excision Repair (BER); this process involves a glycosylase that removes the damaged base, causing the production of an apurinic or apyrimidinic site. Subsequently, this site can be filled by the appropriate base or processed further (Demple and Harrison, 1994); DNA polymerase fills the resulting gap, and bind it to the parental DNA.

The Nucleotide Excision Repair (NER) system provides the ability of the cells to remove bulky lesions from DNA; damage recognition, incision, excision, repair synthesis, and ligation are the basic steps (Preston and Hoffmann, 2001).

A specific system is present in eukaryotic cells to repair DNA double-strand breaks, the Double-Strand Break Repair; it act by two general pathways, the homologous recombination and the nonhomologous end-joining (Haber, 2000).

The DNA mismatch repair system operates to repair mismatched bases formed during DNA replication, genetic recombination, and as a result of DNA damage induced by chemical and physical agents. Damage recognition by a specific protein that binds to the mismatch, stabilization of the binding by the addition of one or more proteins, cut of DNA at a distance from the mismatch, excision past the mismatch, resynthesis and ligation are the principal steps in all cells from prokaryotes to humans (Jiricny, 1998).

The 0^6-methylguanine-DNA methyltransferase (MGMT) protects the cells from the cytotoxic effect of alkylating agents. The MGMT-mediated repair mechanism involves a one-step reaction, in which the alkyl group at the 0^6 position of guanine is transferred to a cysteine residue in MGMT. This process results in guanine being restored in DNA (direct reversal) and MGMT being rendered inactive, which is an irreversible process. Therefore, MGMT is often referred to as a "suicide enzyme" (Christmann and Kaina, 2013).

The particular repair pathway, the rate of the damage-repair and the fidelity and completeness of the repair can influence the probability that induced DNA damage can be converted into a genetic alteration.

Genotoxicity Tests

In vitro and *in vivo* test systems have been developed to study the effects of chemicals on cellular DNA and chromosomes (Table 2). Gene mutation systems in bacteria and gene and chromosome damage systems in cultured rodent cells are the most frequently used *in vitro* tests for routine screening; regarding as *in vivo* test, chromosome damage is typically measured in bone marrow cells of mice or rats.

Table 2. Principal assays in Genetic Toxicology (Preston and Hoffmann, 2001).

I. **Pivotal assays**
   A. A well-characterized assay for gene mutations
      The *Salmonella*/mammalian microsome assay (Ames test)
   B. A mammalian assay for chromosome damage *in vivo*
      Metaphase analysis or micronucleus assay in rodent bone marrow

II. **Other assays offering an extensive database or unique genetic endpoint**
   A. Assays for gene mutations
      E. coli WP2 tryptophan reversion assay
      TK or HPRT forward nutation assays in cultured mammalian cells
      Drosophila sex-linked recessive lethal assay
   B. Cytogenetic analysis in cultured Chinese hamster or human cells
      Assays for chromosome aberrations and micronuclei
      Assays for aneuploidy
   C. Other indicators of genetic damage
      Mammalian DNA damage and repair assays
      Mitotic recombination assays in yeast and *Drosophila*
   D. Mammalian germ-cell assays
      Mouse-specific locus tests
      Assays for skeletal or cataract mutations in mice
      Cytogenetic analysis and heritable translocation assays
      DNA damage and repair in rodent germ cells
      Dominant lethal assay

Bacterial assays are commonly used to detect and study the molecular mechanisms of mutations in bacteria and also to establish priorities for other testing approaches. Also, fungi have been used in genotoxicity assays. The yeasts belonging to the genus Saccharomyces and Schizosaccharomyces, as well as the molds Neurospora and Aspergillus, have been utilized in forward mutation tests, which are similar to the Salmonella histidine revertant assays.

In mammalian cells, mutagenesis *in vitro* assays are used to measure the induction of mutants that are resistant to the cytotoxicity of toxic drugs. One of the most frequently employed is the assay detecting mutation conferring 6-thioguanine resistance in the Chinese hamster ovary (CHO) cell line.

Many *in vitro* assays have been also developed to measure the ability of specific agents to induce damage at chromosomal level, as chromosomal aberrations, micronuclei formation and unscheduled DNA synthesis (UDS) (Landolph, 2005).

The *in vivo* mutagenicity tests in mammalian systems are the most relevant methods to evaluate mutagenicity in humans; rats or mice offer insights into human physiology, metabolism, and reproduction that cannot be duplicated in other tests. Furthermore, the way of administration of a chemical to a test animal can be selected taking into account the human environmental or occupational conditions of exposure. Moreover, human epidemiologic findings may also be compared with the results of tests done in animals. Time required and the costs are the

disadvantages of *in vivo* mammalian test systems (Teaf and Middendorf, 2000). The ICH and the OECD/EPA guidelines provide the scientific basis of the genetic toxicology tests, the rationale for the selection of test batteries, test procedures and guidance for data interpretation (EMEA, 1998).

The usual approach in detecting genotoxic agents is to carry out a battery of *in vitro* and *in vivo* tests. According to EMEA (1998), to detect relevant genetic changes and the most of genotoxic rodent carcinogens, it is appropriate to assess genotoxicity *in vitro* in a bacterial reverse mutation test or Salmonella (Ames) test. Furthermore, it should be evaluated *in vitro*, in mammalian cells, chromosomal damage, gene mutations and clastogenic effects that can not adequately measured in bacteria. A variety of *in vitro* mammalian cell tests are proposed, such as L5178Y test (MLA), chromosome aberrations assay by using CHO or V79 cell lines and micronucleus test in human lymphocytes or in peripheral blood erythrocytes (Kirkland et al., 2005). Finally, an *in vivo* test for genetic damage should usually be a part of the test battery to give a test model in which additional relevant factors (absorption, distribution, metabolism, excretion) that may influence the genotoxic activity of a compound, are taking into account. A sufficient level of safety is defined for compounds that giving negative results in the standard 3-test battery. Compounds giving positive results may need to be tested more extensively. The suggested standard set of tests does not imply that other genotoxicity tests are generally considered as inadequate or inappropriate; such tests serve as options in addition to the standard battery for further investigations. Furthermore, molecular techniques to study mechanisms of genotoxicity in the standard battery systems may be useful for risk assessment (EMEA, 1998). In this study we describe in detail the Ames test.

Ames Test

An usually accepted short-term bacterial assay for identifying substances which can produce genetic damage that leads to gene mutations is the "Ames Test", also named "Bacterial Reverse Mutation Test" or "*Salmonella typhimurium*/microsome assay" (Mortelmans and Zeiger, 2000). This assay has been described for the first time in 1971 by Bruce Ames. Over the years the Bacterial Reverse Mutation Test was improved to increase its sensitivity to several types of mutagens. An exogenous mammalian metabolic activation system was included, since the bacteria cells are unable to metabolize chemicals via cytochromes P450 (CYP450) (Ames et al., 1973). Simultaneously, the plate incorporation assay procedure was developed to replace the spot test method. It was a major contributing factor to the success of the Ames test, because it made the test easier to perform, more sensitive and reduced its cost. Afterwards, a pre-incubation

methods was introduced to test some mutagens poorly detected by the standard procedure (Gatehouse et al., 1994). In the years, several mutant strains were introduced to obtain major information on the mechanism of action of chemicals tested. For example, YG descendants of TA98 and TA100, in which the introduction of a further plasmid expresses the genes codifying for nitro-reductase and O-acetyl-transferase enzymes, are strains high sensitive to the mutagenic action of specific chemicals such as nitroarenes, nitro-aromatic compounds and aromatic amines (Aufderheide and Gressmann, 2008). Finally, the Ames test was also used to assess the antimutagenic activity as described by Edenharder et al. (1997).

International guidelines have also been developed from several regulatory agencies, such as the Organisation for Economic Co-operation and Development (OECD, 1997), the International Commission on Harmonization (ICH, 1995) and the European Medicines Agency to ensure uniformity of testing procedures (EMEA, 1996). The test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained (Zeiger et al., 1990).

Plate-incorporation vs. pre-incubation method

The pre-incubation test has been widely accepted as a sensitive method to detect the mutagenicity of carcinogenic azo dyes, nitrosamines, alkaloids and several volatile chemicals (Maron and Ames, 1983) (Table 3). The fact that test compound, S9 or phosphate buffer and bacteria are incubated at higher concentrations than in the standard plate incorporation enhance the method sensitivity. The pre-incubation procedure seems to posses some advantages: the pre-incubation time reduce the possibility of non-specific binding of the "active mutagen" to top agar; the bacterial suspension is exposed to a higher concentration of test compound; it is used a higher concentration of S9, so there is a more efficient metabolism, useful to detect some indirect mutagens that require metabolic activation; in presence of S9 mix, the lag phase of the growth curve of the bacterial strains is shortened (~ 1-2 hr) due to the nutritive effects of the S9 proteins. The rapid bacterial growth allows to detect the mutagenicity of some relatively unstable or short-lived "mutagenic species" (Gatehouse et al., 1994).

Table 3. Compounds more efficiently detected by using the pre-incubation method (Gatehouse et al., 1994).

COMPOUND (class)	Possible mechanism of mutagenicity masking	Reference
Aliphatic nitrosamines (Short chain)	Agar-binding of metabolites More efficient metabolism in pre-incubation	*Araki et al., 1984* *Bartsch et al., 1976*
Divalent metals	Inhibition by components of media	*Pagano and Zeiger, 1992*
Aldehydes	Volatility	*Ashby et al., 1985* *Dillon et al., 1992*
Azo-dyes	Metabolic requirements	*Prival et al., 1984* *Lefevre and Ashby, 1981*
Pyrollizidine alkaloids	More efficient metabolism in pre-incubation	*Yamanaka et al., 1979* *Rubiolo et al., 1992*
Allyl compounds	Volatility	*Neudecker and Henschler, 1985a/b*
Nitro compounds	Agar-binding of metabolites More efficient metabolism in pre-incubation	*Dellarco and Prival, 1989*

A disadvantage of the pre-incubation procedure is the enhanced toxicity at highest concentrations. Some chemicals can be tested at higher non toxic concentrations only by the plate-incorporation method.

The pre-incubation can be used routinely to confirm the results or when inconclusive data are obtained in the standard method. De Serres and Shelby (1979) have recommended the use of this test in screening assay, because of the increased sensitivity. Although on the basis of the published literature certain classes of chemicals were more effectively tested using the pre-incubation procedure, the two methodologies are highlighted as valid approaches at least as initial test.

Bacterial tester strains

Several mutant strains are used, each sensitive to specific genotoxic damage. According to OECD guideline 471 (1997), to detect a mutagenic substance, at least five bacterial strains should be used, particularly *S. typhimurium* TA1535, TA1537 (or TA97), TA98, TA100, and *E. coli* WP2*uvr*A (or WP2*uvr*ApKM101, or *S. typhimurium* TA102). *S. typhimurium* strains are able to detect frameshift and base-substituition mutagen at GC base pairs, while WP2*uvr*A increase the system sensitivity to oxidizing and cross-linking agents at AT base pair.

S. typhimurium and *E. coli* strains carry different mutations that leave the bacteria dependent to histidine (*his*) and tryptophan (*trp*) respectively to grow. New mutations at site of these pre-existing mutations can reverse the gene-function, allowing the cells to synthesize the aminoacid and growing to form the colonies. To make the strains more sensitive to detect specific mutation mechanisms, additional mutations were engineered into them. The specificity of the test strains can provide some information on the types of mutations that are induced by the genotoxic agents; among these, base pair substitution mutagens induce a base change in DNA while frameshift mutagens cause base pair addition or deletion, changing the reading frame in RNA. The table 4 showed the genotype and the kind of mutation induced by the strains used in our study.

Table 4. Genotype and kind of mutation induced by the strains used in our study.

Strain[a]	Genotype	Reversion event
TA1535	*hisG46, Δ(gal-bio-chl-1005) rfa*	Base-pair substitution
TA100	*hisG46 Δ(gal-bio-chl-1005) rfa, ΔuvrB,* pKM101f	Base-pair substitution
TA1537	*hisC3076 Δ(gal-bio-chl-1005) rfa*	Frameshifts
TA98	*hisD3052, Δ(gal-bio-chl-1008), rfa, ΔuvrB,* pKM101	Frameshifts
TA98NR	Same as TA98, but deficient in classical NR[b]	Frameshifts
TA98 1,8DNP	Same as TA98, but deficient in OAT[c]	Frameshifts
WP2*uvr*A	*trpE65, ΔuvrA*	Base-pair substitution
WP2*uvr*A/R	*trpE65, ΔuvrA,* pKM101	Base-pair substitution

[a]TA98 and TA100 (Ames et al. 1973b); WP2*uvr*A (Hill 1958); TA1535 and TA1537 (Mortelmans and Zeiger 2000); TA98NR and TA98 1,8DNP (Hrelia et al. 1999). [b]NR: nitroreductase. [c]OAT: O-acetyltransferase

S. typhimurium is a pathogenic bacterium, causing diarrhoea and food poisoning. The name of this bacterium derives from Daniel Elmer Salmon, an American veterinary pathologist, although it was his partner Conor Fitzpatrick who first isolated the bacterium in 1885 from pigs. *S. typhimurium* LT2 strain is the wild-type parent of the strain used in reversion mutation assays. It is not very virulent and unable to grow in media containing n-histidine as source of nitrogen because it produces a very little histidase and urocanase upon induction by n-histidine.

Ames and other genetists obtained the standard tester strains by modifications of wild-type. Mutant strains present virulence lower by orders of magnitude, due to the deep rough mutation (*rfa*). A mutation at the *hisG* gene, *hisD3052* (TA98, TA98NR, TA98 1,8DNP) and *hisG46* (TA100) alleles determine the histidine-dependence. TA98 and its derivative strains are reverted to wild-type by various frameshift mutagens while TA100 by several mechanisms of base-pair

substitution (DeMarini, 2000). The presence in the genome of a plasmid pKM101, in the strains above mentioned, determines a more sensitivity to chemical mutagens.

E. coli was first identified by the German pediatrician, Theodor Escherich. It is a Gram negative micro-organism (Fam. *Enterobacteriaceae* such as the *Salmonella* genus), predominantly facultative anaerobe and distributed in the intestine of humans and warm-blooded animals. Although most strains of *E. coli* are not regarded as pathogens, they can cause opportunistic infections in immunocompromised hosts. Virulent strains are distinguishable from nonvirulent by possessing genetic elements encoding for virulence factors (Qadri et al., 2005). However, in the genotoxicity assays are used non pathogenic mutant strains. *E. coli* WP2*uvr*A and WP2*uvr*A/pKM101 (WP2*uvr*A/R) strains are mutants used in the reversion mutation assays, carrying a tryptophan-dependence which makes them sensitive to oxidative mutagens, free radical generators and cross-linking agents which preferentially attack the A-T base pair base-pair substitution mutagens (Brusick et al., 1980; Wilcox et al., 1990).

The genotypes of the strains tested, listed in Table 4, are following described, according to Mortelmans and Zeiger (2000).

➢ ***his* or *trp* mutation:** this mutations lead bacteria aminoacid-dependent to grow.

➢ ***bio*-mutation:** it leads to biotin dependence, increasing the sensitivity to mutagens.

➢ ***gal*-mutation:** it encodes to galactose-synthesis and increase bacteria permeability, by reducing the lipolysaccharide (LPS) layer; some chemicals (e.g. crystal violet or benzo[a]pirene) which cannot cross the wild-type bacteria cell, are toxic in the *gal*-mutant strains.

➢ ***chlD*-mutation:** it increase bacteria sensitivity to mutagens by removing the chlorates–resistence.

➢ ***rfa*-mutation o deep rough:** it leads to a defective LPS layer, so making bacteria more permeable to bulky chemicals.

➢ ***uvrB* and *uvrA* mutation:** these deletions reduce the excision repair effectiveness. Therefore more DNA lesions might to be repaired by the error-prone DNA repair system.

➢ **pKM101-mutation:** the plasmid encodes for *muc*-gene (Mutable by Chemicals), which enhance chemical and UV-induced mutagenesis, inducing the error-prone recombinational DNA repair pathway. Additionally, pKM101 confers ampicillin-resistance, which is a marker for its presence.

Specific target DNA sequence

Target site for mutations in *S. typhimurium* TA98 and TA100 is the *hisG* gene, *hisD3052* and *hisG46* alleles respectively, which carry the histidine-dependence. The *hisD3052* allele, carried by strains TA98, TA98NR, TA98 1,8-DNP is a 1-frameshift deletion within a reversion target of at least 76 bases. This allele can be reverted by five classes of mutations: hotspot 2-base deletion (within the sequence CGCGCGCG), deletions, duplications, insertions, or complex mutations consisting of either complex frameshifts (a frameshift with an adjacent base substitution) or concerted (templated) mutations. Mutagens can revert this allele primarily by induction of only two of these classes of mutations: hotspot deletion and complex frameshifts (DeMarini, 2000). Various frameshift mutagens, such as 2-nitrofluorene and other aromatic nitroso derivatives of amine carcinogens, can determine a reversion of the hisD3052 mutation back to the wild-type state (DeMarini, 2000).

The *hisG46* marker in TA100 results from the substitution of a leucine (GAG/CTC) by a proline (GGG/CCC); mutational event consist of a transition or transversion base substitutions primarily at one of the GC pairs (DeMarini, 2000). Mutagens that cause base-pair substitution mutations can revert this mutation.

E. coli WP2*uvr*A and WP2*uvr*A/pKM101 are a tryptophan-dependent strains, which contain a substitution in allele *trpE65*. The excisable and non-excisable misreplications or misrepairs at AT sequence are the main genotoxic events involved in WP2uvrA DNA-damage (Brusick et al., 1980); they are reverted by base-pair substitution mutations.

AIM OF THE STUDY

AND

EXPERIMENTAL DESIGN

AIM OF THE STUDY AND EXPERIMENTAL DESIGN

Present study was aimed at evaluating the potential chemopreventive properties of some natural and naturally-derived compounds, particularly the sesquiterpenes β-caryophyllene (CRY) and β-caryophyllene oxide (CRYO), and the aldehyde α-hexylcinnamal (HCA).

Chemoprevention is an approach which uses natural or synthetic compounds to inhibit, suppress or reverse the development and progression of cancer, by blocking the DNA damage that initiates carcinogenesis or by arresting or reversing the progression of pre-malignant cells in which such damage has already occurred (Hong and Sporn, 1997; Sporn and Suh, 2002). Cancer chemoprevention is an important aspect of biomedical research and provides a practical approach to identify potential useful inhibitors of cancer development and to study the mechanism of carcinogenesis (Wattenberg, 1993). Some compounds exert protective antimutagenic activity by extracellular mechanisms, such as inhibition of the mutagen-uptake, complexation, dilution and/or deactivation of mutagens, affection of the activation and detoxification systems; furthermore, antioxidant effects can also prevent DNA-damage; the mutagen-inhibition by some physical, chemical or enzymatically catalysed interactions with the antimutagen can also occur in the intracellular compartment (De Flora, 1998). Antimutagens, which induces extra- or intracellular inhibiting interferences with the mutagen, are defined as desmutagenic agents (Shamon and Pezzato, 1994). Other antimutagens can prevent the fixation and progression of DNA-damage, acting in intracellular compartment, by stimulating the DNA repair and/or reversion systems: these substances are defined as bioantimutagens (Shamon and Pezzato, 1994).

In addition to the antimutagenicity, some chemopreventive agents can also exert chemosensitizing properties, by increasing the effectiveness of cancer chemotherapy and radiotherapy, when used in combination with chemotherapeutical agents. This approach is very interesting to prevent the development of multidrug resistance (MDR), which makes the cancer cells not-sensitive to a broad range of drugs with different chemical structures and mechanisms of action. A chemopreventive compound can act synergistically with a chemotherapeutic one, also potentiating the anticancer effect at lower doses, so minimizing the chemotherapy-induced toxicity.

In this context, natural substances are of very interest for chemoprevention, because of their widespread exposure and the easy availability. Vitamins, soyabeans, polyphenols, cruciferous vegetables, curcumin, diallyl sulfide and indole-3-carbinol represent some examples of natural compounds with "chemopreventing" properties (Shukla et al., 2003; Shukla and Pal, 2004). In

addition, both curcumin and its major metabolite tertrahydrocurcumin were found to restore drug sensitivity in cancer cells overexpressing ATP-binding cassette (ABC) transporters by directly inhibiting their functions (Wu et al., 2011).

In present study, the chemopreventive potential of CRY, CRYO and HCA has been evaluated in term of antimutagenic activity, which represents the ability of a compound to inhibit DNA-damage induced by known mutagens. At this purpose, the reverse bacterial mutation assay (Ames test), the most widely used assay for assessing the mutagenic and antimutagenic properties of a chemical, has been carried out (Di Sotto et al., 2012). The pre-incubation method, which has been recognized more sensitive than the standard plate-incorporation, has been applied (Maron and Ames, 1983). A metabolic activation system (S9) has been also included to mime the mammalian metabolism. In fact bacterial strains are unable to metabolize inactive chemicals via CYP450-based metabolic system. In this manner substances that require an enzymatic activation to display their mutagenic activity, can also be detect. *S. typhimurium* TA1535, TA1538, TA98, TA98NR, TA98 1,8 DNP and TA100, and *E. coli* WP2*uvr*A and WP2*uvr*A/R were used as tester strains, according to the OECD Guideline (1997). TA98NR and TA98 1,8 DNP were derivative strains, enclosed in our study when necessary, in order to better understand the mechanisms responsible for the inhibition of the mutagens. The strain genotypes were verified by assessing the presence of specific genetic markers in the strain check assay. Before evaluating the antimutagenicity, for each compound preliminary assays were performed in order to identify the concentrations at which no cytotoxic effects (i.e. reduction in the number of revertant colonies and thinning of the bacterial background growth) were produced, and to exclude any potential genotoxic effects.

The compounds 2-nitrofluorene (2NF), sodium azide (SA), methyl methanesulfonate (MMS), 2-aminoanthracene (2AA), benzo[a]pyrene (B*a*P), 4-nitroquinoline N-oxide (4NQO), 1-nitropyrene (1NP), 1,8-dinitropyrene (1,8 DNP) in the Ames test were used as known mutagens (Figure 15). 2-Nitrofluorene (2NF) is an environmental pollutant released from industrial and engine waste (Rosenkranz and Mermelstein, 1983). 2NF is a direct-acting mutagen, bioactivated by cytoplasmic nitroreductase and *O*-acetyltransferase enzymes (codified by *cnr*, *snrA*, and OAT genes) to electrophilic intermediates, which form DNA-adducts with cellular, so inducing frameshift mutations (Rosenkranz and Mermelstein, 1983; Watanabe et al., 1990; Espinosa-Aguirre et al., 1999). Sodium azide is a base-pair substitution mutagen (mainly G:C→A:T transitions), activated into bacterial cells by O-acetylserine (thiol)-lyases to L-azidoalanine, which induce DNA helix distortion (Owais and Kleinhofs, 1988; Koch et al., 1994). Methyl methanesulfonate is a SN2 type DNA alkylating agent, which predominantly methylates nitrogen

atoms of the purine bases guanine and adenine, so causing base mispairing and replication blocks (Lundin et al., 2005). The sensitivity of cells to MMS increases significantly when other DNA repair pathways are compromised (Lundin et al., 2005). In particular in *E. coli*, MMS induces two complex systems, the Ada response, which protect the cells against alkylating agents (Shevell et al., 1990), and the SOS response, which express some proteins enabling DNA replication after the non-coding lesions (Walker, 1984). 4-nitroquinoline N-oxide (4NQO) is a potent mutagen and carcinogenic compound (Endo et al., 1971; McCann et al., 1975b), activated into bacterial cytoplasm, by the enzymatic reduction of its nitro group, to 4-hydroxyaminoquinoline 1-oxide (4HAQO), which forms DNA-adducts (Tada et al., 1976, 1984) and causes oxidative DNA-damage (Ishizawa and Endo, 1967; Hozumi, 1969). 1-nitropyrene (1NP) is a nitrocompound released from engine emissions and hard cooking (Tokiwa and Ohnishi, 1986). Its N-(deoxyguanosin-8-yl)-1-aminopyrene (dG-C8-AP) derivative, formed by bacterial nitroreductase, is responsible for DNA adducts, so inducing mutagenic effects (Tokiwa and Ohnishi, 1986; Wislocki et al., 1986; Howard et al., 1983; Bell et al., 1991; Malia et al., 1996). The *cnr*-codified nitroreductase seem to be mainly involved in the 1NP activation (Salamanca-Pinzón et al., 2010), although the biotransformation O-acetytransferase-mediated has been also reported (Watanabe et al. 1990; Espinosa-Aguirre et al. 1999). Also the pollutant 1,8-dinitropyrene (1,8 DNP) is a potent, direct-acting mutagen, activated by both bacterial nitroreductase and O-acetyltransferase to the nitropyrene-1-nitrenium ion, responsible for DNA-adducts and frameshift type mutations induction (Shah et al., 1990; Lambert et al., 1991; Djuric et al., 1993; Nohmi et al., 1995). Nitroreductases (*snrA*- and *cnr*-codified) seem to be less responsible for the 1,8-DNP activation (Salamanca-Pinzón et al., 2010). In fact, *cnr*-overproducing strains and *cnr*-deficient strain (i.e. TA98NR) are equally sensitive to the mutagenic effect of 1,8-DNP (Rosenkranz et al., 1983; Watanabe et al., 1989).

As an example of procarcinogenic compound, requiring a CYP450-mediated, 2-aminoanthracene (2AA) was also included. It is a common pollutant, released in the environment by the incomplete combustion or burning of organic (carbon-containing) items, e.g., cigarettes, gasoline, and wood (ATSDR, 1995). From a chemical point of view, this mutagen is an aromatic amine and is mainly activated by CYP1A2 and CYP1A1 isoenzymes, respectively (Jemnitz et al., 2004). The reactive derivative of 2AA, N-hydroxyarylamine, is able to attacks DNA, causing frameshift and base pair substitution mutations (Sabbioni and Jones, 2002). Moreover, the increase of the cellular oxidative stress seems to contribute to the 2AA-mutagenicity (Murata and Kawanishi, 2011; Leadon et al. 1988).

The antimutagenic activity was studied against the following mutagens: 2NF (11 µM for TA98 without S9), 2AA (10 µM for TA98 and TA100 with S9), SA (30 µM for TA100 without S9), MMS (5826 µM for WP2uvrA without S9), 4NQO (1 µM for TA98 without S9), 1NP (3 µM for TA98 without S9), 1,8-DNP (30 µM for TA98 without S9), and 2AA (49 µM for WP2uvrA with S9). These concentrations, obtained from the linear part of the concentration-response curve of mutagens, were chosen as they induce a submaximal mutagenic effect (about 70%).

In addition to the mutagens described above, in the present study a sample of cigarette smoke condensed (CSC), obtained from 3R4F reference cigarettes (University of Kentucky, Lexington, KY) was used. CSC was prepared using the standard Federal Trade Commission (FTC) protocol (35 puff volume in ml, 60 inter puff interval in sec and 2puff duration in sec, respectively) (Wan et al., 2009). Reference cigarettes have a circumference and lengths that are typical of cigarettes in the Canadian market; they are homogenous in terms of their physical, chemical and smoke yield characteristics and are intended to be used as references during smoke emissions testing (ISO 10185:2004). Tobacco smoke is one of the greatest threats to human health and the leading cause of preventable death in the industrialized society. Also the indirect or involuntary smoke, called "secondhand smoking" and derived from the smoke released in the environment by smokers, is considered by IARC (International Agency for Research on Cancer) as an important carcinogenic hazard for humans (IARC, 2004). Smoking-damages are numerous and involve mainly the respiratory and cardiovascular system. Although smoking cessation is the better strategy to avoid the development of cancer in the upper aerodigestive tract, former smokers continue to have an elevated risk of cancer for years after quitting (Tong et al., 1996). In this context, chemoprevention represents a highly sought-after approach to reduce the risk of smoking damages. In the present study, CSC was prepared by diluting, in DMSO, the sample provided by the University of Kentucky (40 mg/ml smoke particulates in DMSO). The CSC-concentration of 700 µg/plate was used in the antimutagenicity assay as, in preliminary assays, it resulted inducing a submaximal mutagenic effect (data not shown).

In addition to the antimutagenicity studies, the potential ability of CRY, CRYO and HCA to inhibit or modulate the multidrug resistance in cancer cells by acting as chemosensitizers was evaluated. A chemosensitizer is a compound that makes tumor cells more sensitive to the effects of chemotherapy and a combination of cytotoxic drugs with chemosensitizers represents a new approach to overcome drug resistance (Gottesman et al., 2002). Today, drug combinations are already applied successfully in the treatment of infectious diseases (including AIDS), hypertension, and many types of cancer and rheumatic diseases (Wagner, 2011; Proudman et al., 2000). The advantages of using drug combinations include an increase of therapeutic efficacy, a

decrease in the dose while maintaining the same effect with less toxicity, a reduction of drug resistance development, and potential synergistic effects (Chou, 2006).

The Figure 9 shows the molecular structures of the mutagens used in the Ames test.
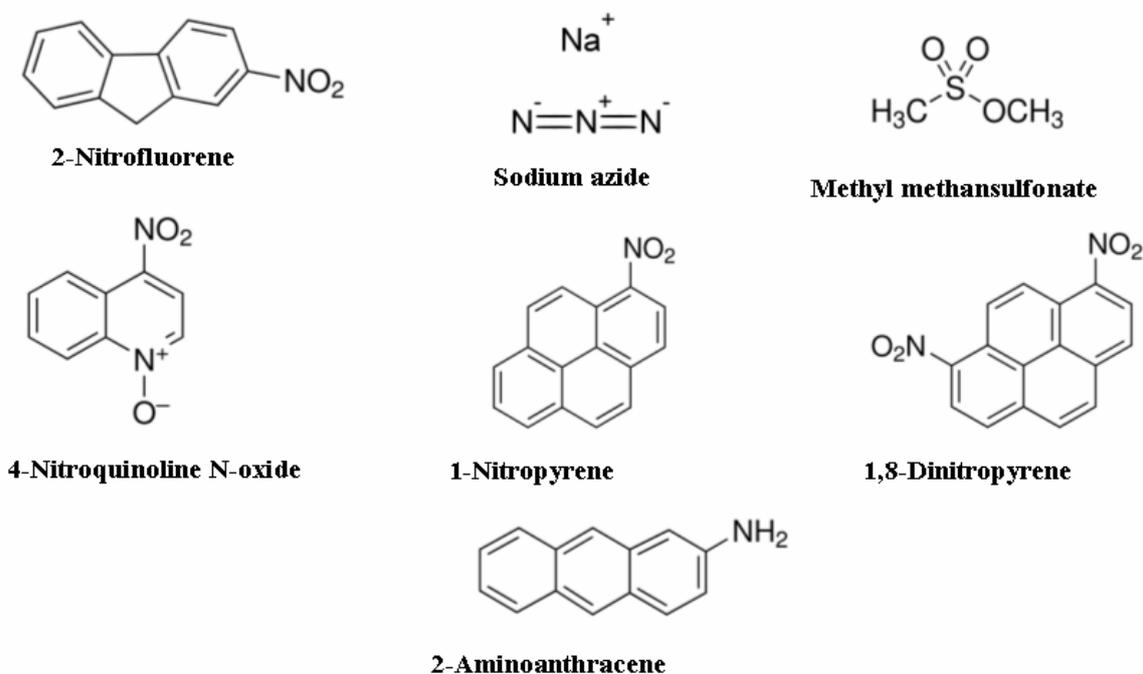

Figure 9. Molecular structures of the mutagens used in Ames test

Multi-resistant tumor cells frequently express different ATP-binding cassette (ABC) transporters simultaneously, e.g. P-glycoprotein (P-gp), multidrug resistance protein 1 (MRP1), breast cancer resistant protein (BCRP), and others (Annereau et al., 2004; Gillet et al., 2004). Because the substrate spectra of ABC transporters only partly overlap, co-expression of transporters might produce more diverse resistance profiles than those of any one member alone. Thus a broad spectrum of reversal agents are needed (Hyafil et al., 1993; Maliepaard et al., 2001; Brooks et al., 2003). At this purpose, in our study different human cancer cell lines, particularly CCRF/CEM (suspended sensitive cells of human T cell leukemia), CEM/ADR5000 (the derived doxorubicin-resistant subline of CCRF/CEM) and Caco-2 (adherent human colon-rectal adenocarcinoma cells) were used. CCRF/CEM cells, poorly expressing P-gp transporter, are studied in comparison with the multidrug-resistant subline CEM/ADR5000, which in contrast over-express P-gp and represent a model for evaluating the modulatory effects of different compounds on P-gp (Efferth et al., 2003; Gillet et al., 2004). Caco-2 cells represents an ideal model for studying MDR, because they highly express the ABC-transporter proteins, including P-gp (synonym MDR1), MRP1, and BCRP.

In regard to the experimental design, preliminarily the cytotoxicity of the test substances in the three different cell lines by methyl thiazol-diphenyl-tetrazolium bromide (MTT) assay was evaluated. This test represents one of major technique for evaluating the tumor cell resistance to anticancer agents, on the basis of the living cell ability (in contrast to the dead ones) to reduce MTT into a formazan derivative, which can be measured spectrophotometrically. It is a rapid, reliable and objective assay, suited for large-scale studies in leukaemia and lymphoma.

Subsequently, if the compounds resulted only weakly cytotoxic, we evaluated its potential ability to act as chemosensitizers. In this context, some combination tests were performed in order to detect the potential additive, synergistic or antagonistic effects between non toxic concentration (IC_{10} or IC_{20}) of the test compounds and doxorubicin (DOX) (Figure 10), used as a common anticancer drug; also combination assays among the test substances were carried out.
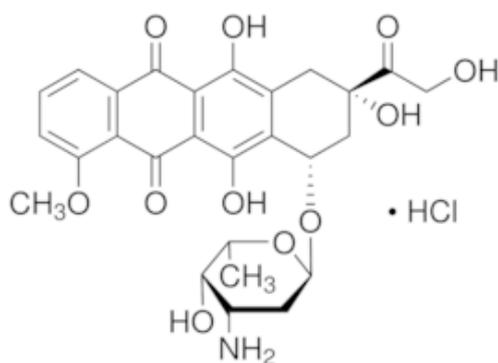
Figure 10. Molecular structure of the anticancer agent doxorubicin.

DOX is an anthracyline drug routinely used in the treatment of several types of cancer (i.e. breast, lung, gastric, ovarian, thyroid, non-Hodgkin's and Hodgkin's lymphoma, multiple myeloma, sarcoma and pediatric tumors) (Arcamone et al., 1969; Weiss, 1992). DOX acts by an unstable semiquinone metabolite, intercalating into DNA, disrupting the topoisomerase-II-mediated DNA repair and increasing the free radicals release, so damaging the cell structures and DNA (Doroshow, 1986; Gewirtz, 1999). Although doxorubicin is a valuable clinical antineoplastic agent, the major limits for the use of doxorubicin are cardiotoxicity and the development of cancer cell resistance (Carvalho et al., 2009; Swain et al., 2003). The mechanism of resistance involves ABCB1 (MDR1, Pgp) and ABCC1 (MRP1) and other transporters (ABCC2, ABCC3, ABCG2, and RALBP1) (Thorn et al., 2011).

In order to study the potential ability of the test compounds to inhibit the ABC transporters, the rhodamine 123 (Rho123) assay was performed. Rhodamine 123 is a fluorescente dye extensively used as both an inhibitor of mitochondrial function and a tracer for membrane transport. It cross

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easily through the membranes, due to its lipophilic nature and accumulates in areas with negative membrane potentials (i.e. mitochondria matrix) (Altenberg et al., 1994). Rho123 is also a known substrate not only for P-gp but also for MRP, so it can be used as a probe to detect the ABC transporters inhibitory activity (Versantvoort et al., 1996). The main advantages of the Rho123 use as a biological tracer, include commercial availability, low cost, high quantum yield, non-invasive detection and low interference with metabolic processes. In our experiments, verapamil, which is a MDR1 substrate, has been used as a known P-gp inhibitor (positive control).

OVERVIEW ON THE TEST SUBSTANCES

Overview on the test substances

α-Hexyl cinnamaldehyde

α-Hexylcinnamaldehyde (HCA; FL No. 05.041; Flavouring Group Evaluation, FGE.19, sub-group 3.1) is a synthetic α,β-unsaturated aldehyde (Figure 11), structurally derived from the natural aldehyde cinnamal. It possess a characteristic floral scent and are used as ingredients in many personal care (perfumes, creams, shampoos, etc.) and household products, and as additives in food and pharmaceutical industry (Schnuch et al., 2007). It is enclosed among the most frequently flavouring additives used in UK (Buckley, 2007).
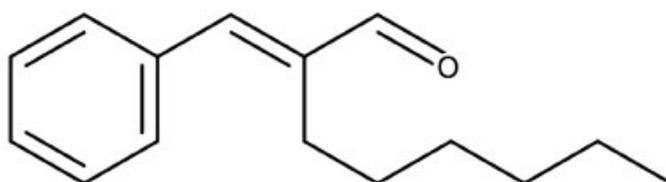
Figure 11. Chemical structure of α-hexyl cinnamaldehyde

It has been also listed by European Food Safety Authority (EFSA) in CEF (Food Contact Materials, Enzymes, Flavourings and Processing Aids) Panel, in order to require additional toxicity data for safety assessment (EFSA, 2012).

From a structural point of view, it is an aldehyde, due to the presence of a polarized carbon–oxygen double bond, able to react with electron-rich biological macromolecules (i.e. DNA and proteins), and to induce adverse health effects, including general toxicity, allergenic reactions, mutagenicity, and carcinogenicity (Feron et al., 1991; Patlewicz et al., 2002; Garaycoechea et al., 2012). HCA is potentially more reactive than a simple aldehyde, as it also possesses an additional double bond between carbons 2 and 3 (α and β respectively).

In spite of its potential reactive structure, in our previous study we found that HCA was not genotoxic, inducing neither point mutations nor primary DNA-damages, nor chromosome abnormalities (Di Sotto et al., 2013).

On the basis of these results, and taking into account that cinnamaldehyde, its natural precursor, has been found to possess many beneficial properties (Chuang et al., 2012), in the present study the potential chemopreventive properties of HCA have been evaluated.

β-CARYOPHYLLENE

β-caryophyllene (FL No. 01.007; FGE. 78), a bicyclic sesquiterpene with a rare cyclobutane ring (Figure 12), is a volatile compounds found in large amounts in the essential oil of many different spice and food plants, such as *Eugenia caryophyllata* (Fam. Myrtaceae) (Zheng et al., 1992) *Salvia* spp. (Fam. Lamiaceae) (Sonboli et al., 2006), *Syzygium aromaticum* Merr. et Perr. (Fam. Myrtaceae) (Prashar et al., 2006), *Piper nigrum* L. (Fam. Piperaceae) (Politeo et al., 2006), *Cannabis sativa* L. (Fam.) (Malingrè et al., 1975), *Citrus aurantifolia* (Fam. Rutaceae) (Gamarra et al., 2006), *Zingiber nimmonii* (J. Graham) Dalzell (Fam. Zinziberacee) (Sabulal et al., 2006), *Origanum syriacum* L. (Fam. Lamiaceae) (Alma et al., 2003), *Marrubium globosum* subsp. *globosum* (Fam. Labiate) (Sarikurkcu et al., 2008), *Comptonia peregrina* L. Coulter (Sylvestre et al., 2007) and in copaiba balsam, *Copaifera reticulata* Ducke (Fam. Fabaceae) (Veiga Junior et al., 2007). It is also one of the major components of the extracts from different species of *Helichrysum* (Lourens et al., 2004) and of *Carum nigrum* seed (Singh et al., 2006).
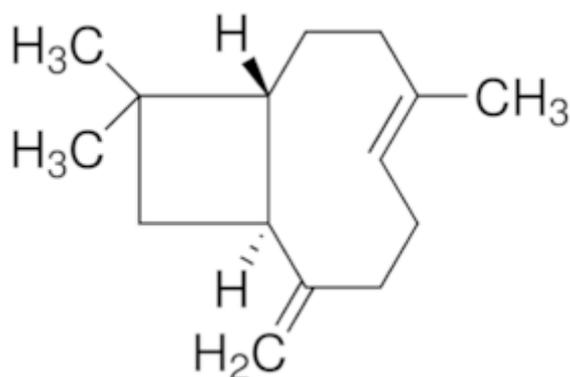
Figure 12. Chemical structure of β-caryophyllene

β-Caryophyllene is also one of the volatiles emitted by maize, by a terpene synthase 23 (TPS23) enzyme, which catalyzes the cyclization of farnesyl diphosphate to β-caryophyllene (Köllner et al., 2008), in response to herbivore damage (Turlings et al., 1998; Gouinguené et al., 2001). It is also an important signal in the attraction of enemies (particularly *Diabrotica virgifera virgifera*, defined as the root-feeding pest western maize rootworm) toward other maize herbivore. It is known that after the damage by this parasite, maize roots release β-caryophyllene, which attracts entomopathogenic nematodes (Rasmann et al., 2005). In nature, β-caryophyllene is usually found together with small amount of its isomers α-caryophyllene (α-humulene) and γ-caryophyllene (isocaryophyllene) or in a mixture with its oxidation product, β-caryophyllene oxide. Because of

its aromatic taste, β-caryophyllene is commercially used as a food additive and in cosmetics since the 1930s (Opdyke, 1973). It was detected in 33% of 300 analyzed cosmetic products on the Dutch market in the beginning of the 1990s (De Groot et al., 1994). In another study carried out on 71 deodorants sold in European market (Rastogi et al., 1998), it has been revealed in the 45% analyzed products. The Federal Food, Drug and Cosmetic Act, section 201 (s) and the Expert Committee of the USA Flavour and Extract manufacturer's Association (FEMA) have recognized β-caryophyllene as safe when used as a food flavouring additive. The Joint FAO/WHO Expert Committee on Food Additives has reported β-caryophyllene as presenting no safety concerns at current levels of intake when used as a flavouring agent (FAO/WHO, 2004). The Council of Europe Committee of Experts on Flavouring Substances has also established that as a flavouring substance it may be used as foodstuffs by at an upper level of 5 mg/kg in other foods, namely candy and confectionary. Moreover, this sesquiterpene appears on the list of "Permitted Additives to Tobacco Products in the United Kingdom" at a maximum level permitted for inclusion in cigarettes of 0.15 % w/w tobacco (Philipp Morris, 2005). Finally, it is considered a rare sensitizer agent. In fact, although caryophyllene is widely used, only few sensitized patients were identified (0.5%, 8 of 1511 tested consecutive dermatitis patients) (Sköld et al., 2006). Sköld et al., 2006 showed that β-Caryophyllene itself was revealed to be non-sensitizing while an oxidation mixture of autoxidized β-caryophyllene showed a weak sensitizing capacity in animal experiments.

β-caryophyllene was investigated for its possible *in vitro* biological activities; only few *in vivo* studies are carried out. This sesquiterpene has showed to be responsible of some antimicrobial (Alma et al., 2003; Lourens et al., 2004; Sabulal et al., 2006; Delamare et al., 2007), antileishmanial (Soares et al., 2013), anti-inflammatory, antiarthritic (Martin et al., 1993; Tambe et al., 1996; Baricevic et al., 2001; Agarwal and Rangari, 2003; Cho et al., 2007) local anaesthetic (Ghelardini et al., 1999) and anti-oxidant activities (Lourens et al., 2004; Ka et al., 2005; Singh et al., 2006). β-caryophyllene also showed anti-spasmodic activity on isolated rat tracheal smooth muscle which could be explained, at least in part, by the voltage-dependent Ca^{2+} channels blockade (Pinho-da-Silva et al., 2012). The anti-inflammatory effect of β-caryophyllene was also be demonstrated *in vivo* studies. and it seems to be mediated by the cannabinoid receptor 2 (CB2) and the PPARγ pathway (Bento et al., 2011). Furthermore, β-caryophyllene has been reported to possess cannabimimetic *in vivo* effects, such a CB2-receptor ligand (Gertsch et al., 2008) and some properties as skin-penetration enhancing (Cornwell and Barry, 1994).

β-caryophyllene has also demonstrated to act as a potential anticarcinogenic agent, due to its capability to induce the detoxifying enzyme glutathione S-transferase (Kubo et al., 1996).

Furthermore, Legault and Pichette (2007) have reported that it increases the anticancer activity of α-humulene, isocaryophyllene and paclitaxel against tumour cell lines, in part by alterating the membrane permeability. In previous studies carried out in my group of research, β-caryophyllene resulted lacking of genotoxic effects both in bacterial and in human eukaryotic cells (Di Sotto et al., 2008; Di Sotto et al., 2010); conversely a strong antimutagenic activity against the mutagen 2-nitrofluorene and anticlastogenic properties were highlighted (Di Sotto et al., 2008; Di Sotto et al., 2010).

On the basis of these results and taking into account that β-caryophyllene appears on the list of "Permitted Additives to Tobacco Products in the United Kingdom" for inclusion in cigarettes (Philip Morris U.S.A., 2005), in the present study we evaluated the potential ability of CRY to inhibit the genotoxic damage induce by cigarette smoke. In addition, its chemosensitizing properties were investigated.

β-CARYOPHYLLENE OXIDE

β-caryophyllene oxide (FL No. 16.043; Flavouring Group Evaluation, FGE. 82) (Figure 13), is a bicyclic sesquiterpene structurally similar to β-caryophyllene. It represents the oxidation product of β-caryophyllene by auto-oxidation due to air exposure (Sköld et al., 2006). It occurs in essential oils from various medicinal and edible plants, such as clove (*Syzygium aromaticum* (L.) Merr. et Perry), citrus (*Citrus* spp.), basil (*Ocimum basilicum* L.) and hop (*Humulus lupulus* L.) (Pino et al., 2001; Njoroge et al., 2006; Sajjadi, 2006; Nance and Setzer, 2011). However, the major amount (43.8 %) is contain in the essential oil of *Melaleuca styphelioides* (Fam: Myrtaceae) (Farag et al., 2004).

These sesquiterpene is used as a food additive and in several consumer products, such as creams, lotions, detergents, and various other personal and household products. Accordingly, the human exposure to this agent is widespread and often involuntary. Obviously, given the ubiquitous distribution of flavourings, it is to be assumed that when used as intended they do not represent a health hazard. In order to define a list of the flavouring substances which may be used in foodstuffs, the European Food Safety Authority (EFSA) asked to provide additional data for the risk assessment of some flavouring substances, reported on the EFSA CEF (Food Contact Materials, Enzymes, Flavourings and Processing Aids) Panel (EFSA 2012). Among these fragrances, β-caryophyllene oxide is representative for the epoxide group. The assessment of the toxicity risk for this flavor was reported to be pending due to its potentially reactive structure. In fact, epoxides, owing to their instability, are considered as reactive electrophilic intermediates, which are able to form covalent adducts with cellular macromolecules, such as proteins and DNA (Laffon et al., 2003). Recently, Di Sotto et al. (2013) have demonstrated that β-caryophyllene oxide is devoid of genotoxic potential, both at gene level, as a frameshift or a base-substitution mutagen, and at chromosomal level, as a clastogenic or aneuploidogenic agent. Our research group also has demonstrated the antimutagenic activity of this compound (unpublished data).

This sesquiterpene has showed to be responsible of some antifungal (Johann et al., 2012), trypanocidal (Polanco-Hernández et al., 2012) and antiviral (Dunkić et al., 2011) activities. Moreover, it recently showed an antimicrobial activities (Ellouze et al., 2012). In the past the antimicrobial activity of β-caryophyllene oxide was supposed. In fact, the sesquiterpene was considered the responsible of the antimicrobial activity of the oil of St. John's wort and the alcoholic extract of *Centaurea ensiformis* Hub.-Mor., being content in large amount, 30.8% and 28.7%, respectively. (Toker et al., 2006; Cavaleiro et al., 2011).
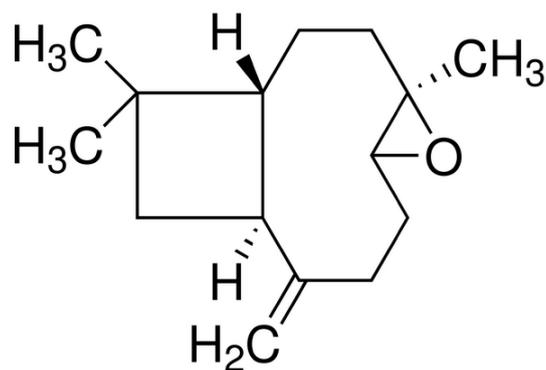
Figure 13. Chemical structure of β-caryophyllene oxide

β-Caryophyllene oxide has also showed to suppress the proliferation of a wide variety of tumor cells, including breast, liver, prostate, multiple myeloma, and lung adenocarcinoma and can thus act as a potent anticancer agent. Also, this compound has attracted interest, especially for its reported potential anti-inflammatory effects (Tung et al., 2008; Chao et al., 2005). The *in vivo* anti-hypernociceptive properties of this sesquiterpene have been demonstrated in inflammatory and neuropathic models of hypernociception in mice (Quintao et al., 2010). β-Caryophyllene oxide ability to control the expression of pro-inflammatory cytokines (IL-1b and IL-6) (Chao et al. 2005), nitric oxide (NO) and prostaglandin E2 (PGE2) production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages (Tung et al., 2008) has been reported. Also, it have recently reported that β-caryophyllene oxide can induce apoptosis, suppress cell proliferation, and inhibit invasion through the modulation of STAT3 (Kim et al., 2013), PI3 K/AKT/mTOR/S6K1 signaling cascades and MAPKs activation (Park et al., 2011). Kim et al. (2013) also have showed that β-caryophyllene oxide potentiated the apoptosis induced by tumor necrosis factor α (TNFα) and chemotherapeutic agents, suppressed TNFα-induced tumor cell invasion, all of which are known to require NF-κB activation. The effects of β-caryophyllene oxide might be mediated through the suppression of NF-κB and NF-κB–regulated gene products. In this study, the possible chemopreventive properties of β-caryophyllene oxide was investigated. Firstly, considering the demonstrated antimutagenic activity of β-caryophyllene oxide and its possible presence in tobacco smoke, being a metabolite of β-caryophyllene, we evaluated the potential protective role against the cigarette smoke condensate. Then, the potential ability of β-caryophyllene oxide to act as chemosensitizer was investigated.

MATERIALS AND METHODS

MATERIALS

Chemicals

The test substances α-hexylcinnamaldehyde (*synonyms* hexyl cinnamal, 2-phenyl methylene octanal; CAS number 101-860; purity ≥ 95%), β-caryophyllene (*synonyms* trans-caryophyllene, trans-4,11,11-trimethyl-8-methylenebicyclo[7,2,0]undec-4-ene; CAS number 87-44-5; purity ≥ 98.5%) and β-caryophyllene oxide (*synonyms* epoxycaryophyllene, (1R,4R,6R,10S)-9-Methylene-4,12,12-trimethyl-5-oxatricyclo[8.2.0.0(4,6)]dodecane; CAS number 1139-30-6; purity 95), the mutagens 2-nitrofluorene (*synonym* 2NF; CAS number 607-57-8; purity 98%), sodium azide (*synonyms* SA, hydrazoic acid sodium salt; CAS number 26628-22-8; purity > 99.5%), methyl methanesulfonate (*synonyms* MMS, ethanesulfonic acid methyl ester; CAS number 66-27-3; purity 99%), 2-aminoanthracene (*synonyms* 2AA, 2-anthramine; CAS number 613-13-8; purity 96%), 4-nitroquinoline N-oxide (*synonyms* 4-NQO, 4-nitroquinoline 1-oxide; CAS number 56-57-5; purity ≥ 98%), 1-nitropyrene (*synonym* 1-NP; CAS number 5522-43-0; purity 99%), 1,8-dinitropyrene (*synonym* 1,8-DNP; CAS number 42397-65-9; purity 98%), 9-aminoacridine hydrochloride hydrate (*synonym* 9AA; CAS number 52417-22-8; purity > 98 %), benzo[a]pyrene (*synonyms* B*a*P, 3,4-benzopyrene, 3,4-Benzpyrene, Benzo[*def*]chrysene; CAS number 50-32-8; purity 96 %), doxorubicin hydrochloride (*synonyms* DOX, adriamycin, hydroxydaunorubicin hydrochloride; CAS number 25316-40-9; purity 98.0-102.0%), rhodamine 123 (*synonyms* Rho123, 2-(6-Amino-3-imino-3H-xanthen-9-yl)benzoic acid methyl ester; CAS Number 62669-70-9), Methylthiazolyldiphenyl-tetrazolium bromide (synonyms MTT, Thiazolyl Blue Tetrazolium Bromide; CAS Number 298-93-1; purity 98%), the solvents dimethylsulphoxide (DMSO; CAS number 67-68-5; purity > 99.5%), ethanol (EtOH; CAS number 64-17-5; purity ≥ 99.5%) and all the other substancess, if not otherwise written, were purchased from Sigma, St. Louis, MO. The 3R4F ccigarette smoke condensate (CSC; batch n. R100404) was obtained from the University of Kentucky.

Media

The media Oxoid Nutrient broth No. 2, Vogel-Bonner medium, and Nutrient Agar were purchased from Sigma-Aldrich Co (St. Louis, MO, USA), while Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 were obtained by Gibco™, Invitrogen (Karlsruhe, Germany). The Oxoid Nutrient broth No. 2 was prepared as a water solution, then sterilized by autoclave and added with the essential aminoacid for the bacterial growth. The Vogel-Bonner agar medium (syn. minimal glucose agar medium), which favours the selective growth of the strains tested,

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contained a sterile solution of bacteriologic agar, glucose and salt solutions, and was poured hot onto sterile disposable plastic Petri disch. The Vogel-Bonner plates are prepared a few days before the experiments and left to solidify and dry for about seven days. Likewise, a hot solution of Nutrient agar, previously sterilized by autoclave, was poured onto sterile Petri plates. The Top agar, was prepared as a sterile water solution of bacteriological agar (0.6%) and NaCl (0.5%). During experiments, the medium was freshly prepared and maintained liquid at 45°C in a thermal bath, added with a 10% (v/v) solution of histidine and biotine (0.5 mM) (for the strains TA1535, TA1537, TA98, TA98NR, TA98 1,8-DNP and TA100) or tryptophan 0.5% (for the strains WP2, WP2uvrA and WP2uvrA/pKM101), and mixed to the treatment, and poured onto plates. The presence, in the top agar, of aminoacids (histidine, biotin and tryptophan) traces allows the bacteria to start the cell division and the colony formation.

The Dulbecco's modified Eagle's medium (DMEM) was purchased as solution and added with L-glutamine (1% v/v), heat-inactivated fetal bovine serum (FBS; 10% v/v), penicillin (100 U/ml), streptomycin (100 µg/ml), sodium pyruvate (1 mM) and non-essential amminoacids (1% v/v) in order to prepare a complete medium for growing and maintaining Caco-2 (human colon-rectal adenocarcinoma cells) adherent cells. Likewise, the solution of RPMI 1640 was added with L-glutamine (1% v/v), heat-inactivated fetal bovine serum (FBS; 10% v/v), penicillin (100 U/ml), streptomycin (100 µg/ml), and used for the growing and maintaining of both CCRF/CEM (human T cell leukemia) and CEM/ADR5000 (doxorubicin-resistant subline from CCRF/CEM) suspension cells.

Preparation of the solutions

All solutions were prepared in the better solvent, sterilized by autoclave and stored for a just conservation time at recommended temperature, i.e. room temperature (RT) or refrigerated conditions (from 4°C to -80°C). Each solution was labeled, indicating substance identity, solvent, concentration, date of preparation, sell-by date and storage temperature.

The mutagens 2NF, 4-NQO, 1-NP, 1,8-DNP, 9AA, 2AA and BaP were dissolved in DMSO, while SA and MMS in deionised water. The compounds α-hexylcinnamaldehyde, β-caryophyllene and β-caryophyllene oxide were prepared in DMSO for the Ames test; while, to perform the assays on the eukaryotic cell cultures, they were dissolved in EtOH solution (50% v/v) and hence diluted in the appropriate complete medium. Doxorubicin hydrochloride was dissolved in deionised water to obtain a 2 mg/ml stock solution, hence diluted in the medium and added to cultures. The stock solutions of Rho123 (1 mg/ml) and MTT (5 mg/ml) were prepared by dissolving the powder in DMSO and phosphate buffer saline (PBS 1X v/v), respectively. In

order to avoid the solvent cytotoxicity, DMSO and EtOH were used at a maximum of 2% and 1% concentration in bacteria and eukaryotic cells, respectively.

Stock solutions used in the Ames test

- Voger Bonner Salts 50X (VBS 50X): addition in succession (when the previous one was completely dissolved) of $MgSO4 \times 7$ $H2O$ (1%), citric acid (10%), K2HPO4 (50%), NH4NaHPO4 $\times$ 4 H2O (17.5%); sterilization by autoclave; RT storage.
- 40% glucose solution: powder dissolution in 45° C hot deionised water; sterilization by autoclave and RT storage.
- Ampicilline solution (8 mg/ml): ampicilline dissolution in NaOH 0.02 N; sterilization by filtration; 4° C storage.
- Phosphate buffer solution 0.2 M (pH 7.4): mixture of 19.7% KH2PO4 solution (0.2 M) and 80.3% KH2PO4 solution (0.2 M); sterilization by autoclave; RT storage.
- Phosphate buffer solution 0.1 M (pH 7.4): 1:2 (v/v) dilution of 0.2 M phosfate buffer solution; sterilization by autoclave; RT storage.
- KCl 330 mM: powder dissolution in 45°C hot deionized water; sterilization by autoclave; RT storage.
- MgCl2 100 mM, powder dissolution in 45°C hot deionized water; sterilization by autoclave; RT storage.
- Tryptophan (trp) solution 1 mM: powder dissolution in 45°C hot deionised water; sterilization by autoclave; 4°C storage.
- Histidine (his) 1 mM: powder dissolution in 45°C hot deionised water; sterilization by autoclave; 4°C storage.
- Biotin (bio) 1 mM: powder dissolution in 45°C hot deionised water; sterilization by autoclave; 4°C storage.
- Trp 0.5 mM: 1:2 (v/v) dilution of 1 mM trp; sterilization by autoclave; 4°C storage.
- His + Bio 0.5 mM: mixture of 1 mM histidine and 1 mM bio (1:1); sterilization by autoclave; 4°C storage.
- Glucose-6-phosphate (G6P) 0.1 M: powder dissolution in deionised water; sterilization by filtration; -20°C storage.
- Nicotinamide adenine dinucleotide phosphate (NADP) 0.1 M: powder dissolution in deionised water; sterilization by filtration; -20°C storage.

Stock solutions used for the test on eukaryotic cells

- Phosphate buffered saline (PBS) 10X (pH 7.4): dissolution of NaCl (80 g), KCl (2 g), Na_2HPO_4 (14.4 g), KH_2PO_4 (2.4 g) in 800 ml deionized water; sterilization by autoclave; RT storage.
- Phosphate buffered saline (PBS) 1X (pH 7.4): 1:10 dilution of PBS 10X; sterilization by autoclave; RT storage.

- Fetal bovine serum (FBS): thawed in a water bath at 37 °C, inactivated under shaking in 56 °C hot water for 30 min; -20 °C storage.

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml): powder dissolution in PBS; sterilization by filtration; - 20°C storage.

- Rhodamine 123 (Rho123) (1 mg/ml): powder dissolution in DMSO; -20 °C storage.

S9-BASED METABOLIC ACTIVATION SYSTEM

S9 fraction is the supernatant of a postmitochondrial liver homogenate obtained from rats treated with chemicals which induce the hepatic microsomal enzymes, particularly the mixture phenobarbital/β-naphthoflavone (PB/NF). The treatment with PB/NF mixture is able to induce a wide variety of CYP450 enzyme families, so that the S9 fraction results very suitable to detect numerous carcinogens requiring metabolic activation. In comparison with other tissues, the liver seems to be the most convenient source of activating enzymes.

In our experiments, we chosen to use a S9 fraction obtained from PB/NF-treated rats, purchased and certified from Moltox (Molecular Toxicology, Boone, NC, USA). According to SRI Internation, the S9-fraction was stored at -80°C, in order to preserve its activity.

The S9-based metabolic activation system was prepared just before use by mixing phosphate buffer (0.2 M; 500 μl), deionised water (130 μl), KCl (0.33 M; 100 μl), MgCl2 (0.1 M; 80 μl), S9 fraction (100 μl), G6P (0.1 M; 50 μl) and NADP (0.1 M; 40 μl). The mixture was kept in ice during testing.

METHODS

AMES TEST

This test detects mutations, which can revert previous mutations giving the strains depending by an aminoacid to grow; thus, the functional capability of the bacteria to synthesize this aminoacid was restored. All procedures have been carried out by using clean sterile glasswares and solutions, and inside of a sterile cabinet (model VBH48, Steril S.p.a., Mazzo di Rho, MI, Italy).

Bacterial strains and procedures for growing cultures

The strains *Salmonella typhimurium* TA1535, TA1537, TA98, TA98NR, TA98 1,8-DNP and TA100 and *Escherichia coli* WP2*uvr*A and WP2*uvr*A/pKM101 were used. All strains were kindly provided by the Research Toxicological Centre (Pomezia, Rome, Italy) and by University "Alma Mater Studiorum" of Bologna (Italy). In our laboratory, the tester strain genotypes have been verified every two years by a Strain Check assay.

Some aliquots of the tester strains (working cultures) are grown in glass cultures bottles (100 ml) having loose fitting caps and containing sterilized Oxoid nutrient broth No. 2 (NB; 11.5 ml) added with sterile histidine or tryptophan solution (1 mM) (500 μl). The capacity of the vessel should be 3-5 times the volume of the culture required. The cultures are incubated overnight (16 h) at 37° C to reach the late exponential or early stationary phase of growth (concentration approximately 1×10^9 cells/ml). To ensure an adequate aeration, cultures are shaken in a Dubnoff waterbath with horizontal oscillation (model 750, Asal s.r.l., Cernusco S.N., MI, Italy) at approximately 100 rpm. During the experimental procedures, the cultures are maintained at room temperature to avoid thermal shock to the bacteria when they are placed in 45 °C top agar. In order to assere that the cultures used contained a high titre of viable bacteria, in each experiment the number of viable cells was evaluated by a plating experiment, according to OECD (1997). Some aliquots of the tester strains (permanent cultures; Figure 14) were stored in sterile cryotubes at -80° C.

The permanent cultures are used only for the renewal of the strains. At this purpose, a fresh overnight culture, diluted 1:1000 in sterile NB, is added with the cryoprotective agent DMSO (9:100), distributed into 1.7 ml cryotubes and stored at -80° C. For TA98, TA98NR, TA98 1,8-DN, TA100 and WP2*uvr*A/pKM101 strains, ampicillin (25 μg/ml) has been added to NB medium in order to allow the growth of the strains retaining the R-factor in the genotype.

The working cultures, prepared from the frozen permanent ones, are used to prepare the fresh overnight culture for the experiments. A periodical Strain Check assay (about every six months)

of these cultures has been carried out. During the experiments, the bacterial cultures must be stored at controlled temperature to maintain constant the plateau of growth.
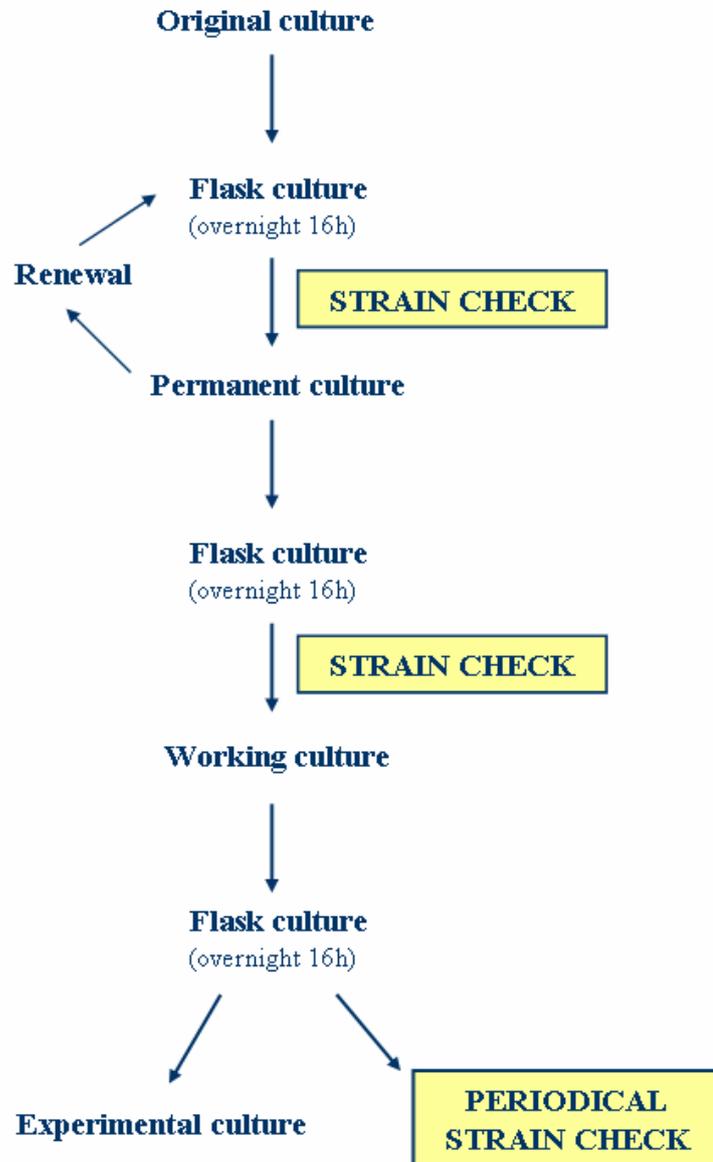
Figure 14. Preparation and check of the strains.

Strain Check Assay

The Strain Check assay allows to confirm the genotypes of tester strains, by studying specific genome markers (Ames, 1975). Both permanent and working cultures were checked, after preparation of renewed copies, during storage and when the spontaneous revertants or the strain-sensitivity to standard mutagens fall out of the normal range. Fresh overnight cultures (1×10^9) or the progressive (1:10) dilutions A (1×10^8), B (1×10^7) and C (1×10^6) are used. According to Mortelmans and Zeiger (2000), the following steps have been followed.

Spontaneous revertant colonies

The strains TA1535, TA1537, TA98, TA98NR, TA98 1,8-DNP and TA100, and WP2*uvr*A and WP2*uvr*A/pKM101 require the aminoacids histidine (his-) as well as tryptophan (trp-) to grow; a genotype mutation can revert to his or trp independence. The spontaneous revertant colony frequence is characteristic for each strain. The number of spontaneous revertants depends on his/trp concentrations. To evaluate this parameter, the overnight culture (100 μl), added to phosphate buffer (500 μl; 0.1 M) or S9 metabolic activator system, and to 45 °C hot top agar (2 ml), was poured onto Vogel Bonner Agar plates. After incubation at 37 °C for 72 hr, the number of revertant colonies per plate was scored. Acceptable ranges of spontaneous revertants should be consistent with the mean (μ) of historical values (acceptable limit is μ±2σ).

Mutagen-induced revertant colonies

The frequence of revertants/plate induced by specific mutagen is characteristic of each tester strain, in absence and in presence of metabolic activator S9. To evaluate this parameter, the overnight culture (100 μl) and the mutagen (100 μl), added to phosphate buffer (500 μl; 0.1 M) or S9 metabolic activator system, and to 45 °C hot top agar (2 ml), were poured onto Vogel Bonner Agar plates. After incubation at 37 °C for 72 hr, the number of revertant colonies per plate were scored. Acceptable ranges of spontaneous revertants should be consistent with the mean (μ) of historical values (acceptable limit is μ ± 2σ).

Preexistent mutants

They are colonies independent on the presence of the essential aminoacids and existing before the mutagen agent exposition. They can proliferate because of a *his* or *trp* inactivity, causing false positive results. To evaluate this parameter, the overnight culture (100 μl) and biotin (100 μl), were poured onto Vogel Bonner Agar plates. After incubation at 37 °C for 72 hr, the number of revertant colonies per plate were scored. Acceptable ranges of spontaneous revertants should be consistent with the mean (μ) of historical values (acceptable limit is μ±2σ).

Viability

The number of the viable colonies is verified because a reduction of the strain culture-growth could cause false results. For each strain, the C dilution of bacterial culture (100 μl), added to 45 °C hot top agar (2 ml), were poured onto Nutrient Agar plates. After incubation at 37 °C for 12-24 hr, plates were examined and the colonies were scored. Acceptable ranges of viability should be of 100-500 colonies.

R-factor (pKM101 gene) or ampicillin-resistence

The presence of this factor was evaluated by determining the the viability of the strains in the presence of a ampicillin solution. For each strain, the C dilution of bacterial culture (100 μl) and the ampicillin solution (8 mg/ml; 100 μl), were added to 45 °C hot top agar (2 ml) and poured onto Nutrient Agar plates. After incubation at 37 °C for 12-24 hr, plates were examined and the colonies were scored. Results were expressed as ampicillin-resistent colonies/viable colonies per plate. Acceptable values should be represented by about 70% of ampicillin-resistance.

rfa mutation

The number of the viable colonies in presence of the toxicant crystal violet is defined to verify the presence of rfa mutation. For each strain, the overnight culture (100 μl) was added to 45 °C hot top agar (2 ml) and poured onto Nutrient Agar plates; hence we pipet 10 μl of a crystal violet solution (0.1 %) to the centre of a sterile filter paper disc (Whatman 6 mm AA disc), placed on the plates using sterile forceps. After incubation at 37 °C for 12-24 hr, the plates were examined for the presence of a clear zone of inhibition around the disc, indicating the killing of bacteria. Acceptable values of inhibition-zone diameter must be > 10 mm.

uvrB mutation

The UV sensitivity of the strains tested, due to uvrB (TA98 and TA100) or uvrA (WP2uvrA and WP2uvrA/pKM101) deletion was evaluated by exposing bacteria to UV germicide radiations. For each strain, the B dilution of overnight culture (100 μl) was added to 45 °C hot top agar (2 ml) and poured onto Nutrient Agar plates; Half of the plate was covered with a piece of cardboard, hence irradiated with a UV germicide lamp ($\lambda = 254$ nm) at a distance of 33 cm, for 8-10 second. After incubation at 37 °C for 12-24 hr, plates were examined. The strains carrying the uvrB deletion will grow only on the not-irradiated side of the plate.

Morfological analysis

Morfology of the strain-colonies was examined at microscopy (40x magnification): *S. typhimurium* colony appears like an egg, containing a dense nuclear zone, while *E. coli* is rounded and with a no much clear nuclear zone.

Preliminary assays

Solubility and selection of test concentrations

Solubility of the test substances in the final mixture poured onto plate was preliminarily assessed in order to identify the highest concentration suitable to be tested. The concentration at which a precipitate of the substance was evident to the unaided eye, in the top agar final mixture or onto the plate after pouring the mixture, was discarded and the maximum concentration used in the successive assays was that inducing no precipitate (OECD, 1997).

Cytotoxicity and viability assay

The cytotoxicity of the substances was evaluated as reduction in the number of revertant colonies and as change in the auxotrophic background of bacterial growth (background lawn) compared with the control plates (Ames, 1975). If the massive cell death occurred, the background lawn on the plates test appears sparse in comparison to control plates. In this case, a more quantity of histidine will be available to the surviving bacterial cells, which will undergo more cell divisions and will appear as small colonies, named "micro-colonies". These colonies can be confused with the revertants if one has failed to observe the absence of a normal background lawn. Micro-colonies can be discriminated from the revertants, by verifying the selective growth on minimal glucose agar containing biotin but not histidine: only the revertants, containing his+ character will grow in these conditions (Maron and Ames, 1983).

To perform the test, different concentrations of the substances (100 μl) were added to the overnight culture (100 μl), the S9 mixture or phosphate buffer (0.1 M) (500 μl). The mixture was pre-incubated under shaking at 37 °C for 30 min, then added with top agar (2 ml), containing his/ bio or trp (10% v/v; 0.5 mM) for *S. typhimurium* and *E. coli* strains respectively, and poured onto a minimal agar plate. In addition, to evaluate the cell viability, a dilution of the overnight culture (about 10^6 fold, to yield approximately 200 cells/100 μl) was added to sterile top agar (2 ml) and the mixture was poured onto nutrient agar plates (Maron and Ames, 1983). When necessary, to evaluate the survival of the bacteria during treatment procedures, a viability assay in the presence of various concentrations of the test substances was also made. For both cytotoxicity and viability assay, the plates were incubated at 37 °C for 48 hr and then the presence of the bacterial background lawn was verified, and the numbers of histidine- or tryptophan-independent revertant and surviving colonies were scored. Results are reported as number of revertant and surviving colonies per plate (Maron and Ames, 1983).

Mutagenicity assay

Mutagenicity was assessed as reported by Maron and Ames (1983) and Green and Muriel (1976), by using the pre-incubation method. Test solutions were prepared by serial dilution (1:1.4) in the solvent DMSO; at least five different concentrations for each substance were tested, both with or without metabolic activator; when necessary, further concentrations were tested in order to obtain a concentration-response curve. Concurrent positive (mutagen) and negative (vehicle) controls, both with and without metabolic activation, were included. The positive control was used to verify the bacterial sensitivity to known mutagenic agents. The plates of the positive controls were treated with a reference mutagenic substance, at exposure levels expected to give a reproducible and detectable increase of the number of revertant colonies. The mutagens used in our experiments were: 2-nitrofluorene (2NF in DMSO, 3.6 μM for TA98 without S9); sodium azide (SA in deionised water, 5.8 μM for TA100 without S9 and 380 μM for TA1535 without S9); methyl methanesulfonate (MMS in deionised water, 1.7 mM for WP2uvrA without S9); 9-Aminoacridine (9AA in DMSO, 97 μM for TA1536 without S9); 2-aminoanthracene (2AA in DMSO, 1.9 μM for TA98 and TA100 with S9 and 19 μM for TA1535, TA1537 and WP2uvrA with S9) and benzo[a]pyrene (BaP in DMSO, 18.9 mM for TA98, TA100 and WP2uvrA with S9 and 37.7 mM for TA1535 and TA1537 with S9). The negative control was used to determine the spontaneous revertant colonies per each strain in the presence of the vehicle used to dissolve the test compounds. In addition, an untreated control (lacking solvent) was used to verify if vehicle induced deleterious or mutagenic effects.

According to Maron and Ames (1983), to perform this assay, an aliquot of the overnight culture (100 μl), test solution (50 μl) and S9 mixture or phosphate buffer (0.1 M; 500 μl), mixed and gently vortexed in a sterile tube, were incubated under shaking at 37 °C for 30 min; then the tubes were added to top agar (2 ml), containing his/bio or trp (10% v/v; 0.5 mM) (for TA1535, TA1537, TA98, TA98NR, TA98 1,8-DNP and TA100 and WP2uvrA and WP2uvrA/pKM101 respectively), vortexed and poured onto a minimal agar plate. The plates were incubated at 37 °C for 72 hr, then the histidine- or tryptophan-independent revertant colonies and viable cells were scored and the bacterial background lawn was observed (Figure 15). The experiments were repeated at least twice and each concentration was tested in triplicate.
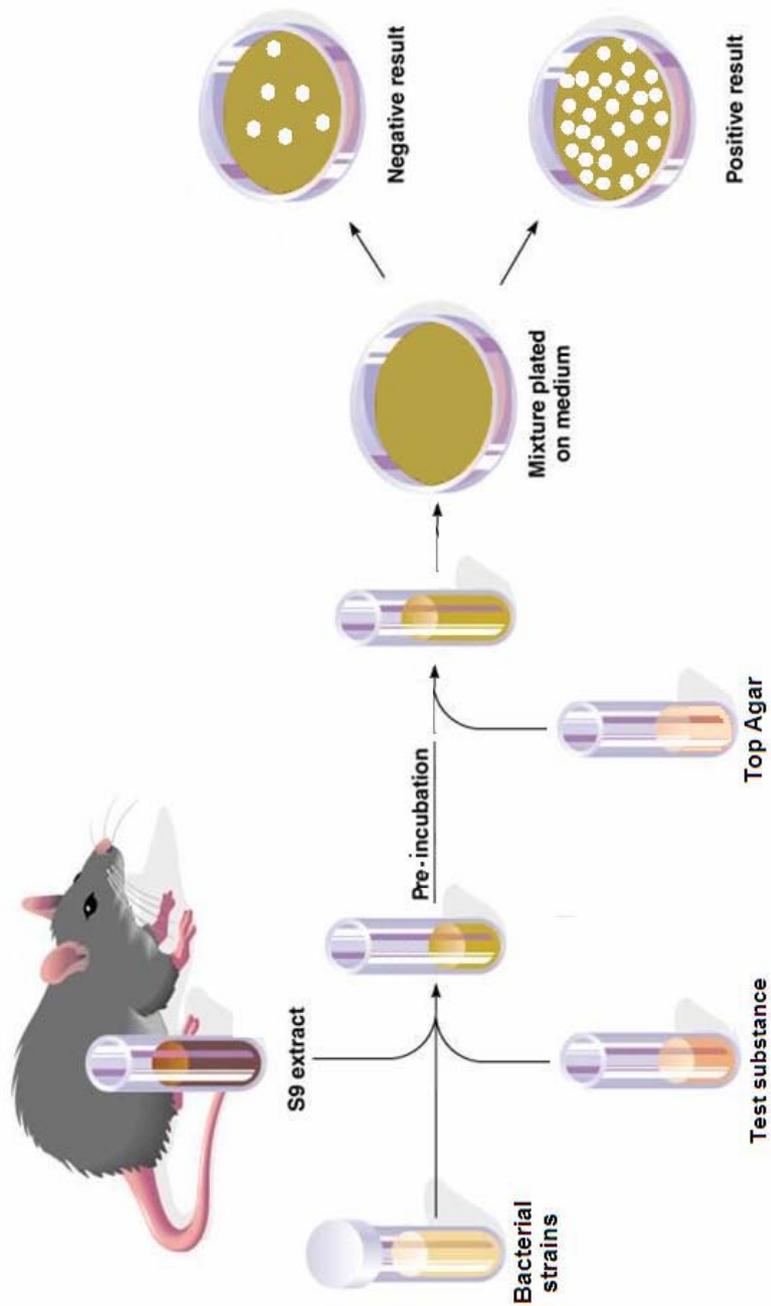
Figure 15. Mutagenicity assay

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Antimutagenicity assay

Antimutagenicity was assayed as previously described by Edenharder et al. (1993), by using the preincubation methods. Plates containing strain-specific mutagen (100% of mutagenic activity) or vehicle (lack of mutagenic activity) were included. In order to discriminate cytotoxicity from true antimutagenesis, plates containing the strain with the test substance were also included. Finally, sterility control plates were prepared in each experiment. The test substance solutions were prepared by serial dilution (1:1.4) in the solvent DMSO. Antimutagenic activity was evaluated against the mutagens 2-nitrofluorene (10.7 μM, for TA98 without S9); sodium azide (29 μM, for TA100 without S9); methyl methanesulfonate (5.8 mM, for WP2uvrA without S9); 1-nitropyrene (3 μM, for TA98, TA98NR and TA98 1,8-DNP without S9); 1,8-dinitropyrene (0.03 μM for TA98 without S9); 4-nitroquinoline N-oxide (1 μM for TA98 without S9); 2-aminoanthracene (9.8 μM for TA98 and TA100 with S9); 2-aminoanthracene (49 μM for WP2uvrA with S9) and cigarette smoke condensate (264 μg/ml for TA98, TA100, WP2uvrA and WP2uvrA/pKM101 with S9).

To perform the test, the bacterial strains were treated by using three different treatment protocols, pre-, co- and post-treatment, in order to study the potential mechanism occurred in the antimutagenic activity. The pre- and co-treatments allows to evaluate the ability of the substance to protect from the genotoxic damage mutagen-induced, or to the directly interfere with the mutagen.

Conversely, the post-treatment highlight the capability of the substance to repair the genotoxic damage mutagen-induced.

1. Pre-treatment

The bacterial overnight culture (100 μl), the test substance solution (25 μl), and S9 mixture or phosphate buffer (0.1 M) (500 μl), were mixed and gently vortexed in a sterile tube and incubated under shaking at 37 °C for 30 min. After this time, the mutagen solution (25 μl) and top agar (2 ml), containing his/bio or trp (10% v/v; 0.5 mM) were added to the sterile tubes. The mixture was vortexed and poured onto a minimal agar plate.

2. Co-treatment

Tge bacterial overnight culture (100 μl), the mutagen solution (25 μl), the test substance solution (25 μl), and S9 mixture or phosphate buffer (0.1 M) (500 μl), were mixed and gently vortexed in a sterile tube and incubated under shaking at 37 °C for 30 min. Then the tubes were added with top agar (2 ml), containing his/bio or trp (10% v/v; 0.5 mM), vortexed and poured onto a minimal agar plate.

3. Post-treatment

The bacterial overnight culture (100 μl), the mutagen solution (25 μl) and S9 mixture or phosphate buffer (0.1 M) (500 μl), were mixed and gently vortexed in a sterile tube and incubated under shaking at 37 °C for 30 min. Then the tubes were added with the test substance solution (25 μl) and with top agar (2 ml), containing his/bio or trp (10% v/v; 0.5 mM), vortexed and poured onto a minimal agar plate.

For each protocol, control plates were prepared by adding the mutagen or the vehicle of mutagen (25 μl) to the vehicle of test substance (25 μl). After incubation at 37 °C for 72 hr, plates were scored for his or trp independent revertant colonies and viable cells; the bacterial background lawn was also examined (Figure 16). The experiments were repeated at least twice and each concentration was tested in triplicate.

Cell survival assay

In order to exclude that the treatment could reduce cell viability by inducing cytotoxicity, the same experimental protocol used for the antimutagenicity assay was repeated in cell survival studies. To perform this test, the bacterial overnight culture (100 μl), the mutagen solution (25 μl), the test substance solution (25 μl) and S9 mixture or phosphate buffer (0.1 M; 500 μl) were mixed in a sterile tube, gently vortexed, and preincubated under shaking at 37 °C for 30 min. At the end of pre-incubation, each treatment was diluted to obtain a concentration of 2×10^3 cells/ml, then added to 2 ml of top agar, and plated onto nutrient agar plates. The positive control was prepared by adding the solvent of the test substance to the mutagen (25 μl + 25 μl), while the negative control was obtained by adding the solvent of the test substance to that of the mutagen (25 μl + 25 μl); then both controls underwent the experimental procedure described above. The resulting plates were incubated at 37 °C for 72 h. After incubation, plates were scored for the colonies originating from viable cells. The reduction of cell viability induced by the treatment was evaluated by comparing the number of viable cells of the negative control and those of each treatment. A treatment was considered cytotoxic when the cell viability was less than 70% with respect to the control.
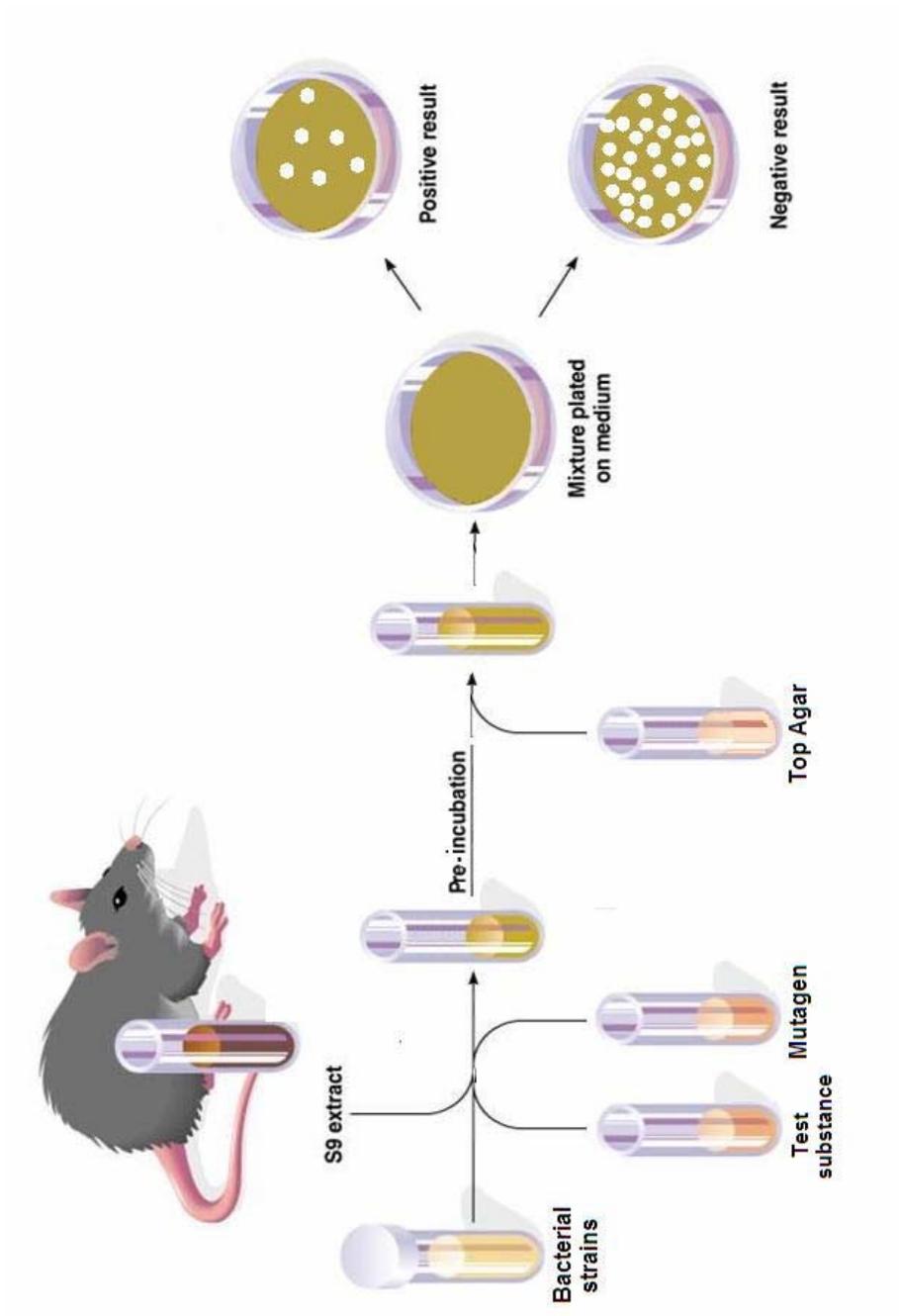
Figure 16. Antimutagenicity assay

Statistical analysis

Results of mutagenicity assay has been expressed as number of revertant colonies per plate. A positive response was defined as an increase (at least two-fold above the vehicle) in *his-* or *trp-* independent revertant colonies in each strain, with or without metabolic activation (Figure 17) (Ames et al., 1975).
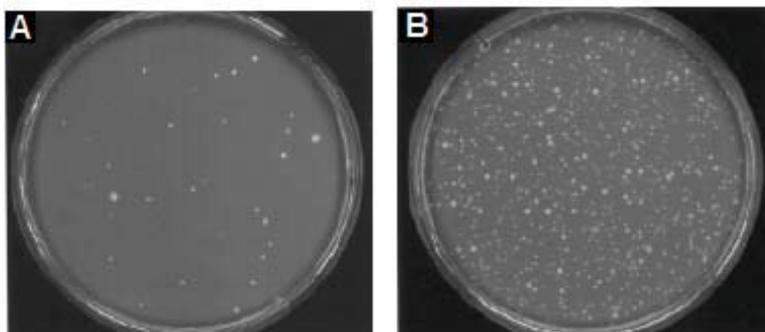
Figure 17. Image of the plates obtained in the Ames test. (A) Plate treated with the vehicle (negative control). (B) Plate treated with the mutagen.

Due to the high variance in the revertant plate-count, several statistical procedures (i.e. 2-fold rule, t-test, ANOVA + multiple comparison, slope of the initial linear regression etc.) have been used to data analyze (Kim and Margolin, 1999). In our experiments, we employed the "2-fold rule" method combined with the "ANOVA + multiple comparison Post-test". When the revertant count exceeded twice the spontaneous background rate, according to the two-fold rule, the results of the mutagenicity assay were considered positive. The "ANOVA + multiple comparison Post-test" method allows to confirm the results and to detect false positive results. The potency of a mutagen has been defined, if possible, by the concentration-response curve.

Results of the antimutagenicity assay have been reported as number of revertant colonies per plate treated with the test substance. The number of revertant colonies grown in plates containing the mutagen without the test substance was defined as 100 % of mutagenic effect, while this of the plate containing the vehicle represented the lack of mutagenicity. The percentage of inhibition of mutagenic effect was calculated according to the formula: $100 - [(T/M) \times 100]$ where T is the number of revertant colonies per plate in presence of mutagen and test substance, and M is the number of revertant colonies per plate in plates containing mutagen without test substance. According to Negi et al., (2003) the antimutagenic effect was considered as weak or absent (inhibition up to 25 %), moderate (25–40 % inhibition) or strong (inhibition higher than 40%). The statistically significance was evaluated by using the "ANOVA+ Dunnett's Multiple Comparison Post Test".

STUDIES ON EUKARYOTIC CELLS

Human cancer cell lines

Several cell lines were used:

- CCRF/CEM (DSMZ Nr. ACC 240), suspended cells from human T cell leukemia, obtained from the German Collection of Microorganisms and Cell Cultures GmbH;

- CEM/ADR5000, doxorubicin-resistant subline from CCRF/CEM, which overexpress MDR1 gene, provided by Prof. Thomas Efferth (DKFZ, Heidelberg).

- Caco-2 (DSMZ Nr. ACC169), adherent human colon-rectal adenocarcinoma cells, which are epithelial like and grow as monolayer. Caco-2 cells represent an ideal model for studying drug transporters, because they highly express ATP-binding cassette (ABC) transporters including P-gp/MDR1, MRP1 and BCRP.

Cell cultivation procedures

All cell lines were cultivated at $0.1\text{-}0.2 \times 10^5$ cells/cm^2 (25 and 75 cm^2 culture flasks) and were incubated at 37 °C with 5% CO_2. All steps requiring to open the culture bottles, plates or media were carried out under sterile conditions. The cells were morphologically investigated under light microscope, before and after each subculture, in order to determine the cell growth rate.

The adherent cell lines Caco-2 reached the confluence normally after 3-4 days. After that, the cells were washed with PBS, and incubated with trypsin-EDTA solution at 37 °C for about 4 min (until the cells detached from the bottom of the culture flask). Then, the fresh medium was added to detach the cell suspension and to stop trypsin action. Thus, the cells were collected and centrifuged at 2000 rpm, and the fresh medium was added again. In order to separate the cell clumps into single cells, the solution was pipetted up and down several times. After counting, the cells were seeded in a new culture flask.

The CCRF/CEM and CEM/ADR5000 cells, were subcultured in suspension every 2-3 days, by 1:5 dilution in RPMI 1640 medium.

Cell cryopreservation

For a long term storage, the cells were cryopreserved at low sub-zero temperature. Cryopreservation was performed initially by storing the cells at -20 °C for 2 h, then by cooling overnight up to -80 °C, thus by introducing into liquid nitrogen (-196 °C). This procedure effectively stops any biochemical and biological activity without cell death. Cryopreservation was used exclusively for cells that were in their logarithmic growth phase. To perform the

procedure, the cell suspension was harvested and centrifuged at 2000 rpm for 10 min. After the cell counting, one equal volume of the cell suspension was centrifuged at 2000 rpm. The pellets were resuspended in the ice-cold freezing medium and the cell clumps were separed by pipetting gently up and down. Some aliquots of the cell suspension were prepared in pre-cooled cryo-vials (for each cell lines, one milliliter/cryovial cointained 1-5 x 10^6 cells). The freezing medium, consisting of 70% culture medium (corresponding to the cell line), 20% inactivated FBS and 10% DMSO, was freshly prepared and sterilized before the use.

In order to re-cultivate the cells, the cryogenic cultures were thawed in a water bath (37 °C) and then rapidly mixed with a 10 ml fresh and warm (37 °C) medium.

To remove DMSO, the cell suspension was centrifuged at 2000 rpm, the supernatant was aspirated and 10 ml of fresh medium was added to the cell pellets. Then the culture was gently pipetted up and down to separate the clumps. The cell suspension was transferred into a culture flask (25 cm^2) and incubated at 37 °C with 5% CO_2.

Periodically, in order to exclude any mycoplasma contamination of cell cultures, a specific test was carried out by using a VenorGeM® Mycoplasma Detection Kit and according to the manufacturer's instructions. The method reveals, with high sensitivity, the presence of mycoplasma and acholeplasma contamination by the polymerase chain reaction (Hopert et al., 1993).

Trypan blue viability assay

The Trypan blue measures the percentage of viable cells; this method is based on the principle that live (viable) cells, with an intact membrane, are able to exclude the dye while dead (non-viable) cells, without an intact membrane, take up the staining agent. Trypan Blue is one of several stains recommended for the use in dye exclusion procedures for viable cell counting. Staining facilitates the visualization of cell morphology.

To perform the assay, trypan blue stain (100 μl) was added to cell suspension (100 μl), mixed thoroughly and shortly incubated. Then, a small amount (10 μl) of the trypan blue-cell suspension mixture was carefully transferred on a Neubauer chamber (Figure 18), by touching the edge of the cover-slip with the pipette tip and allowing that each chamber filled by capillary action. The Neubauer chamber counting grid (3 mm x 3 mm in size) is structured by a reticulum (network) drawn in the ridge and divided in nine square subdivisions (1 mm width). Since the distribution of the trypan blue-cell suspension mixture on the reticulum is not homogeneous, we obtained the value of cell counts from the mean value of the cells counted in at least five squares (middle, top left, top right, bottom right, bottom left), as showed in Figure 18.
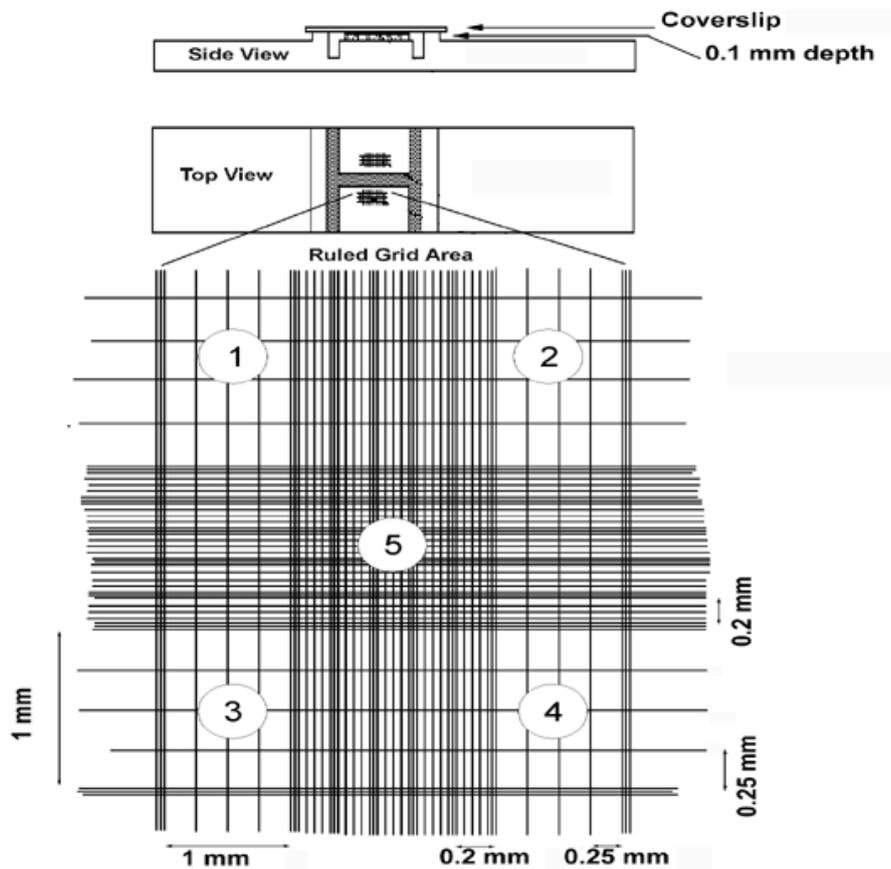
Figure 18. Neubauer chamber

The cell number per ml was obtained by multiplying the count mean by the dilution factor (which is 2) and by 10^4, according to the following equation:

Viable cells/ml = (viable cell average) x (dil. with trypan blue) x 10^4

Preliminary cytotoxicity evaluation by MTT assay

The 3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazolium bromide (MTT) assay is a colorimetric test used to assess the cell viability. The assay is based on the conversion of the yellow water soluble tetrazolium dye MTT into formazan, an insoluble purple compound, due to the action of the living cell mitochondrial dehydrogenases (Figure 19). This conversion can take place only in the cell populations that have not been damaged at the mitochondrial level. The amount of MTT-formazan produced can be determined spectrophotometrically once solubilized in a suitable solvent. The adherent cell line was seeded in the fresh media, incubated at 37 °C with 5% CO_2 for 24 h, so that it was in a logarithmic growth phase, then treated with various concentrations of the test substances. The adherent cells were pre-incubated for one day after

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seeding, in order to achieve a good cell adhesion; otherwise, the results would not indicate the actual effect on attached cells. So the medium was aspirated, replaced by medium containing the test substance, and further incubated.
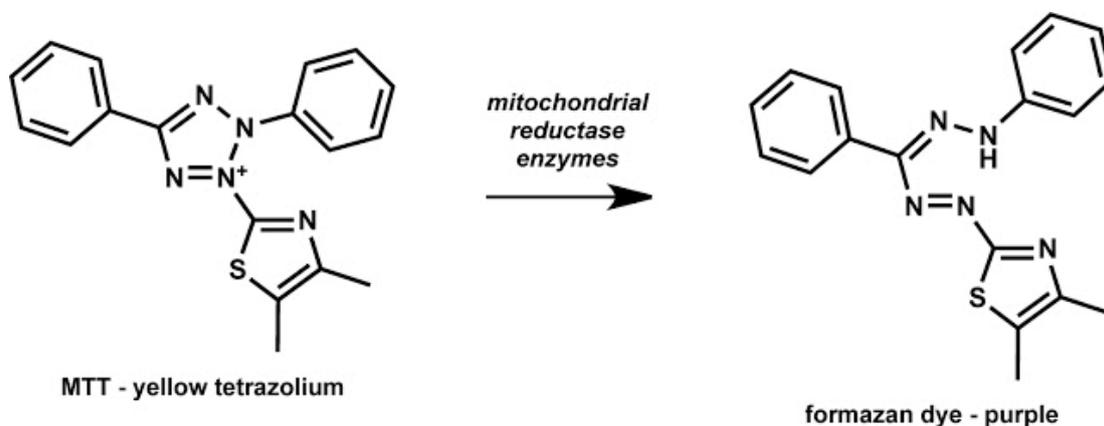
Figure 19. Reduction of MTT in viable cells. NAD(P)H-dependent cellular oxidoreductase mitochondrial enzymes may, reduce the tetrazolium dye MTT to its insoluble purple product formazan.

To perform the MTT assay, different experimental procedures were applied.

Adherent cell line: Caco-2 cells were grown in complete media (see paragraph "MEDIA"), seeded in 96-well plates (2×10^4 cells/well density) and incubated for 24 h. The cells were treated, in 96-well plates (100 µl/well of a medium), with different concentrations of the test substances (up to 10 mM) for a further 24 h. Then, the MTT solution (0.5 mg/ml) was added to each well and the plate was incubated again for 4 h. The formed formazan crystals were dissolved in DMSO and its absorbance was detected at 570 nm, by a Tecan Safire II™ microplate reader (Crailsheimm Germany).

Suspension cell lines: CCRF/CEM and CEM/ADR5000 were seeded in 96-well plates at about 3×10^4 cells/well density and incubated with the test substances for 48 h. Then, the MTT assay was carried out as mentioned before.

For each procedure, the concentrations were tested at least in triplicate, also including a vehicle control. The cell viability was determined as follow:

viability % = [(OD treated cells-OD medium control)/(OD untreated cells-OD medium control)]%

Multidrug resistance (MDR) reversal assay

The ability of the test substances to restore the cancer cells sensibility to chemotherapeutic drugs, i.e. to reverse the multidrug resistance phenomenon, was determined in Caco-2, CCRF/CEM and CEM/ADR5000 cells by the MTT assay (see paragraph above). Low concentrations, particularly IC_{10} and IC_{20} (concentration at which a 10 and 20% cytotoxicity was produced), of the potential reversal agent was tested in combination with doxorubic at very, respectively. To perform the assay, fully differentiated cells were harvested and seeded in flat-bottom 96-well plates (2×10^4 cells/well density for Caco-2 cells; 3×10^4 cells/well density for CCRF/CEM and CEM/ADR5000 cells). Caco-2 cells were incubated for 24 h at 37 °C to allow their attachment, before the addition of the drugs. Different concentrations of doxorubicin or the test substances were added to cells, in the absence and presence of the potential reversal agent, then the plates were incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 for 24 h. Conversely, CCRF/CEM and CEM/ADR5000 cells were directly incubated with doxorubicin or the test substances. Then the cytotoxicity was evaluated.

The combination index (CI) and the isobologram analysis (IB) were used to determine the nature of the interaction among the substances tested; CI was calculated as follow:

$$CI = \frac{C_{A,X}}{IC_{X,A}} + \frac{C_{B,X}}{IC_{X,B}}$$

in which $C_{A,X}$ and $C_{B,X}$ are the concentrations of the drugs A and B used in combination to produce a mean effect X (IC_{50}). $IC_{X,A}$ and $IC_{X,B}$ are the IC_{50} values for the compounds A and B alone. The interaction is synergistic when CI is less then 1, additive if the value is 1, and antagonistic when CI is higher than 1 (Colombo et al., 2010).

The isobologram analysis defines the interaction extent between the potential reversan agent (A) and chemotherapeutical drug (B). The IC_{50} concentrations of drugs A and B are plotted on the x and y axes in a two-coordinate plot, corresponding to (C_A, 0) and (0, C_B), respectively. The line connecting these points represents an additive interaction. The concentrations of the drugs used in combination, denoted as C_A and C_B, are placed in the same plot. The effect was synergistic when C_A and C_B are located below the line, while antagonistic when the values are above the line (Zhao et al., 2004). Furthermore, the reversal ratio (RR, *synomym* cytotoxicity enhancement ratio), determined in order to quantify the drug efficacy enhancement in the presence of the reversal agent, was calculated as follow:

$$RR = \frac{IC_{50} \text{ of TS alone}}{IC_{50} \text{ of TS in combination with CS}}$$

where TS is the substance alone, while CS is the potential chemosensitiser.

Interaction between reversal agents and ABC-transporters

The functional assay for ATP-binding cassette (ABC) transporters measures the active efflux of ABCt substrate across the cell membrane. It detects the amount of substrate taken up by the cells, after a period of incubation, by fluorimentric measurement. In our experiments, Rhodamine 123 (Rho123), a fluorescent dye which is accumulated by mitochondria and is subject to P-gp (P-glycoprotein) and MRP- (multidrug resistance protein) dependent extrusion, was used as a molecular probe. Thus, Rho123 accumulation in cells provided a measure of P-gp- and MRP-dependent transport activity. Accordingly, P-gp and MRP inhibitors, such as verapamil, lead to enhanced accumulation of the dye in cells, as they interfere with dye extrusion. For this reason, verapamil, at the concentration of 20 μM which completely blocks the P-gp- and MRP-mediated efflux of Rho123 from the cells, was used as a positive control (100% of inhibition). Moreover, the functional assay requires viable tumor cell suspensions, which are difficult to obtain from many adherent cells such as Caco-2. Here we describe a method analogous to P-gp functional assay in leukemia. Cell seeding and growth conditions were the same as the combination experiments. The use of Rho123 was considered as a useful approach to determine P-gp and MRP activity in human cancer cells.

To perform the test, Caco-2 cells (2 x 10^3 cells/well density) were seeded in 96-multiwell plates and incubated at 37 °C and 5% CO_2, until a confluent monolayer was formed (after 4–6 days). Then cells were washed twice with PBS and pre-incubated for 30 min at 37 °C with different concentrations (from 20 to 1250 μM) of the test substances. The cells were then incubated for 90 min with Rho123 (1 μg/ml), then washed twice with cold phosphate buffer saline (PBS). Rho123 fluorescence was measured at excitation/ emission wavelengths of 500/535 nm using a Tecan Safire II™ spectrofluorometer (Tecan Crailsheim, Germany).

As regard CEM/ADR5000, the use of flow cytometer is the method of choice. Fluorescence measurement of individual cells was performed using a FACSCalibur™ (Becton-Dickinson) fluorescence-activated cell sorter (San Jose. CA) equipped with an ultraviolet argon laser (excitation at 488 nm, emission at 530/30 and 570/30 nm band-pass filters). Analysis was gated to include single viable cells on the basis of forward and side light-scatter and based on acquisition of data from 10000 cells. Log fluorescence was collected and displayed as single-parameter histograms.

To perform the test, CEM/ADR5000 cells (1 × 10^4 cells/ml density) were seeded in 24-multiwell plates (1 ml/well of a medium). The test substances were added in various final concentrations (10, 50, 100, 250 and 500 μM), and incubated for 2 h at 37 °C. Then the cells were washed twice with cold phosphate-buffered saline (PBS), treated with Rho123 (10 μg/ml final concentration)

and incubated for 2 h at 37 °C. Afterward, the cells were washed twice and resuspended in PBS for measurement.

The fluorescence intensity of treated cells was normalized by calculating the relative fluorescence intensity (inhibitory efficiency) percentage as follow:

$$\text{Inhibitory efficiency} = [(RFU_{substances}-RFU_{control})/(RFU_{verapamil}-RFU_{control})] \times 100$$

where $RFU_{substances}$ is the fluorescence in presence of test substance, $RFU_{verapamil}$ is the fluorescence in presence of verapamil, and $RFU_{control}$ is the fluorescence in the absence of the drug. Only values higher than 10% was considered significant.

All assays were repeated at least three times.

Statistical analysis

All data are expressed as mean ± standard error. The IC_{50} value (substances concentration required to produce a 50% inhibition of the ABC-transporter) was determined from dose-response curves. The dose-response curves were calculated with a four parameter logistic curve (Sigma Plot® 10.0), while the graphs were drawn using GraphPad Prism® software (GraphPad Prism® 4.0, GraphPad Software, Inc., CA, USA). The one-way analysis of the variance plus the Bonferroni's post test was used to analyze the differences among the sets of data. A p-value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

ANTIMUTAGENICITY STUDIES ON α-HEXYLCINNAMALDEHYDE (HCA)

RESULTS

When assessed in absence of S9, HCA was cytotoxic at the concentration of 90 μM in TA1535 and TA1537, and at the concentration of 9000 μM in TA98, TA100 and WP2uvrA strains (Table 5). In presence of S9, the cytotoxicity was highlighted at 90 μM in TA1535, at 900 μM in TA1537 and at 9000 μM in TA98, TA100 and WP2uvrA strains (Table 5).

Table 5. Cytotoxicity of α-hexylcinnamaldehyde (HCA) on *Salmonella typhimurium* TA1535, TA1537, TA98 and TA100, and on *Escherichia coli* WP2uvrA, in absence and presence of the metabolic activator S9. Values are expressed as means ± SEM ($n = 6$ plates).

Substance	μM	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2uvrA
Absence of S9-metabolic activator						
HCA	0 [a]	133.0 ± 2.6	41.0 ± 7.4	37.3 ± 4.5	84.0 ± 6.9	55.3 ± 5.2
	18	148.0 ± 28.0	40.5 ± 1.5	-	-	-
	90	51.3 ± 7.8 [t]	40.0 ± 6.3 [t]	35.2 ± 3.6	87.0 ± 3.0	45.5 ± 3.5
	180	40.5 ± 0.5 [t]	24.0 ± 4.0 [t]	38.7 ± 4.8	82.0 ± 8.3	46.7 ± 3.5
	900	42.5 ± 8.5 [t]	25.0 ± 1.9 [t]	36.0 ± 2.0	87.3 ± 4.7	53.3 ± 3.5
	1800	29.7 ± 5.4 [t]	18.0 ± 3.5 [t]	38.0 ± 5.1	93.0 ± 5.0	52.7 ± 4.0
	9000	[at]	[at]	26.3 ± 3.0 [t]	[at]	41.7 ± 7.2 [t]
Presence of S9-metabolic activator						
HCA	0	120.3 ± 5.2	120.0 ± 3.2	50.7 ± 1.3	116.0 ± 6.2	54.7 ± 5.8
	18	121.3 ± 6.9	121.0 ± 11.0	-	-	-
	90	66.5 ± 13.5 [t]	111.0 ± 3.3	52.5 ± 1.3	121.0 ± 3.3	53.0 ± 3.4
	180	30.0 ± 7.0 [t]	112.0 ± 6.0	53.3 ± 5.8	120.4 ± 4.2	52.0 ± 2.3
	900	88.0 ± 8.0 [t]	98.5 ± 9.5 [t]	41.3 ± 3.5	124.1 ± 2.3	62.7 ± 2.7
	1800	71.0 ± 7.0 [t]	74.5 ± 9.5 [t]	43.5 ± 5.4	116.2 ± 2.8	48.3 ± 4.7
	9000	[at]	[at]	32.0 ± 5.3 [t]	[at]	61.0 ± 14.1 [t]

[a] In absence of HCA, strains have been exposed to the vehicle DMSO (50 μl/plate). [t] Toxicity (evaluated as a statistically significant reduction in the number of revertant colonies or as a change in the auxotrophic background growth *vs.* vehicle). [at] Advanced toxicity. - not tested.

On the basis of these preliminary results, as a maximum concentration to test in the mutagenicity assay, we have chosen: 35 μM for TA1535 and TA1537 without S9, 35 and 70 μM for TA1535 and TA1537 respectively, with S9, and 3500 μM for TA98, TA100 and WP2uvrA both in the absence and presence of S9. In the mutagenicity assay, HCA did not increase the number of revertant colonies in all strains tested, both in the absence and presence of the metabolic activator S9 (Tables 6 and 7). Conversely, the mutagens 2NF, SA, 9AA, MMS, 2AA and BaP increased the number of revertant colonies (from 2 to 11 times) with respect to the vehicle (Tables 6 and 7), showing that the system was suitable to detect different mechanisms of mutagenicity (i.e. frameshift mutations, base substitutions, oxidative damages). Only TA1535 strain was not sensitive to the BaP-mutagenicity: no increase in the number of revertant colonies was highlighted up to the concentration of 762 μM, while cytotoxic effects were produced at higher concentrations (Table 7).

Table 6. Effect of α-hexylcinnamaldehyde (HCA) on the number of spontaneous revertant colonies of *Salmonella typhimurium* TA1535, TA1537, TA98 and TA100, and of *Escherichia coli* WP2*uvr*A, in absence of the metabolic activator S9. Values are expressed as means ± SEM (*n* = 6 plates).

Substance	μM	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
HCA	3.5	144.5 ± 3.5	40.3 ± 2.5	-	-	-
	7	136.0 ± 20.0	34.7 ± 5.8	-	-	-
	18	139.0 ± 8.2	38.5 ± 3.5	-	-	-
	25	129.0 ± 18.3	36.0 ± 6.3	-	-	-
	35	65.0 ± 7.5 [t]	41.3 ± 5.8 [t]	34.7 ± 0.9	79.3 ± 9.4	44.0 ± 4.0
	70	-	-	38.3 ± 5.6	87.0 ± 2.4	41.7 ± 2.3
	350	-	-	33.3 ± 2.3	86.0 ± 5.2	53.3 ± 8.1
	700	-	-	38.7 ± 2.4	86.7 ± 4.8	60.0 ± 6.9
	3500	-	-	37.3 ± 2.2	63.0 ± 2.0 [t]	49.5 ± 5.5
2-NF	3.5	-	-	117.3 ± 9.4 [**]	-	-
SA	6	1477.0 ± 65.3 [**]	-	-	197.7 ± 7.2 [**]	-
9-AA	100	-	173.0 ± 22.0 [**]	-	-	-
MMS	1746	-	-	-	-	132.7 ± 11.4 [**]
vehicle [a]		137.5 ± 3.3	39.8 ± 3.4	40.7 ± 2.5	86.5 ± 7.2	52.2 ± 2.2

[a] DMSO 50 μl/plate. [**] $p < 0.01$ *vs.* vehicle. - not tested

Table 7. Effect of α-hexylcinnamaldehyde (HCA) on the number of spontaneous revertant colonies of *S. typhimurium* TA1535, TA1537, TA98 and TA100, and of *E. coli* WP2*uvr*A, in presence of the metabolic activator S9. Values are expressed as means ± SEM (n = 6 plates).

Substance	µM	Number of revertant colonies				
		TA1535	TA1537	TA98	TA100	WP2*uvr*A
HCA	3.5	111.0 ± 8.0	117.0 ± 9.0	-	-	-
	7	116.3 ± 5.2	110.5 ± 5.5	-	-	-
	18	121.3 ± 6.9	126.0 ± 8.5	-	-	-
	25	123.2 ± 7.3	119.0 ± 5.3			
	35	120.3 ± 7.7	114.0 ± 6.0	53.3 ± 7.4	107.3 ± 8.7	50.7 ± 1.3
	70	-	116.0 ± 5.3	42.5 ± 1.3	116.0 ± 6.3	51.2 ± 4.4
	350	-	-	48.5 ± 3.8	115.0 ± 5.3	42.7 ± 3.5
	700	-	-	45.1 ± 2.7	120.6 ± 3.2	58.3 ± 6.1
	3500	-	-	39.6 ± 1.3	108.0 ± 2.0[t]	55.7 ± 3.5
2-AA	2	-	-	170.7 ± 15.7[**]	250.7 ± 10.2[**]	-
	20	804.0 ± 20.0[**]	446.0 ± 26.0[**]	-		198.7 ± 21.8[**]
BaP	76	126.9 ± 1.9	-	430 ± 11.0[**]	693 ± 49.6[**]	132.5 ± 6.0[**]
	152	116.1 ± 2.7	354.0 ± 42.0[**]	-	-	-
	762	135.0 ± 2.2	-	-	-	-
Vehicle [a]		117.9 ± 8.6	113.3 ± 6.2	43.7 ± 3.3	128.0 ± 4.7	51.9 ± 2.8

[a] DMSO 50 µl/plate. [**]$p < 0.01$ *vs.* vehicle. - not tested.

The same concentrations, at which neither cytotoxic nor mutagenic effects were registered, were assayed for the antimutagenicity (i.e. the ability of the test substance to reduce the number of revertant colonies induced by known mutagens), in the strains TA100, WP2*uvr*A, TA98, TA98NR and TA98 1,8-DNP, by using three different experimental protocols: pre-treatment, co-treatment and post-treatment. HCA did not significantly affect the mutagenicity of SA in TA100 strain in the three protocols: a very weak inhibition (12.5% at the highest concentration of 900 µM) was registered only in the post-treatment (Table 8). A weak even if statistically significant inhibition was registered against 2AA reaching, at highest concentration of 900 µM, the maximum value of 24.2% and 24.9% in the co- and post-treatment respectively. Conversely, in the pre-treatment protocol HCA did not significantly affect the mutagenicity of 2AA (Table 8). The test substance also produced a null or weak inhibition of the MMS- and 2AA-mutagenicity in WP2*uvr*A strain in all protocols.

Only in the post-treatment, HCA produced a moderate (25% < value < 40%) inhibition of MMS-mutagenicity (34.8% maximum inhibition at highest concentration of 900 µM) (Table 9).

Table 8. Effect of HCA on the number of revertant colonies in *Salmonella typhimurium* TA100 both with and without metabolic activator (S9). Means ± SEM ($n = 4$ plates).

Substance	[μM]	Number of revertant colonies					
		PRE-TREATMENT		CO-TREATMENT		POST-TREATMENT	
		-S9	+S9	-S9	+S9	-S9	+S9
HCA	9	1877 ± 123.1	465.3 ± 14.8	1519 ± 59.0	449.3 ± 5.3	1888 ± 64.7	637.3 ± 4.8
	18	1861 ± 114.7	453.3 ± 9.6	1501 ± 29.7	446.0 ± 14.5	1867 ± 10.7	530.7 ± 7.1***
	90	1801 ± 89.7	454.7 ± 65.1	1477 ± 98.8	408.5 ± 22.8	1803 ± 28.2	530 ± 10.4***
	180	1797 ± 96.3	384.0 ± 8.3	1457 ± 42.3	404.0 ± 22.3	1771 ± 34.8	520.0 ± 4.6***
	900	1652 ± 6.9	365.3 ± 11.6	1451 ± 27.0	347.0 ± 39.1*	1671 ± 34.8*	481.3 ± 5.8***
Vehicle[a]		178.7 ± 29.1	30.7 ± 4.4	230.0 ± 34.9	51.83 ± 10.81	197.3 ± 2.7	36.0 ± 3.1
Mutagen		1968 ± 46.9[b]	481.3 ± 5.3[c]	1588 ± 55.6[b]	457.9 ± 16.7[c]	1909 ± 29.7[b]	642.7 ± 2.7[c]

[a] DMSO 50 μl; [b]sodium azide (30 μM); [c]2-aminoanthracene (10 μM). * $p < 0.05$ and *** $p < 0.001$ *vs.* mutagen (Anova + Dunnett's Multiple Comparison Post Test).

Table 9. Effect of HCA on the number of revertant colonies in *Escherichia coli* WP2*uvrA* both with and without metabolic activator (S9). Means ± SEM (*n* = 4 plates).

Substance	[µM]	Number of revertant colonies					
		PRE-TREATMENT		CO-TREATMENT		POST-TREATMENT	
		-S9	+S9	-S9	+S9	-S9	+S9
HCA	9	458.7 ± 66.4	170.7 ± 7.7	484.0 ± 26.6	336.8 ± 9.9	528.0 ± 6.1**	294.7 ± 5.8
	18	451.3 ± 27.7	166.7 ± 17.5	485.3 ± 8.1	330.7 ± 7.09	524.0 ± 14.4**	292.0 ± 6.9
	90	436.0 ± 26.6	152.3 ± 13.2	466.7 ± 11.6	334.3 ± 19.8	517.3 ± 7.0**	308.7 ± 20.8
	180	376.0 ± 24.4	147.0 ± 12.1	448.0 ± 29.5	318.7 ± 4.9	488.0 ± 24.4**	302.7 ± 5.8
	900	365.3 ± 11.6	141.3 ± 1.8	402.7 ± 2.7	293.5 ± 2.8**	474.0 ± 30.1**	304.3 ± 8.0
Vehicle[a]		53.33 ± 6.7	62.7 ± 1.3	61.33 ± 5.2	126.7 ± 23.4	54.7 ± 12.7	72.00 ± 2.3
Mutagen		466.7 ± 40.4[b]	178.7 ± 11.8[c]	485.3 ± 17.5[b]	366.0 ± 10.3[c]	726.7 ± 12.7[b]	308.0 ± 4.0[c]

[a] DMSO 50 µl; [b]methyl methane sulfonate (5826 µM); [c]2-aminoanthracene (49 µM). ** $p < 0.01$ *vs.* mutagen (Anova + Dunnett's Multiple Comparison Post Test).

HCA showed to produce a statistically significant and concentration-dependent reduction in the number of TA98-revertant colonies induced by 2AA: the inhibition was weak (< 25%) in the pre- and post-treatments, while strong in the co-treatment, reaching the maximum value of 61.9% at the highest concentration (Figure 20).
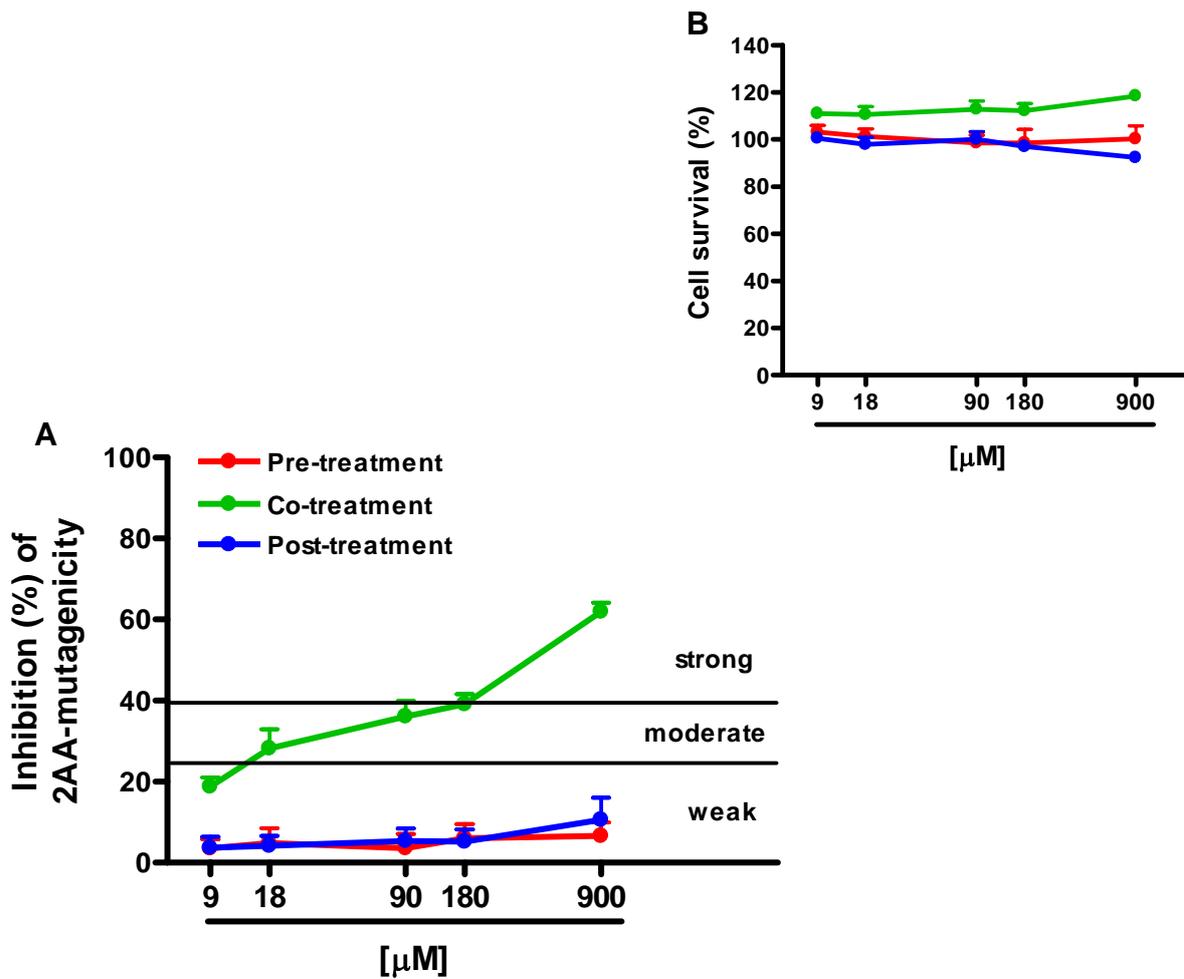
Figure 20. Inhibition (%) by HCA of the 2-aminoanthracene-induced mutagenicity in TA98 strain. Mutagen: 2AA (2-aminoanthracene). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

HCA also reduced, in statistically significant (p < 0.01 or p < 0.001) and concentration-dependent manner, the number of revertant colonies induced by 2NF in TA98 strain (Figure 21). At the highest concentration, the inhibition values were 64.6%, 72.6% and 63.1 in the pre, co- and post-treatment respectively. According to Negi et al. (2003), the antimutagenicity was considered strong (Figure 21).
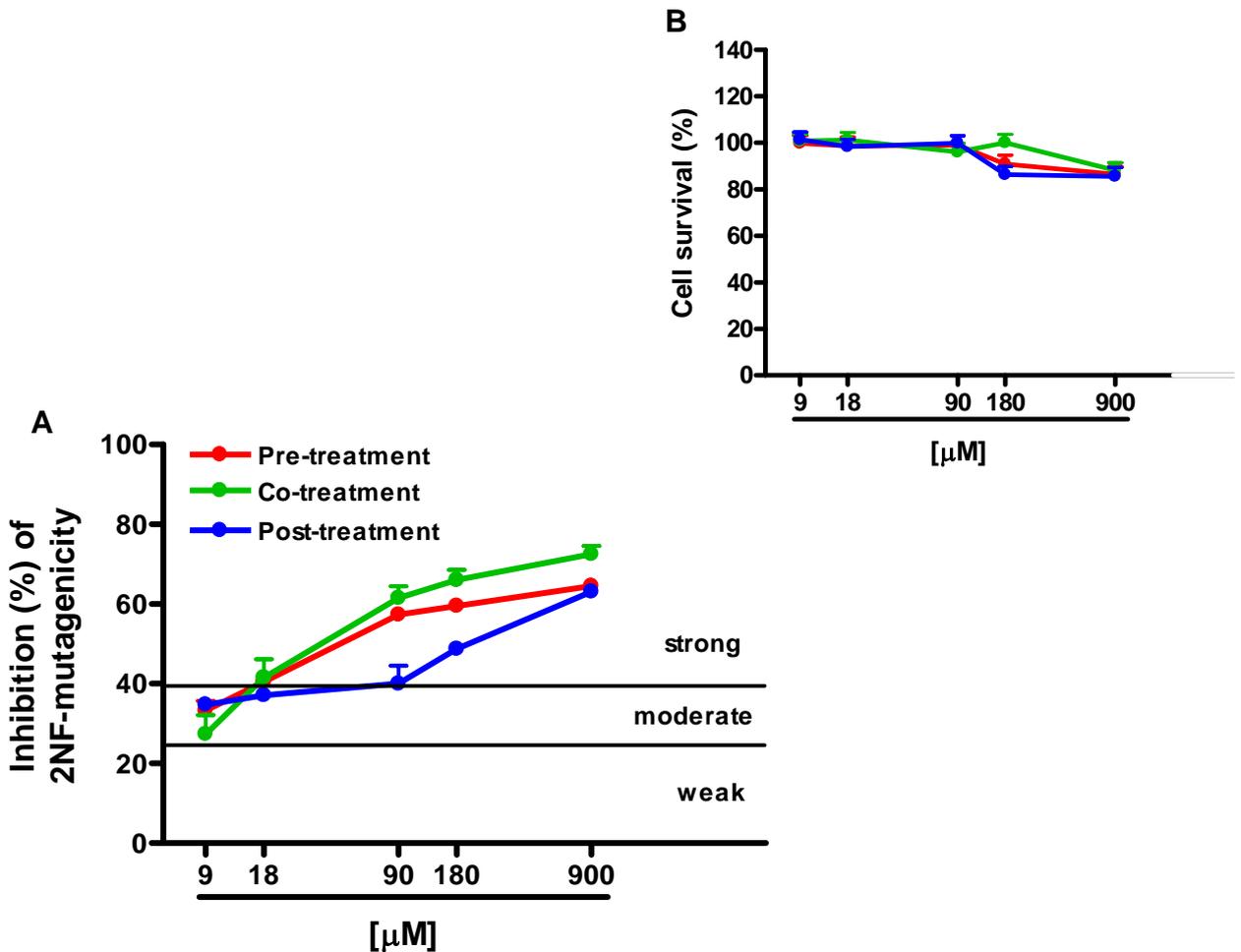
Figure 21. Inhibition (%) by HCA of the 2-nitrofluorene-induced mutagenicity in TA98 strain. Mutagen: 2NF (2-nitrofluorene). The effect was reported in absence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

Conversely, the substance produced a null or weak inhibition of 4NQO-mutagenicity in all treatments: a statistically significant but weak inhibition was registered in the pre-treatment, reaching the maximum value of 20.6% (Figure 22).
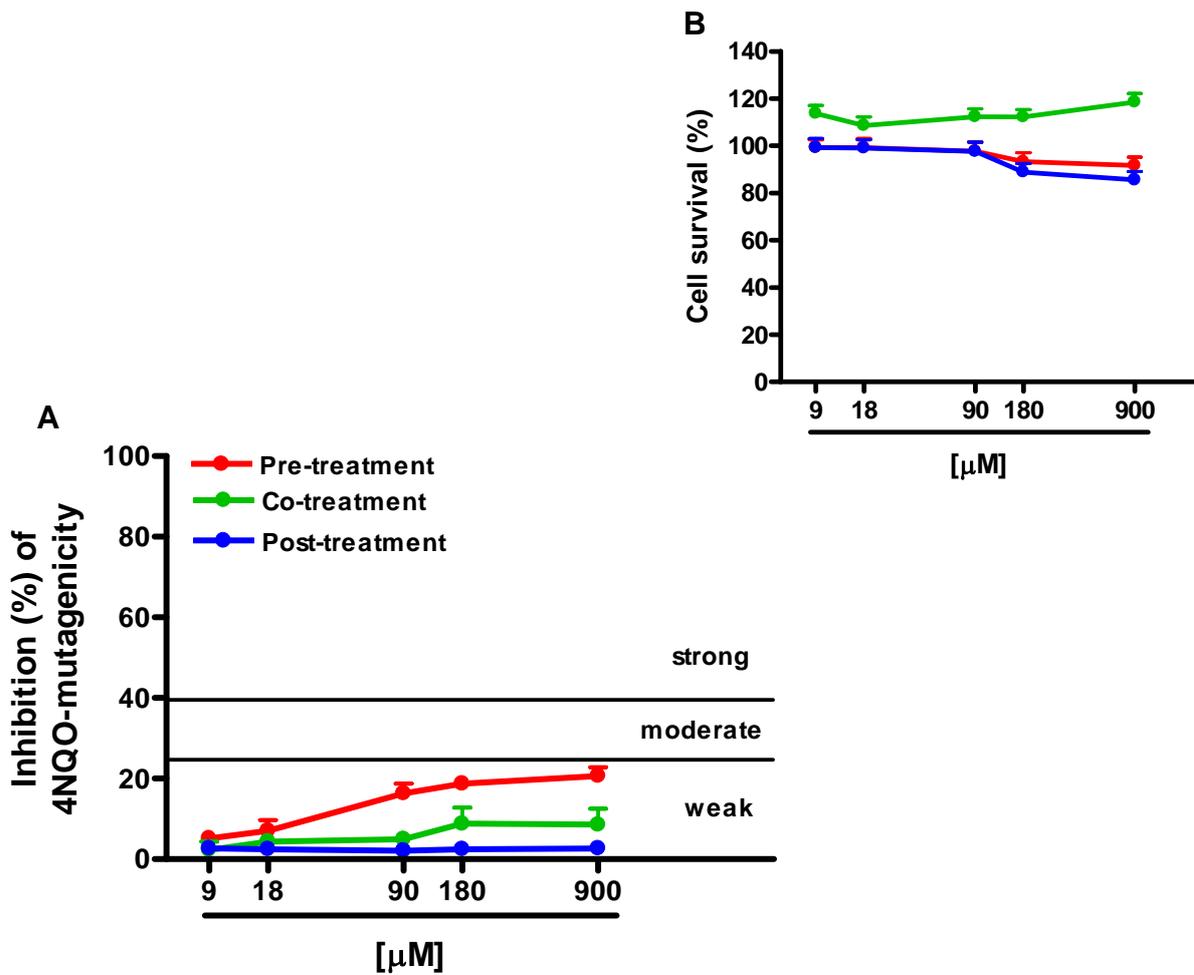
Figure 22. Inhibition (%) by HCA of the 4-nitroquinoline *N*-oxide-induced mutagenicity in TA98 strain. Mutagen: 4NQO (4-nitroquinoline N-oxide). The effect was reported in absence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

A statistically significant reduction of revertant colonies (p < 0.001) was registered against 1-nitropyrene (1NP) in TA98 strain (Figure 23). The inhibitory effect was concentration-dependent, reaching, at the highest concentration of 900 μM, the maximum value 57.98%, 82.06% and 65.76% in the pre-, co- and post-treatment, respectively.
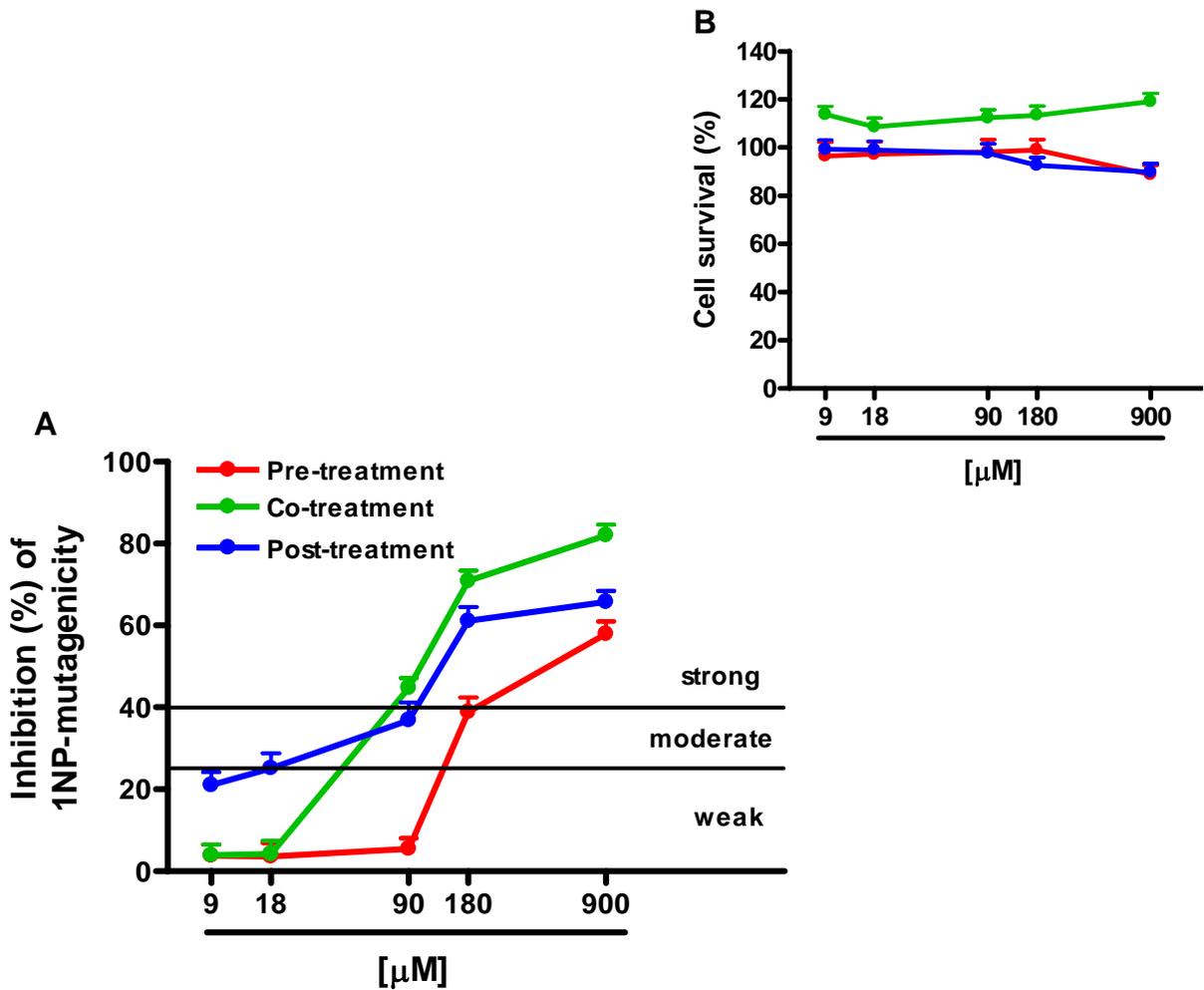
Figure 23. Inhibition (%) by HCA against the 1-nitropyrene-induced mutagenicity in TA98 strain. Mutagen: 1NP (1-nitropyrene). The effect was reported in absence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

The treatment with HCA also significantly reduced the number of TA98-revertant colonies induced by 1,8-dinitropyrene (1,8-DNP) (Figure 24). The effect was moderate in the pre- and post-treatments, reaching the maximum inhibition of 27.96% and 37.35%, at highest concentration of 900 µM, respectively; while, a strong inhibition (50.7%) was produced in the co-treatment, at 900 µM.
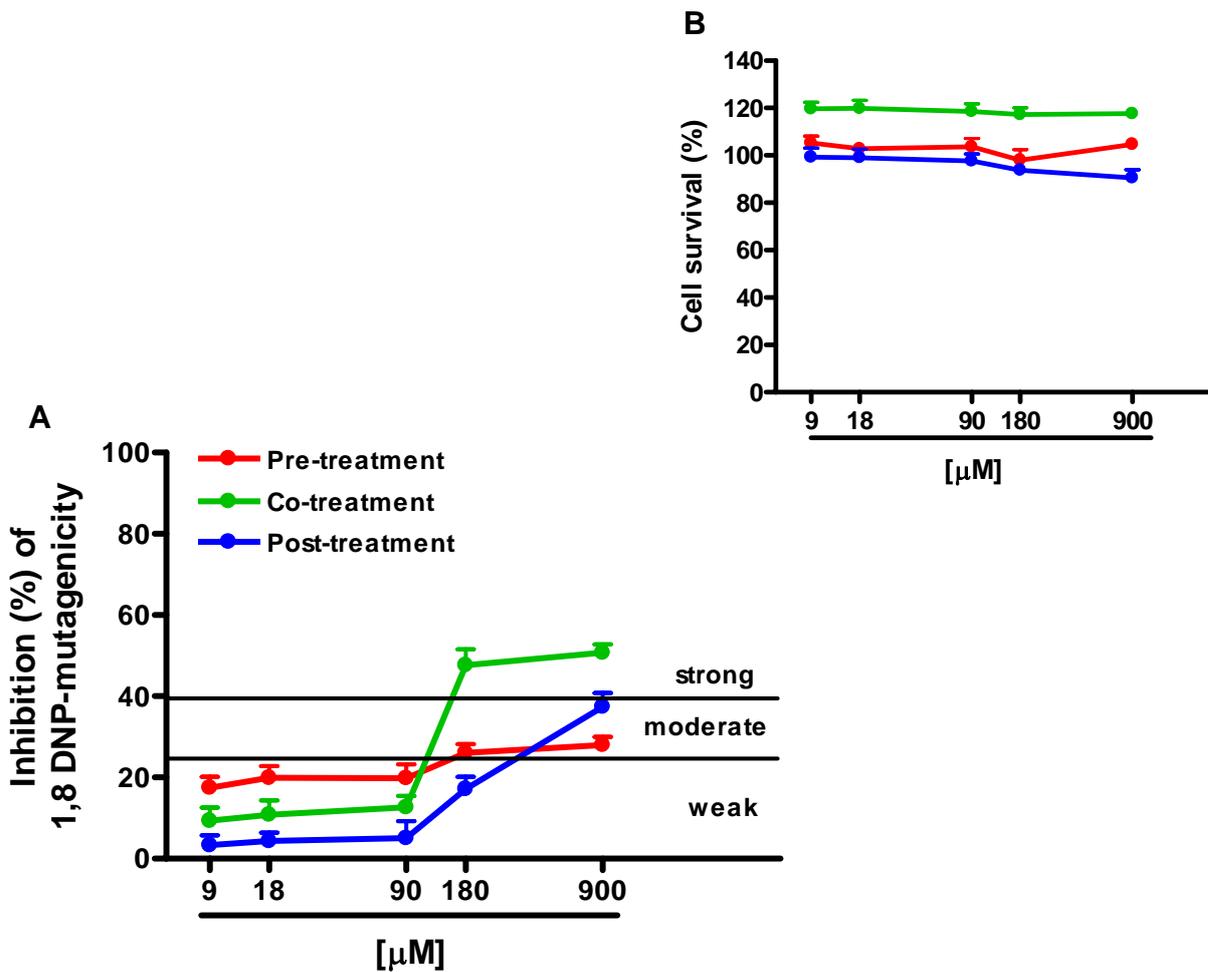
Figure 24. Inhibition (%) by HCA of the 1,8-Dinitropyrene-induced mutagenicity in TA98 strain. Mutagen: 1,8-DNP (1,8-Dinitropyrene). The effect was reported in absence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

In our experiments, we also included TA98NR and 1,8-DNP strains, in order to better define the mechanisms (particularly the role of the mutagen-bioactivation mediated by nitroreductase and O-acetyltransferase enzymes) involved in the strong antimutagenicity observed in TA98. In TA98NR, HCA produced a statistically significant ($p < 0.001$) inhibition of 1NP-mutagenicity in absence of S9. The maximum effect was registered at the highest concentration of 900 μM, reaching the values of 46.9%, 85.6% and 42.1% in the pre-, co- and post-treatment protocols, respectively (Figure 25): so the antimutagenicity was considered strong.
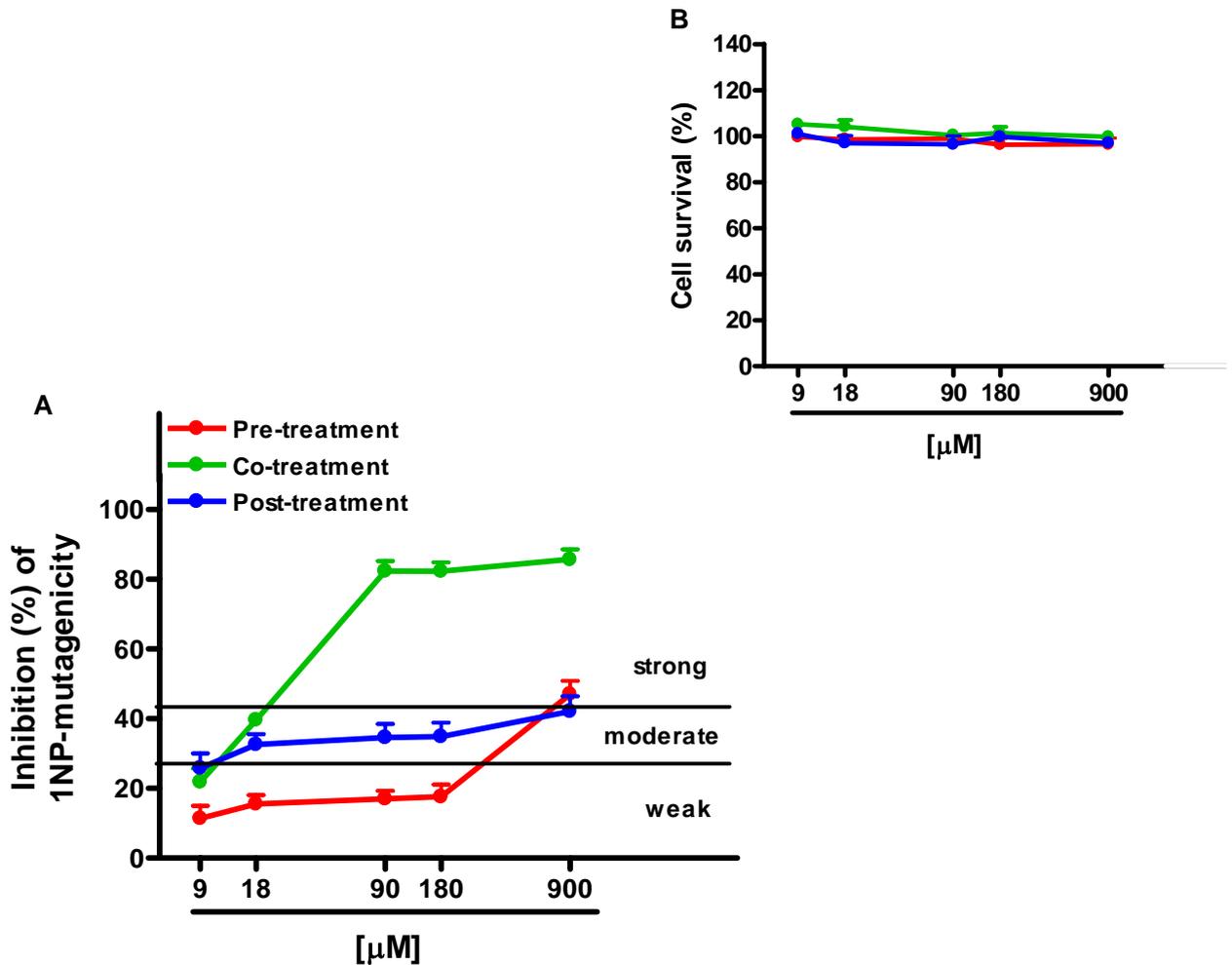
Figure 25. Inhibition (%) by HCA of the 1-Nitropyrene-induced mutagenicity in TA98NR strain. Mutagen: 1-NP (1-Nitropyrene). The effect was reported in absence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

The 1NP-mutagenicity was also significantly inhibited in TA98 1,8-DNP strain. The maximum effect was registered at the highest concentration of 900 µM, resulting moderate (31.3%) in the co-treatment and strong (86.3%) in the post-treatment (Figure 26). In the pre-treatment the inhibition was almost null.

In the cell survival experiments, none of the concentrations tested was cytotoxic in the presence of mutagens, being the viability, with respect to the control, from 85% to 120% for TA98, from 96% to 105% for TA98NR and from 97% to 116% for TA98 1,8-DNP (Figures 20, 21, 22, 23, 24, 25 and 26).
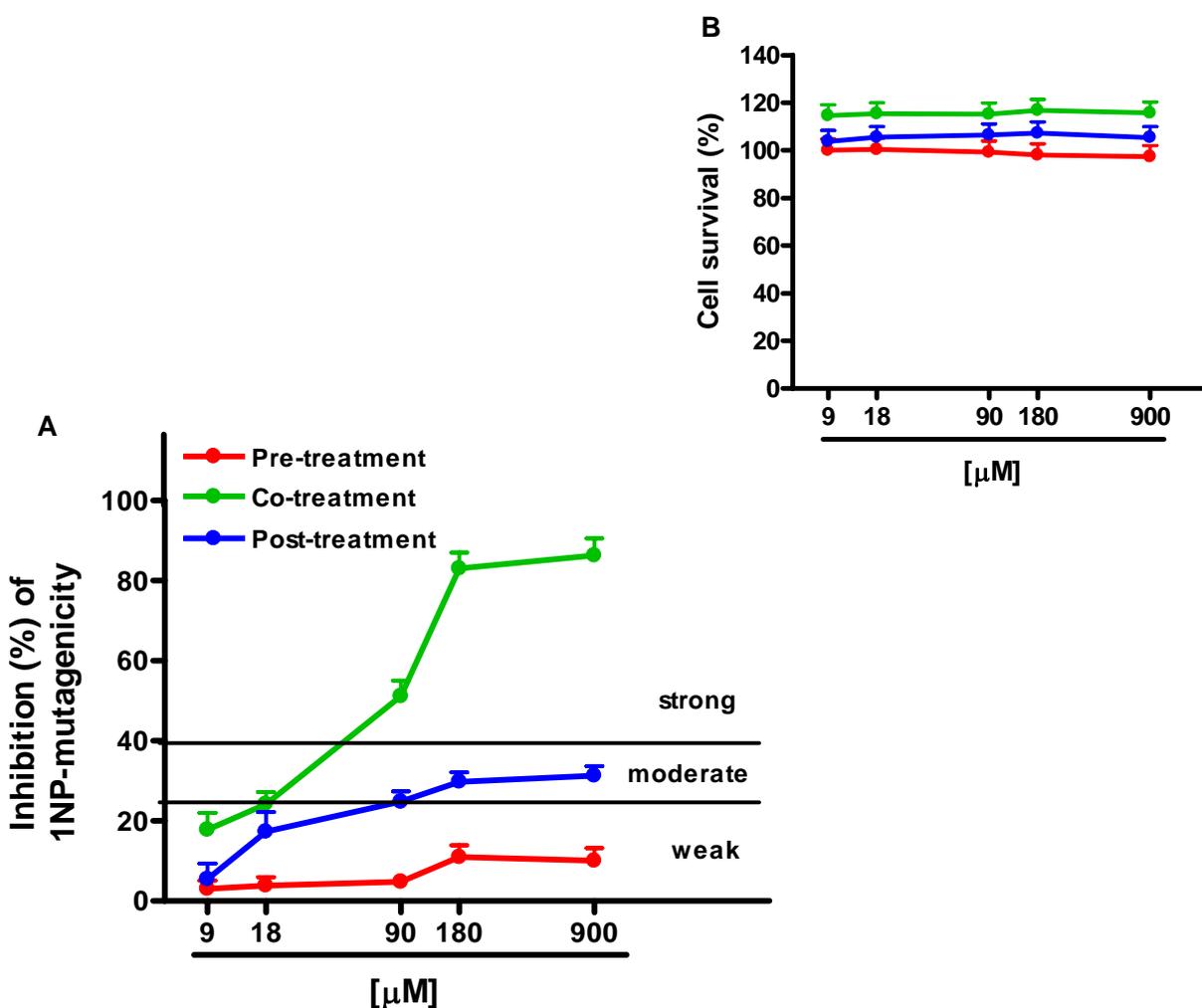
Figure 26. Inhibition (%) by HCA of the 1-Nitropyrene-induced mutagenicity in TA98 1,8-DNP strain. Mutagen: 1-NP (1-Nitropyrene). The effect was reported in absence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

DISCUSSION

α-Hexylcinnamaldehyde is a compound widely used as additive in medical and consumer products, in foods, beverages, and sweetmeat. Chemically, it is a synthetic aldehyde, characterized by the presence of a carbonyl group containing a polarized carbon–oxygen double bond. The marked difference in the electronegativity between the oxygen and the carbon atoms makes this group able to react with electron-rich biological macromolecules (i.e. DNA and proteins), and to induce adverse health effects, including general toxicity, allergenic reactions, mutagenicity, and carcinogenicity (Feron et al., 1991; Patlewicz et al., 2002; Garaycoechea et al., 2012).

HCA is potentially more reactive than a simple aldehyde, as it possesses a double bond between carbons 2 and 3 (α and β respectively). The conjugation of the unsaturated function with the carbonyl group makes the α-carbon positively polarized and consequently the preferred site for a nucleophilic attack (Feron et al., 1991). Because of its potentially reactive α,β-unsaturated structure, HCA has been listed on the EFSA CEF Panel, in order to collect additional genotoxicity data for safety assessment (EFSA, 2008). Several aldehydes are found to be released as pollutants in the environment during combustion process, such as car exhausts, tobacco smoke, and flue gases, so increasing the carcinogenic hazard for humans (Seaman et al., 2007; Abraham et al., 2011; Kabir and Kim, 2011). For instance, acetaldehyde, a typical indoor air pollutant, and crotonaldehyde, a component of the cigarette smoke, are potential genotoxic carcinogens (Stein et al., 2006), while the flavouring agent isobutyraldehyde resulted inactive as carcinogen (Benigni et al., 2005). Among α,β-unsaturated aldehydes, although cinnamaldehyde and citral were found to be not cancerogenic (Benigni et al., 2005), acrolein and its alkyl derivatives, p-nitro cinnalmaldehyde and o-methoxycinnamaldehyde are classified as potentially genotoxic/mutagenic compounds (Eder et al., 1991; Eder and Deininger, 2001; Adams et al., 2004). Due to the widespread and often involuntary human exposure to HCA, it is very important to verify that when used as intended, this aldehyde does not represent a health hazard for humans and environment. In present study, the potential genotoxic effects of this aldehyde have been evaluated by the bacterial reverse mutation assay to study point mutations in bacteria. Moreover, including TA100 bacterial strain increases the sensitivity of the test to the aldehydes mutagenicity (Dillon et al., 1998).

Present results show that HCA does not induce genotoxic effects in *S. typhimurium* TA1535, TA1537, TA98 and TA100 strains and in *E. coli* WP2*uvr*A strain, also in presence of the exogenous metabolic activation system, suggesting that no genotoxic derivatives were produced by the CYP450-mediated biotransformations. The presence of the metabolic activator reduced in

some cases the toxicity of the tested substances: a partial detoxification of the aldehydes by reaction with the nucleophilic components of the S9 could occur. To our knowledge no evaluation of the potential genotoxic effects has been carried out for HCA up to now; thus our results can contribute to assess the risk of exposition to this aldehyde and its safety profile.

HCA also exhibited interesting antimutagenic properties. Antimutagens are defined as chemicals, both natural and synthetic agents, able to block the effects of mutagens, acting in the extracellular or intracellular compartment, by physical, chemical or enzymatically catalysed reactions with the mutagen or by stimulating the DNA repair systems (De Flora, 1998). The antimutagens, which induce extra- or intracellular inhibiting interferences with the mutagen, are defined as desmutagenic agents (Shammon and Pezzato, 1994), while those acting in intracellular compartment as bioantimutagens (Shammon and Pezzato, 1994). Under our experimental conditions, test substance strongly inhibited the mutagenic effect of 2NF and 1NP in all experimental protocols and of 1,8-DNP particularly in the post-treatment. In addition, a singular antimutagenicity was highlighted only in TA98 strain and in the co-treatment.

2NF is a direct-acting mutagen for *Salmonella* strains; it is indeed metabolized by the cytoplasmic bacterial nitroreductases to electrophilic intermediates which form adducts with cellular DNA, and induce mutations of frameshift type (Rosenkranz and Mermelstein, 1983).

HCA could protect against the mutation 2NF-induced by chemical deactivation of the mutagen or by inhibiting the nitroreductases and/or O-tranferases enzymatic activation of the mutagen (Horn and Ferrao Vargas, 2003), and thus blocking the DNA-damage. Also, HCA could interfer with the frameshift mutations at GC sequence level (DeMarini, 2000). Alternatively, HCA, being antimutagen also in the post-treatment protocol when the mutation has already occurred, could act as bioantimutagens: it might stimulate the DNA repair systems, so interfering with the fixation process of mutation; this hypothesis seems to be unlikely because TA98 is a plasmid-containing strain with an enhanced error-prone DNA repair.

Noteworthy, in *S. typhimurium* strains employed in the Ames test, nitroreductases play an important role in the metabolic activation of several nitrocompounds (McCoy et al., 1981). Two nitroreductases are identified in *S. typhimurium*: s*nrA* and *cnr*. They belong to the type of oxygen-insensitive NRs involved in nitrocompound bioactivation into mutagens (Koder et al., 2002). The strains employed in the Ames test are not identical in relation to the presence of *snrA* and *cnr*. Moreover, it has been proposed that *snrA* and *cnr* might exhibit different activity towards nitro-substituted compounds (McCoy et al., 1981). Yamada et al. (1997) showed that *cnr* nitroreductase was the major enzyme involved, although *snrA* activated almost all the compounds tested. Accordingly, the mutagenicity of a given chemical may differ in the various

Ames tester strains, these being not isogenic in relation to the presence of *snrA* and *cnr* genes. Even more, Porwollik et al. (2001), showed that *hisD3052* (TA1538 and TA98) strains possessed a *snrA* gene deletion. Thus, the 1,8-DNP mutagenicity observed in our experiments was due probably by *O*-acetyltransferase enzymes-mediated activation. As regard 1NP, Salamanca-Pinzón et al. (2010) demonstred that it was more efficiently activated by *cnr* nitroreductases. Furthermore, results with O-acetyltransferase-overproducing and O-acetyltransferase-deficient strains (Watanabe et al., 1990; Espinosa-Aguirre et al., 1999) indicate that 1-NP also undergo O-acetylation facilitating the formation of a DNA-reactive intermediate.

In this context, in order to better define the involvemnent of the enzymatic inhibition (particularly, the cytoplasmic enzymes nitroreductases and *O*-acetyltransferases) in the HCA-antimutagenicity, the substance was also tested in the isogenic TA98NR and TA98 1,8-DNP strains against the mutagen 1NP. HCA was able to strongly inhibit the 1-nitropyrene-mutagenicity in both strains and in almost all treatments. However, the strongest antimutagenic activity of HCA was observed in the co-treatment protocol for both strains. According to the results obtained in TA98NR (which lacks the "classical" nitroreductase) and TA98 1,8-DNP (which lacks O-acetyltransferase) strains, we can suppose that HCA protect against the mutagenicity of 1NP mainly by inhibiting both nitroreductase and *O*-acetyltranferase enzymes, but also by chemical reaction with the mutagen. Furthermore, HCA could exert its antimutagenic effects by alterating the bacterial membrane permeability (Di Sotto et al., unpublished data).

At present, further experiments are necessary to explain the mechanisms involved in the antimutagenicity of HCA. Taking into account that it has been little investigated and no evaluation of the potential effects on DNA have been carried out up to now, our results obtained by the bacterial reverse mutation assay can be considered as a starting point for evaluating the protective properties of this substance.

ANTIMUTAGENIC ACTIVITY OF β-CARYOPHYLLENE (CRY) AND β-CARYOPHYLLENE OXIDE (CRYO) AGAINST THE MUTAGENICITY OF THE CIGARETTE SMOKE CONDENSED (CSC)

RESULTS

In line with previuos published study (Di Sotto et al., 2008), the substances was assayed in TA98, TA100, WP2uvrA and WP2uvrA/pKM101 strains, in presence of S9, by three different experimental protocols (pre-, co- and post-treatment). In preliminary studies, at 700 μg/plate (265 μg/ml), the CSC-mutagenicity was submaximal (about 70% mutagenicity), so this concentration was used for the antimutagenicity test. β-caryophyllene was able to reduce, in statistically significant (p < 0.01 *vs.* mutagen) and concentration dependent manner, the number of CSC-induced revertant colonies in TA98 strain in all treatments (Figure 27).
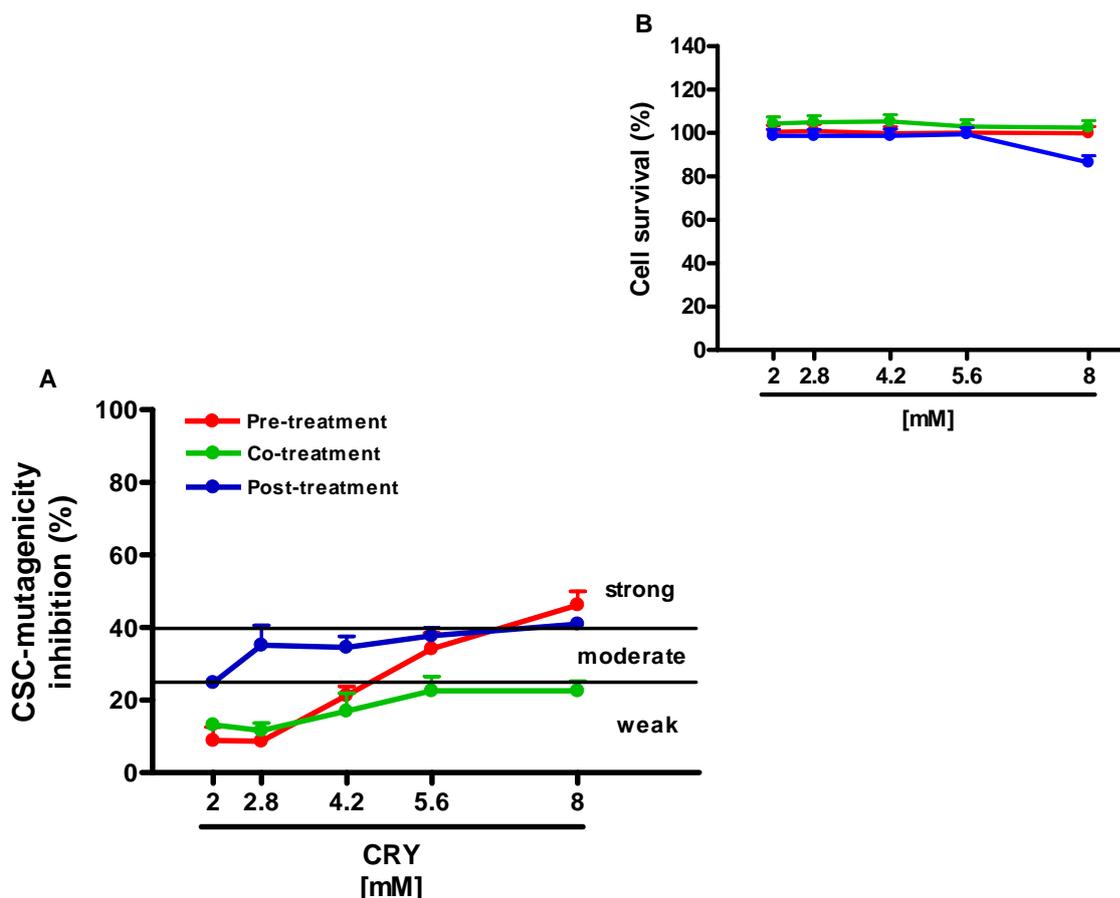

Figure 27. Inhibition (%) by CRY of the CSC-induced mutagenicity in TA98 strain. Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

According to Negi et al. (2003), the effect was strong in pre- and post-treatment (maximum inhibition of 46.2% and 40.9% at the highest concentration of 8 mM), while weak (22.5% maximum inhibition) in co-treatment.

The substance also significantly inhibited the CSC-mutagenicity in TA100 strain; the effect was particularly strong in the post-treatment, being the inhibition between 47.09% and 65.96% in the range of concentrations tested; the antimutagenicity was also strong, at the highest concentration of 8 mM, in the pre- and co-treatment, reaching the inhibition values of 46.2% and 46.4%, respectively (Figure 28).
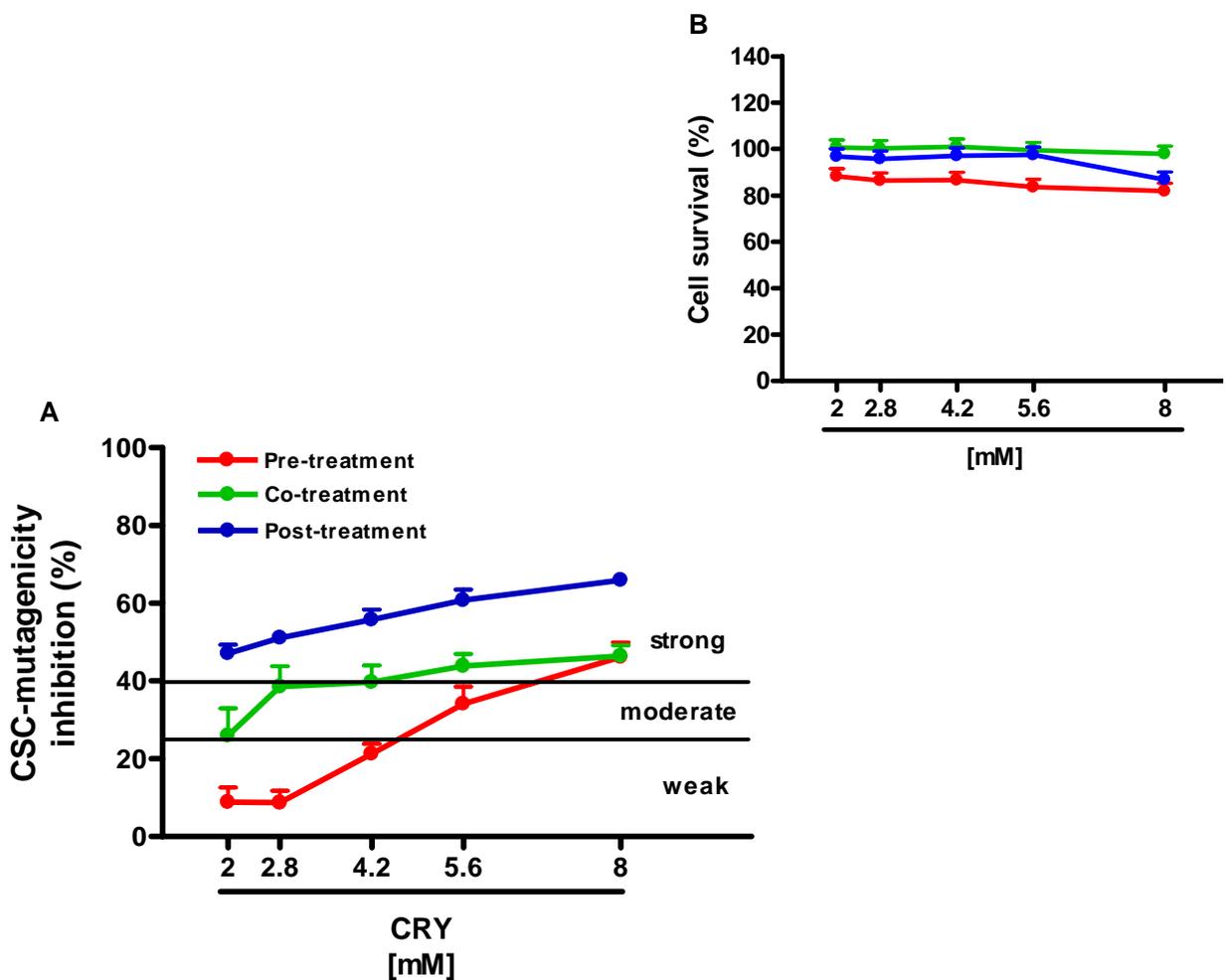

Figure 28. Inhibition (%) by CRY of the CSC-induced mutagenicity in TA100 strain. Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

A statistically significant (p < 0.05 or p < 0.01 vs. mutagen) inhibition of CSC-mutagenicity was also registered in WP2*uvr*A: the effect was strong in all experimental conditions, reaching, at the highest concentration of 8 mM, the inhibition values of 40.9%, 48.0% and 42.2% in the pre-, co- and post-treatment, respectively (Figure 29).
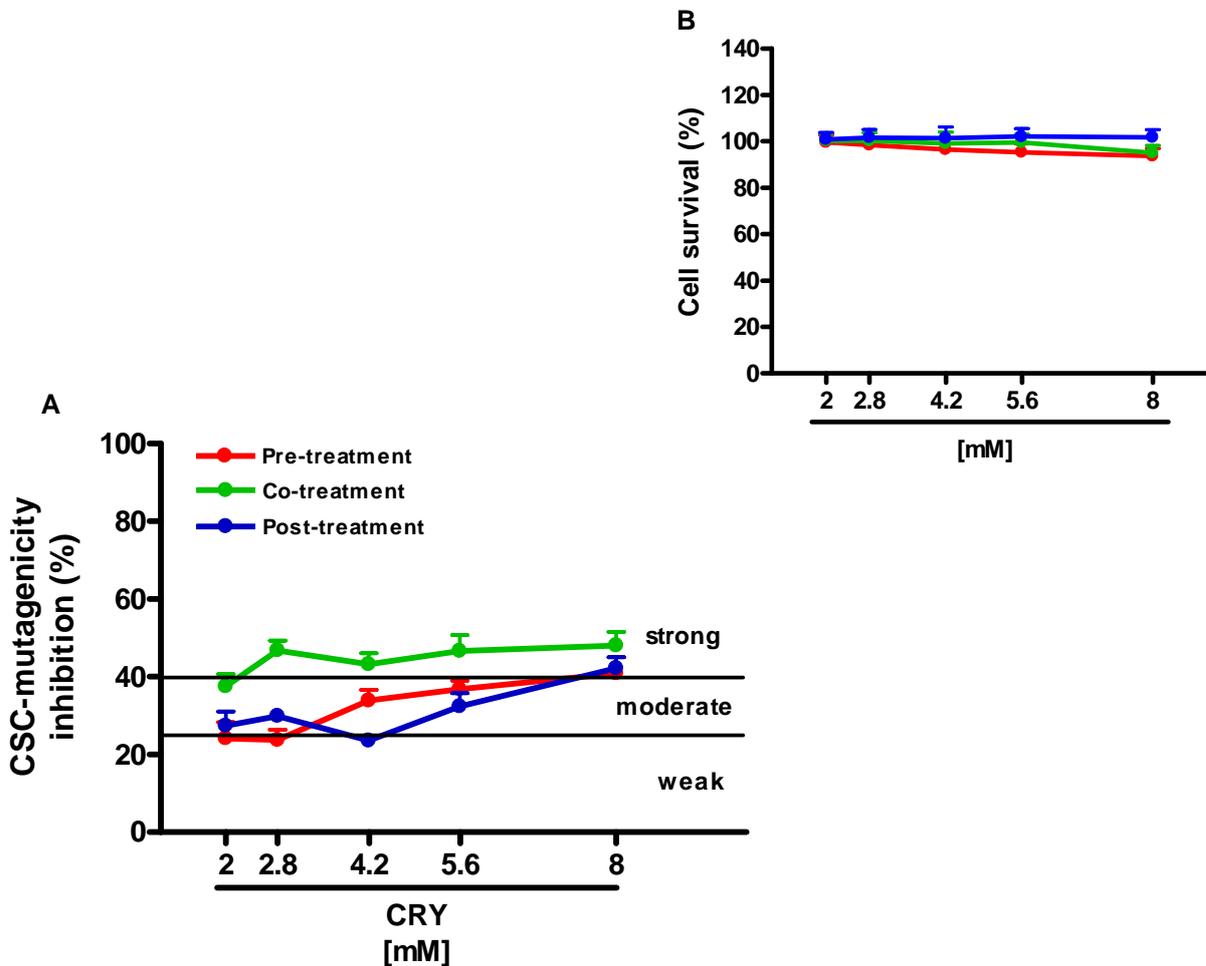

Figure 29. Inhibition (%) by CRY of the CSC-induced mutagenicity in WP2uvrA strain. Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

Finally, β-caryophyllene significantly inhibited the CSC-mutagenicity in WP2*uvr*A/R strain, (Figure 30): the antimutagenic effect was strong in all experimental conditions, reaching, at the highest concentration of 8 mM, the inhibition values of 58.9%, 57.9% and 52.1% in the pre-, co- and post-treatment respectively.

In cell survival experiments, none of the concentrations tested were cytotoxic in the presence of mutagens, being the viability, with respect to control, from 86% to 104% for TA98, from 82% to 101% for TA100, from 94% to 102% for WP2*uvr*A, and from 96% to 104% for WP2*uvr*A/R (Figures 27, 28, 29 and 30).
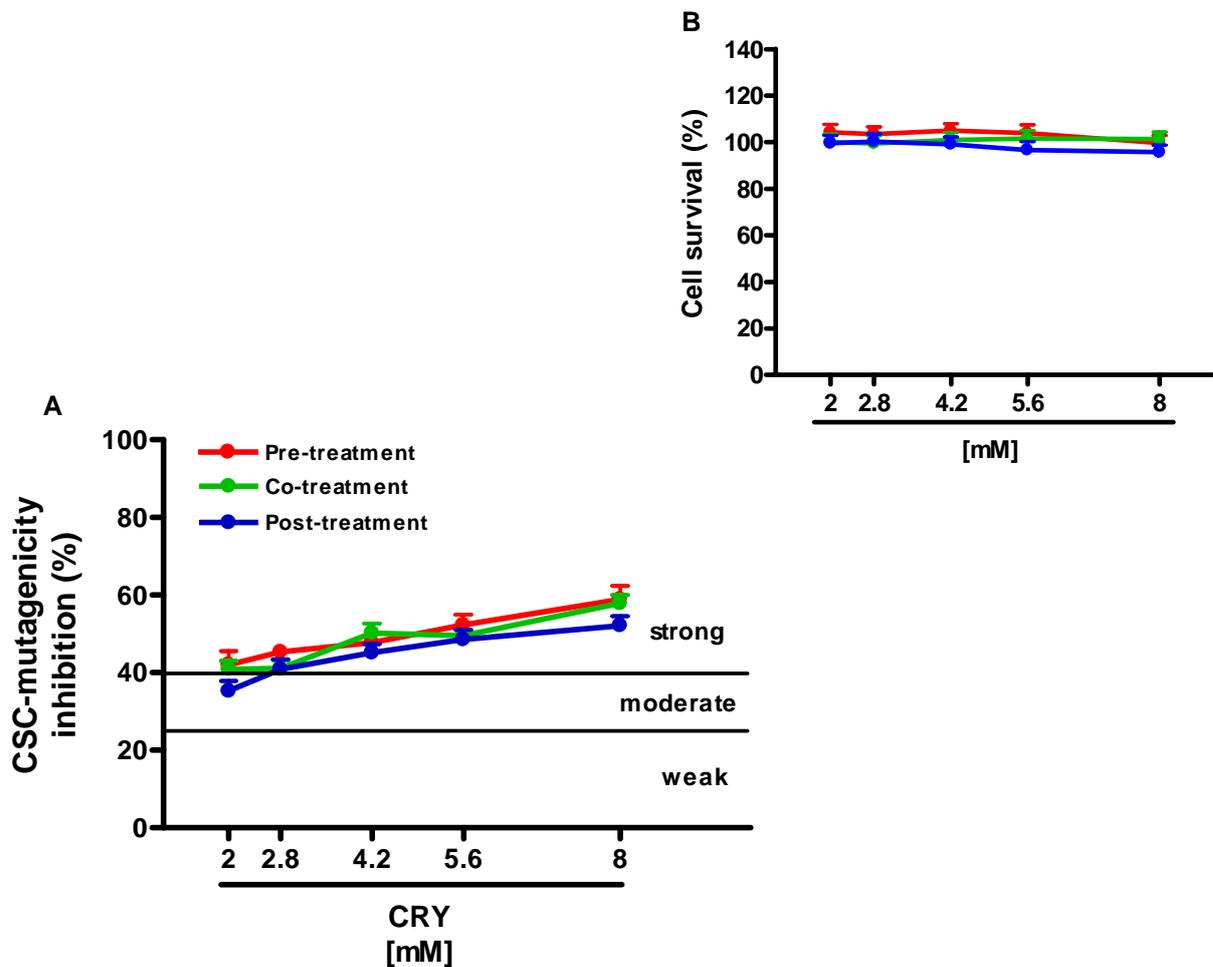
Figure 30. Inhibition (%) by CRY of the CSC-induced mutagenicity in WP2uvrA/R strain.
Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

Also the ability of β-caryophyllene oxide (CRYO) to inhibit the mutagenicity of the condensed smoke from standard 3R4F cigarettes (CSC) in TA98, TA100, WP2*uvr*A and WP2*uvr*A/R strains, in the presence of the S9-metaboli activator and by using three different experimental protocols (pre-treatment, co-treatment and post-treatment), was evaluated.

CRYO was able to reduce, in statistically significant and concentration dependent manner, the number of CSC-induced revertant colonies in TA98 strain in all treatments (Figure 31). According to Negi et al. (2003), the effect was strong in the post-treatment (maximum inhibition of 49.0% at the highest concentration of 1.5 mM), while moderate in the pre- and co-treatments (at the highest concentration of 1.5 mM, 34.4% and 25.6% maximum inhibition respectively).
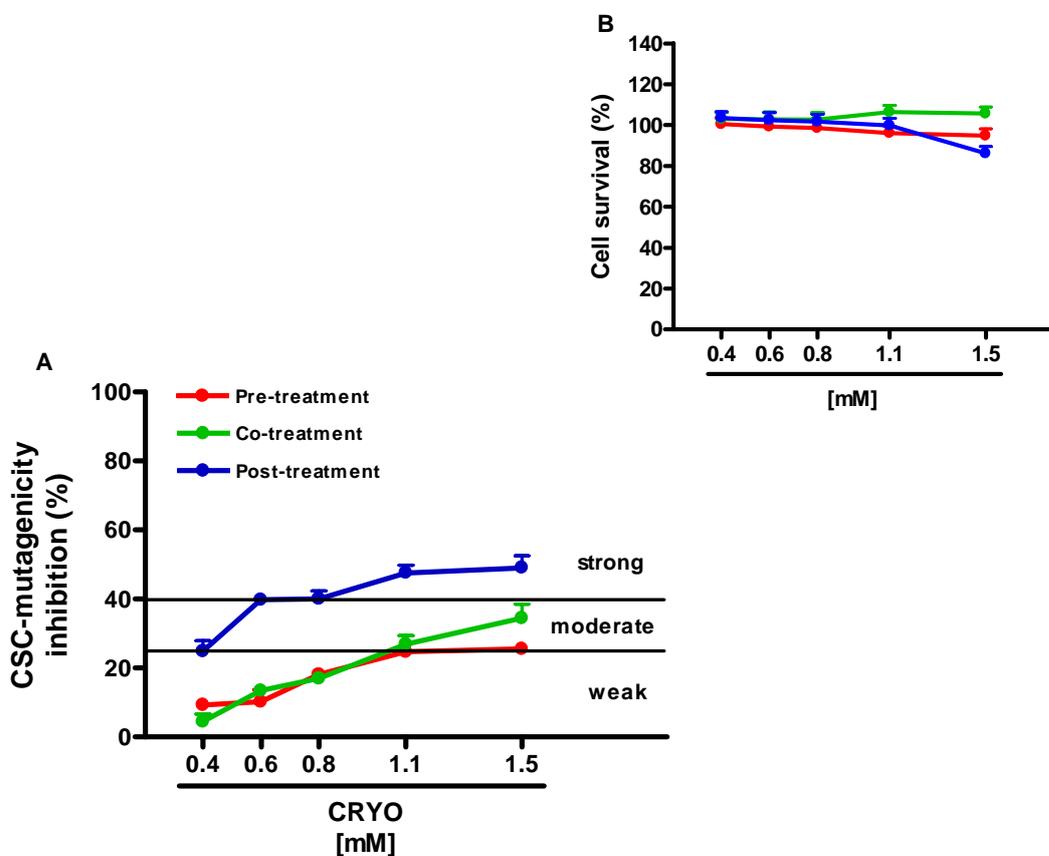
Figure 31: Inhibition (%) by CRYO of the CSC-induced mutagenicity in TA98 strain.
Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

The substance also significantly inhibited, the CSC-mutagenicity in TA100 strain; the effect was strong in all experimental conditions, reaching, at the highest concentration of 1.5 mM, the maximum inhibition values of 59.0%, 52.8% and 68.4% in the pre-, co- and post-treatment respectively (Figure 32).
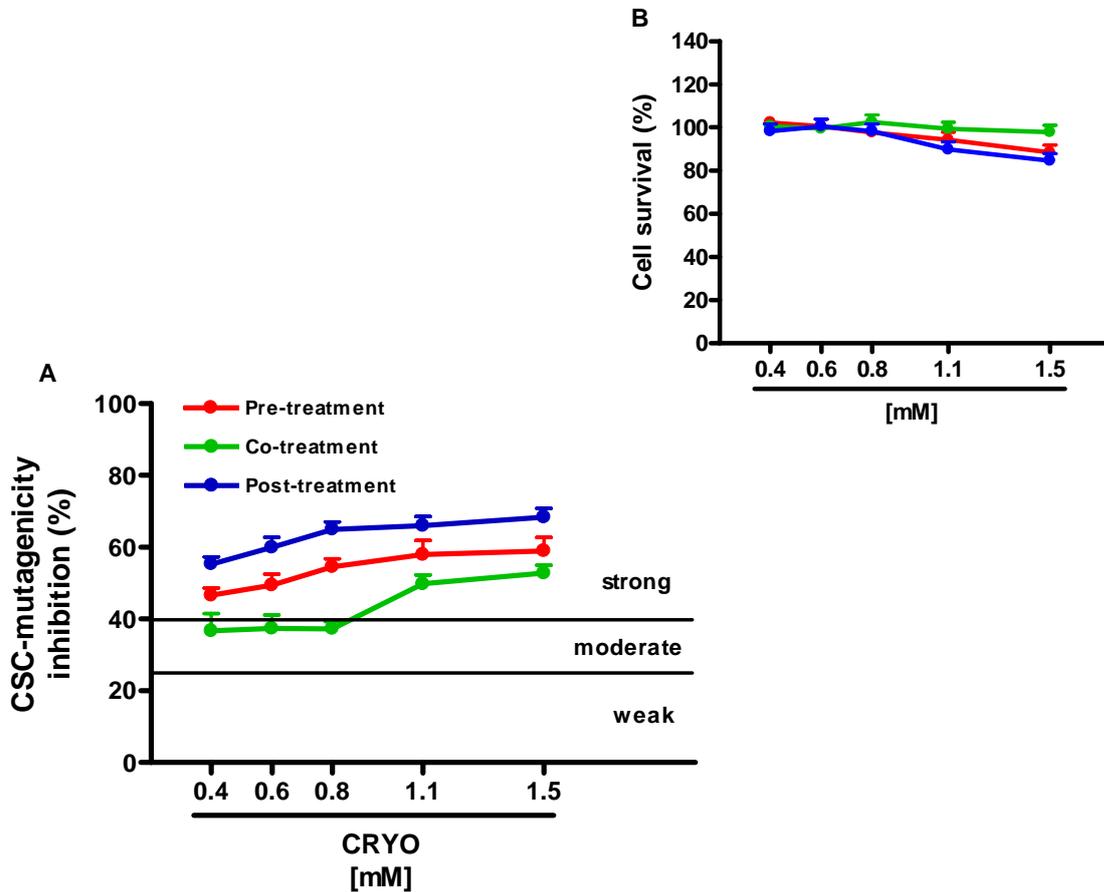
Figure 32: **Inhibition (%) by CRYO of the CSC-induced mutagenicity in TA100 strain.** Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

A statistically significant inhibition of CSC-mutagenicity was also registered in WP2*uvr*A: the effect was strong in all experimental conditions, reaching, at the highest concentration of 1.5 mM, the inhibition values of 50.8%, 68.4% and 55.4% in the pre-, co- and post-treatment, respectively (Figure 33).
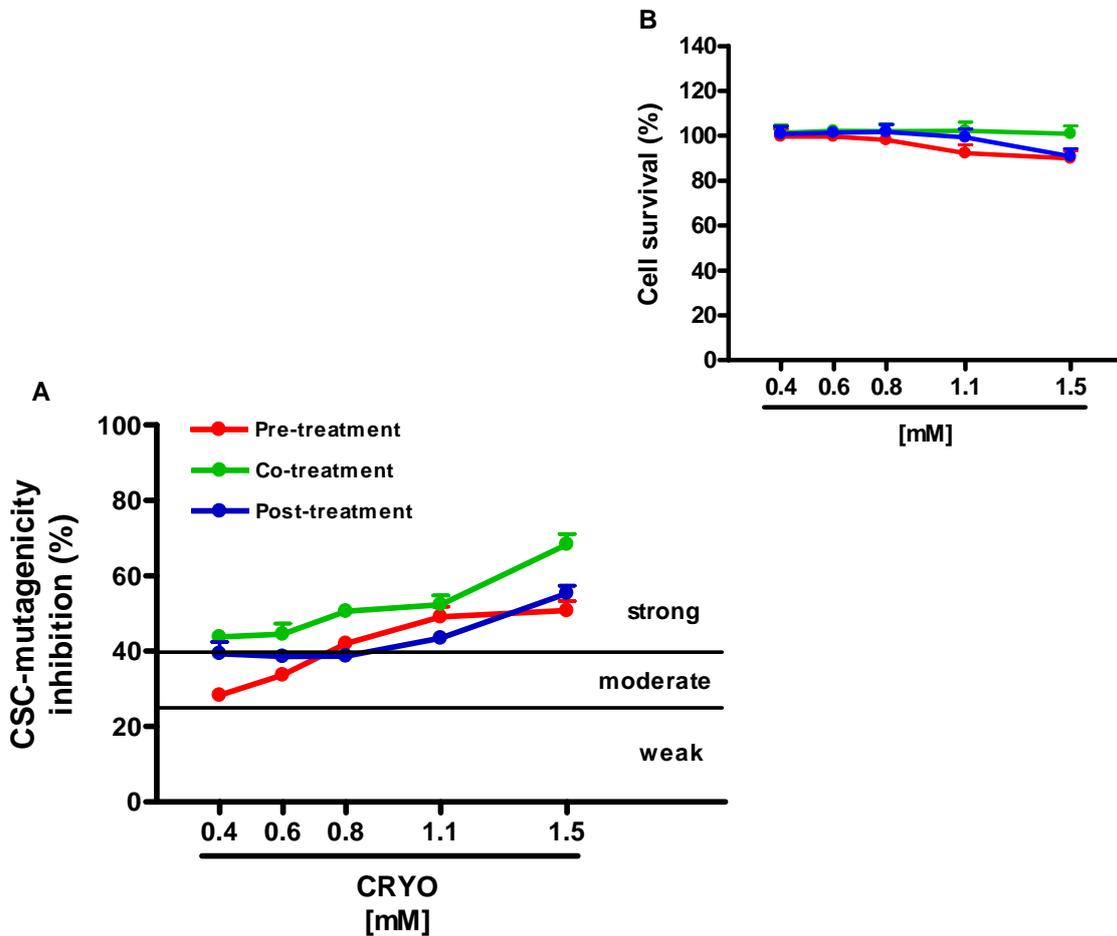
Figure 33: Inhibition (%) by CRYO of the CSC-induced mutagenicity in WP2uvrA strain. Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

Finally, β-caryophyllene oxide significantly inhibited the CSC-mutagenicity in WP2*uvr*A/R strain, (Figure 34): the antimutagenic effect was strong in all experimental conditions, reaching, at 1.5 mM highest concentration, the inhibition values of 67.4%, 60.3% and 56.4% in the pre-, co-, and post-treatment respectively.

In cell survival experiments, none of the concentrations tested were cytotoxic in the presence of mutagens, as the viability, with respect to control, was from 86% to 106% for TA98, from 85% to 102% for TA100, from 90% to 101% for WP2*uvr*A, and from 86% to 109% for WP2*uvr*A/R (Figures 31, 32, 33 and 34).
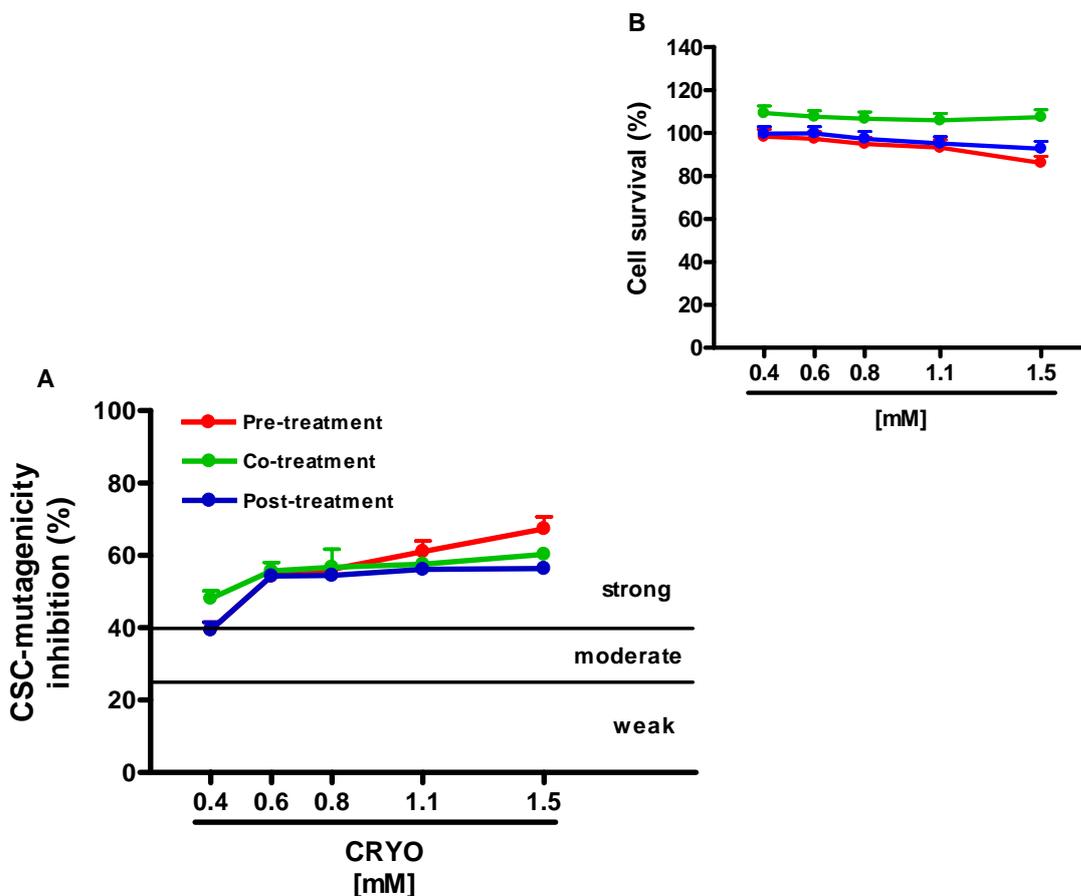
Figure 34: Inhibition (%) by CRYO of the CSC-induced mutagenicity in WP2uvrA/R strain. Mutagen: CSC (cigarette smoke condensed). The effect was reported in presence of the S9 metabolic activation system and in the three different experimental protocols. Values are expressed as mean ± SEM (n = 6). (A) Percentage of inhibition. (B) Cell survival. Strong: inhibition >40%; moderate: inhibition between 25% and 40%; weak: inhibition <25%.

DISCUSSION

Tobacco smoke is one of the greatest threats to human health as it is responsible for malignancies and precancerous lesions in different organs and tissues (Huang and Chen, 2011). Although smoking cessation is the better strategy to avoid the development of cancer, former smokers continue to have an elevated risk for years after quitting. In this context, chemoprevention represents a highly sought-after approach to reduce the risk of smoking damage, and the identification of new chemopreventive agents is a very desirable goal.

Considering that β-caryophyllene and β-caryophyllene oxide are used as cigarette ingredients and that, in our previous studies, they showed to be able to inhibit the genotoxicity of some environmental carcinogens, such as 2-nitrofluorene and 2-amino anthracene (Di Sotto et al., 2010, 2011), in present study, the natural sesquiterpenes β-caryophyllene (CRY) and β-caryophyllene oxide (CRYO) were evaluated for their potential antimutagenic activity against a condensed smoke from standard 3R4F cigarettes (CSC), in the bacterial reverse mutation assay on *Salmonella typhimurium* TA98, TA100 and on *Escherichia coli* WP2*uvr*A and WP2uvrA/pKM101 strains.

Preliminary studies were carried out to identify the concentration of CSC to use in the antimutagenicity assay. At 700 μg/plate (265 μg/ml), the mutagenic effect of CSC was submaximal so this concentration was used for the antimutagenicity test. Three different experimental protocols were used to investigate the potential mechanism involved in the antimutagenicity of CRY and CRYO (pre-, co- and post-treatment). Antimutagens that are active in pre- or co-treatment are defined as desmutagens (De Flora et al., 1988), whereas those active in post-treatment are defined as bioantimutagens (De Flora et al., 1998).

In TA98 strain, β-caryophyllene produced a strong antimutagenic activity against CSC in the pre- and post-treatment protocols. Also β-caryophyllene oxide showed, in the same strain, a strong antimutagenicity against the CSC in the post-treatment protocols, higher than the other sesquiterpene. The antimutagenic effects exerted against CSC by both compounds could be due to a physical-chemical or enzymatic interaction of the sesquiterpenes with the mutagen. Alternatively, they might aid the reparation of DNA-damage, by promoting the excision system repair; however this effect seems unlikely because TA98 is a plasmid-containing strain with an enhanced error-prone DNA repair (DeMarini, 2000). β-Caryophyllene exerted an interesting antimutagenicity in TA100 strain, particularly strong in the post-treatment. In this context, we can hypothesize that CRY was able not only to prevent mutagen-induced damage, but also to interact with the mutagen and to stimulate repairing the mutagen-induced damage. Likewise, a strong antimutagenicity was produced in WP2*uvr*A and WP2*uvr*A/R strains, in protocols

applied. Regarding β-caryophyllene oxide, a strong CSC-antimutagenicity was observed in all experimental protocols in TA100, WP2*uvr*A and WP2*uvr*A/R strains. These results are noteworthy, because they show that CRYO it was able not only to prevent the genotoxic damage, but also to directly interfere with the mutagen at the intra- or extracellular level and to repair the mutagen-induced damage.

It is important to underline that WP2*uvr*A/pKM101 is a mutant strains of WP2, in which the uvrA marker induces a DNA-repair deficiency (Wilcox et al., 1990). In this strain, the susceptibility to mutations is increased by the presence of plasmid pKM101, as this factor enhances error-prone repair. As a consequence, it is very sensitive to damage induced by cross-linking and pro-oxidant mutagens (Mortelmans et al., 2000). The plasmid pKM101 (viz., R factor) is related to an increased susceptibility of the strain to both base substitution and frameshift mutagenesis by a variety of chemicals and by an increased spontaneous mutation rate (Walker, 1985). In WP2*uvr*A/pKM101, both sesquiterpenes showed the highest antimutagenic activity, suggesting a potential protective role of these substances against cross-linking and pro-oxidant mutagens. Moreover, considering the lipophilic nature of these sesquiterpenes it is plausible to hypothesize an interaction with biological membranes by inhibiting the mutagen permeability. The unique structure and the presence of a sesquiterpene epoxide function in the β-caryophyllene oxide could justify an interaction of chemical species with the mutagen. Last, but not least, we can also hypothesize the ability of these sesquiterpenes to inhibit the metabolic activation of pro-carcinogens present in smoke condensate of cigarettes, in particular aromatic amines and nitroarenes (Oda et al., 2001). On the basis of these results, we hypothesize that aspecific mechanisms should be involved in the antimutagenicity of CRY and CRYO, being the substances effective in strains sensitive to different genotoxic damages (i.e. frameshift and base-substitution mutations, oxidative stress and DNA alkylation), and in all experimental conditions, so acting both as desmutagenic and bioantimutagenic agents (Kada and Shimoi, 1987). The hypotheses on the bio-anti-mutagenic mechanisms are more complex and could range from the induction of bacterial systems involved in repair of DNA damage (SOS and Ada) to activation of specific intracellular signaling pathways (De Flora, 1998).

The antimutagenic effect of β-caryophyllene and β-caryophyllene oxide here found deserves attention; if it should be confirmed in *in vivo* studies, it could open up new prospects for the use of this natural substances in the field of human health, as chemopreventer (Wall et al., 1990; Yen and Chen, 1994) or in preventing other diseases linked with occurrence of mutation.

MULTIDRUG RESISTANCE (MDR) REVERSAL ASSAY
AND
ABC–TRANSPORTER INHIBITION

RESULTS

Preliminary cytotoxicity assays

The CCRF/CEM was the most sensitive cell line to the treatment with the test substances. The IC_{50} values were 212.95 ± 6.36, 311.59 ± 14.36 and 235.18 ± 5.18 µM for α-hexylcinnamaldehyde (HCA), β-caryophyllene (CRY) and β-caryophyllene oxide (CRYO), respectively. The positive control doxorubicin showed an IC_{50} value of 0.41 ± 0.02 µM.

Also in P-gp overexpressing CEM/ADR5000 cells, the test substances showed to significantly reduce the viability of cells. In this cell line, the IC_{50} values were 256.52 ± 2.27, 368.48 ± 2.23 and 297.98 ± 3.33 µM for HCA, CRY and CRYO, respectively. It is noteworthy that the resistant cell line CEM/ADR5000 showed a significantly decreased sensitivity when treated with the positive control doxorubicin (IC_{50} = 74.28 ± 0.35 µM) but not when treated with the other test substances. CEM/ADR5000 cells were 181.2 fold resistant to doxorubicin but only 1.2 or 1.3 fold resistant to HCA, CRY and CRYO compared to CCRF/CEM wild-type cells.

Among the different cell lines, Caco-2 were found to be the more resistant cells to the treatment with HCA, CRY and CRYO, but not to the treatment with doxorubicin. Moreover, CRY resulted to be less cytotoxic (IC_{50} = 1103.34 ± 17.32 µM) than HCA (IC_{50} = 315.66 ± 4.75 µM) and CRYO (IC_{50} = 332.30 ± 3.97 µM). The positive control showed an IC_{50} value of 5.24 ± 0.66 µM. Dose-response curves of the various test substances divided for cell lines are reported in Figures 35, 36 and 37.
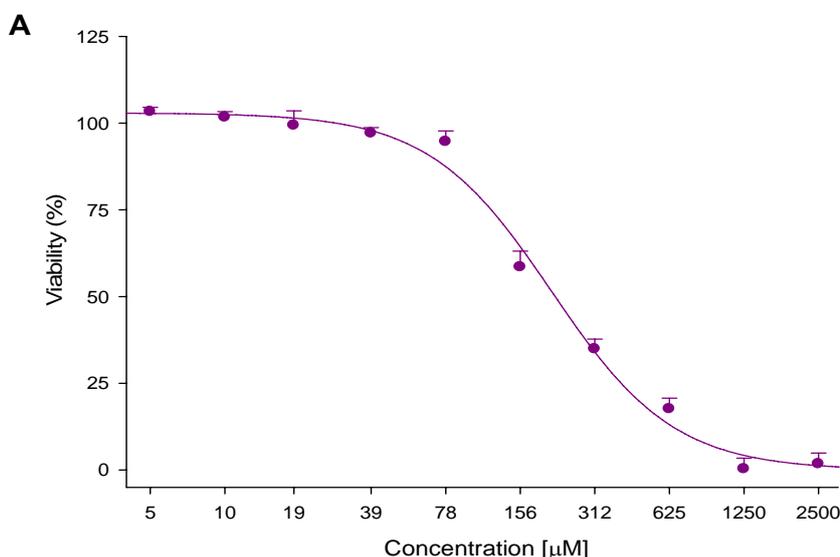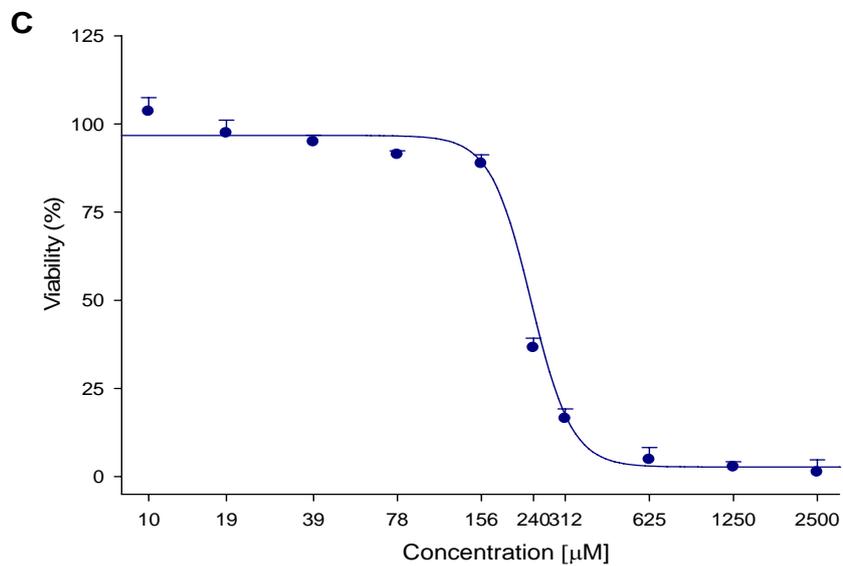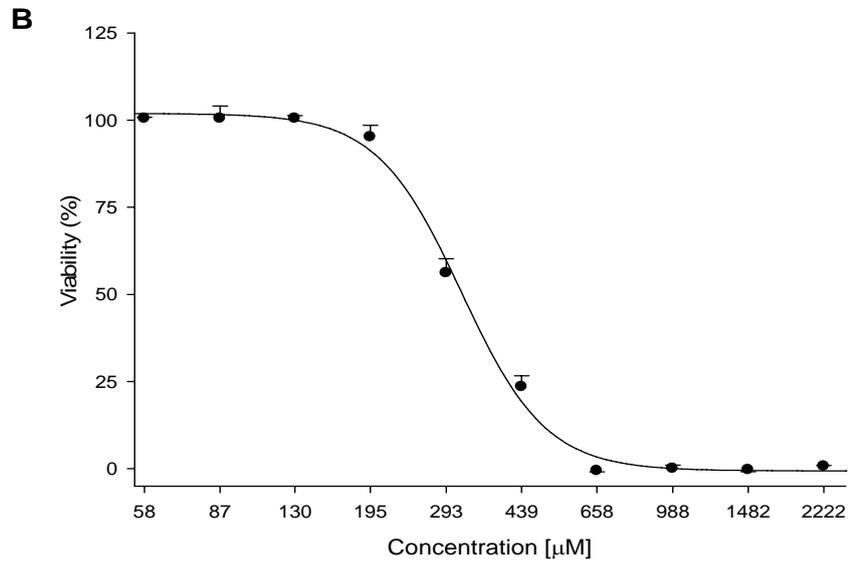
Figure 35. Dose response curve of α-hexylcinnamaldehyde (A), β-caryophyllene (B) and β-caryophyllene oxide (C) on viability of CCRF/CEM cells using MTT assay. Values are expressed as mean ± SEM (n = 9).
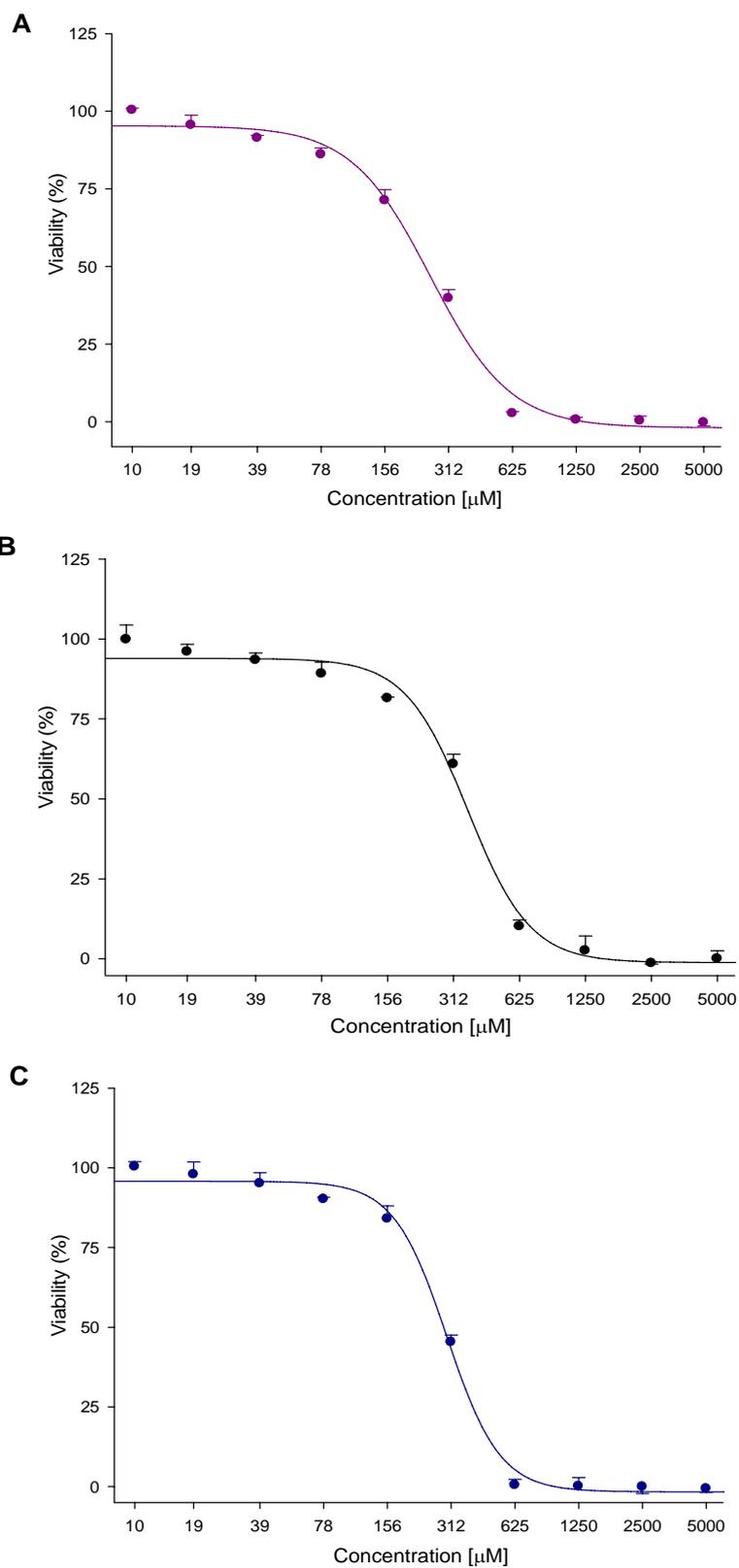
Figure 36. Dose response curve of α-hexylcinnamaldehyde (A), β-caryophyllene (B) and β-caryophyllene oxide (C) on viability of CEM/ADR5000 cells using MTT assay. Values are expressed as mean ± SEM (n = 9).
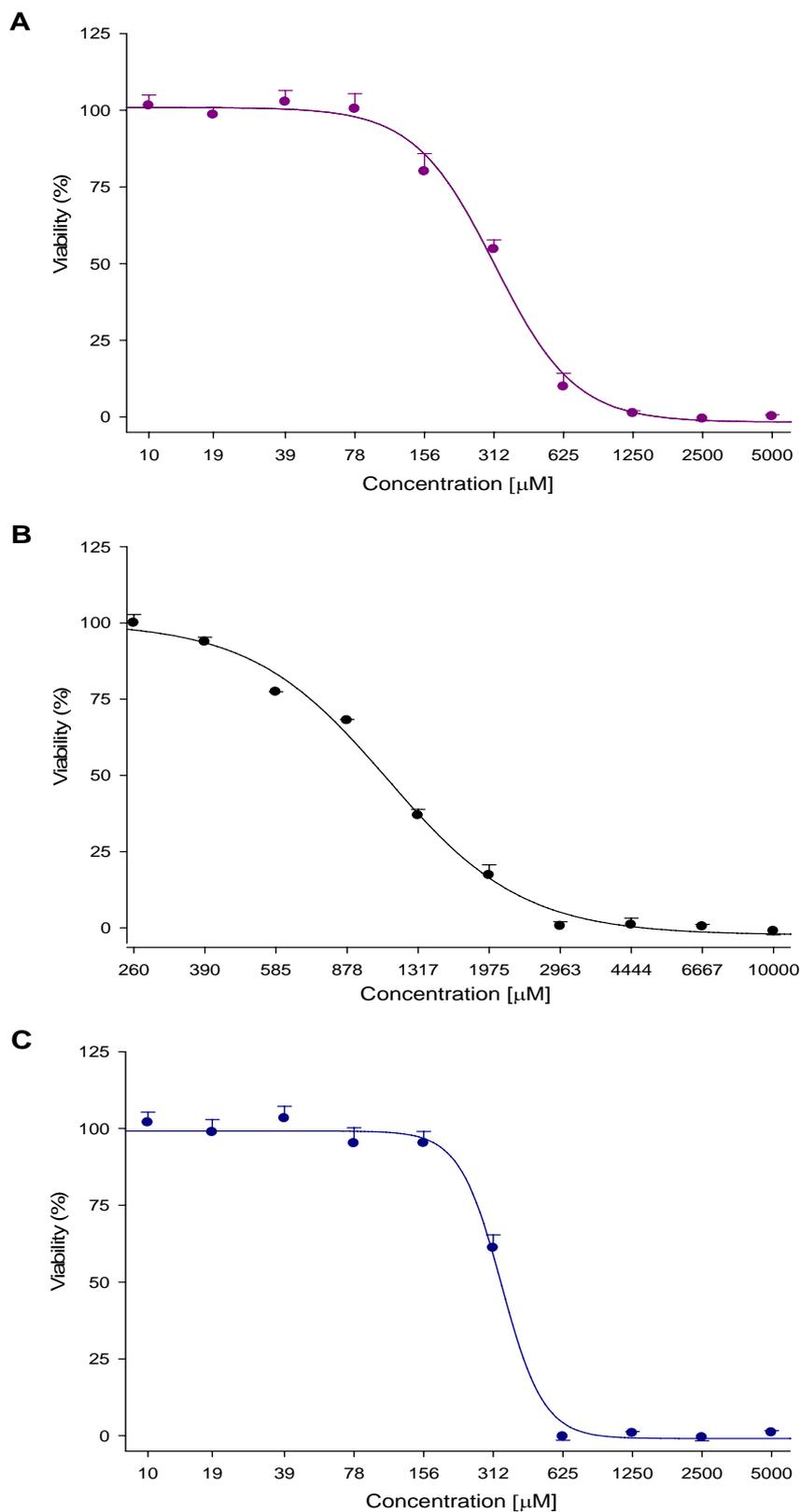
Figure 37. Dose response curve of α-hexylcinnamaldehyde (A), β-caryophyllene (B) and β-caryophyllene oxide (C) on viability of Caco-2 cells using MTT assay. Values are expressed as mean ± SEM (n = 9).

α-Hexylcinnamaldehyde

Co-incubation of doxorubicin with non-toxic concentrations of α-hexylcinnamaldehyde (IC_{10} = 50 μM and IC_{20} = 100 μM) resulted in a significant ($p < 0.001$) and synergistic increase in its cytotoxicity in sensitive CCRF/CEM cell line (Figure 38).

The IC_{50} value of doxorubicin decreased 6-fold (from 0.42 ± 0.02 to 0.06 ± 0.004 μM) and 47-fold (from 0.42 ± 0.02 to 0.008 ± 0.0002 μM) when combined with non-toxic concentrations of α-hexylcinnamaldehyde (Figure 39). The combination index (CI) was 0.41 ± 0.02 and 0.49 ± 0.01 for the lower and highest concentration, respectively. The corresponding isobolograms are reported in Figure 40.
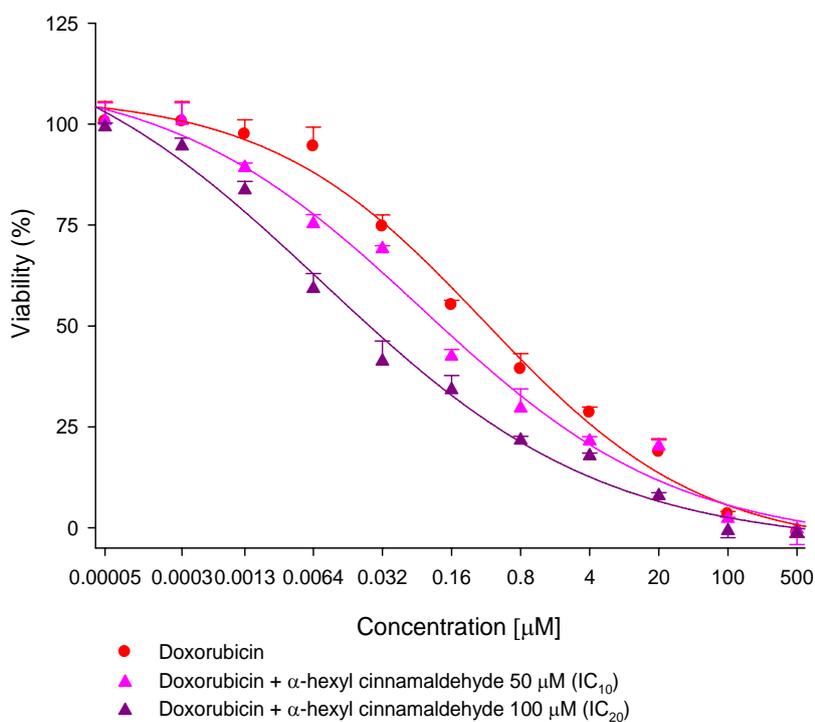
Figure 38. Doxorubicin dose response curve on viability of CCRF/CEM cells with and without α-hexylcinnamaldehyde. Values are expressed as mean ± SEM (n = 9).
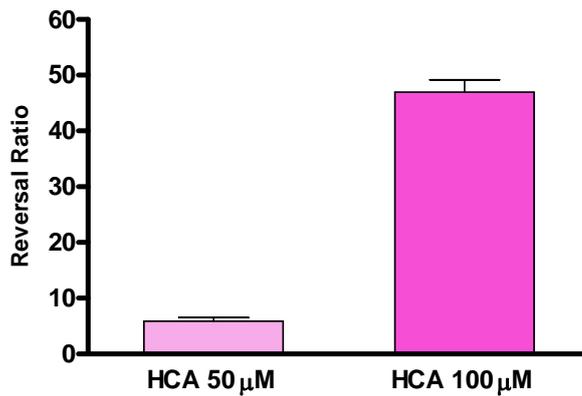
Figure 39. Enhancement of doxorubicin cytotoxicity by combination with α-hexylcinnamaldehyde in CCRF/CEM cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
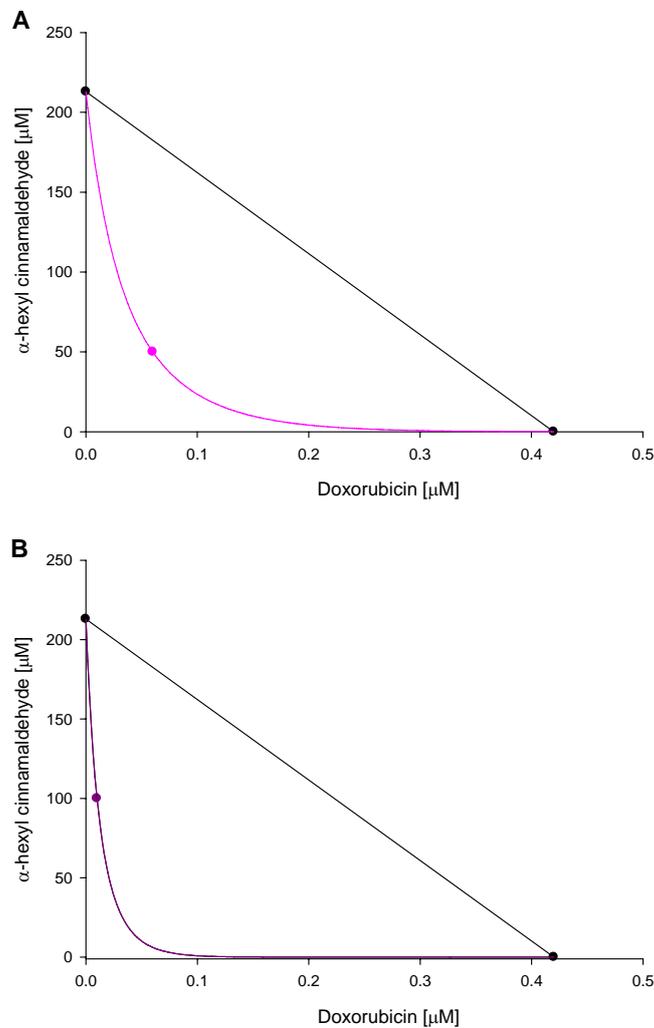

Figure 40. Isobologram analyses in CCRF/CEM cells: IC_{50} concentrations of doxorubicin are plotted on x-axis and IC_{50} values of α-hexylcinnamaldehyde on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

Furthermore, α-hexylcinnamaldehyde was able to sensitize CCRF/CEM cells to the cytotoxicity of β-caryophyllene and β-caryophyllene oxide (Figure 41A and B).

The combination with α-hexylcinnamaldehyde 50 and 100 μM determined a significant ($p < 0.001$) reduction of β-caryophyllene IC_{50} equal to 2-fold (IC_{50} decrease from 311.59 ± 14.36 to 202.44 ± 0.02 μM, CI 0.85 ± 0.03) and 10-fold (IC_{50} decrease from 311.59 ± 14.36 to 31.47 ± 0.50 μM, CI 0.55 ± 0.02), respectively.

Also the β-caryophyllene oxide cytotoxicity was enhanced by combination: the IC_{50} value was reduced from 235.18 ± 5.18 to 81.65 ± 0.85 μM (CI 0.57 ± 0.01) and to 21.38 ± 0.35 μM (CI 0.54 ± 0.01) by the lower and highest concentration.

The corresponding reversal ratio and isobolograms are reported in Figures 42 and 43.
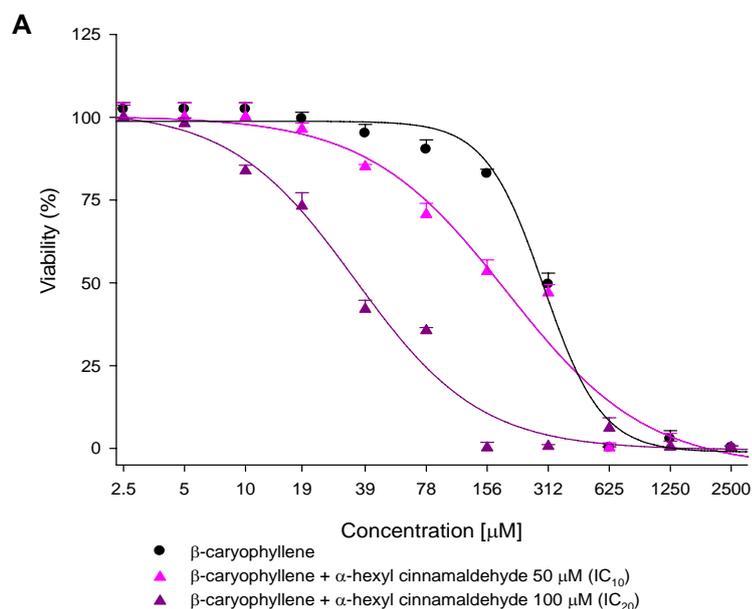
Figure 41. β-caryophyllene (A) and β-caryophyllene oxide (B) dose response curves on viability of CCRF/CEM cells with and without α-hexylcinnamaldehyde. Values are expressed as mean ± SEM (n = 9).
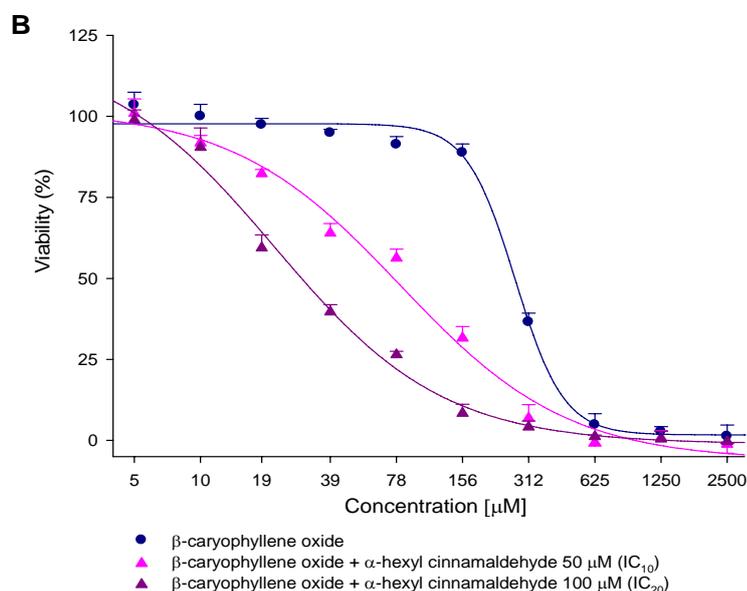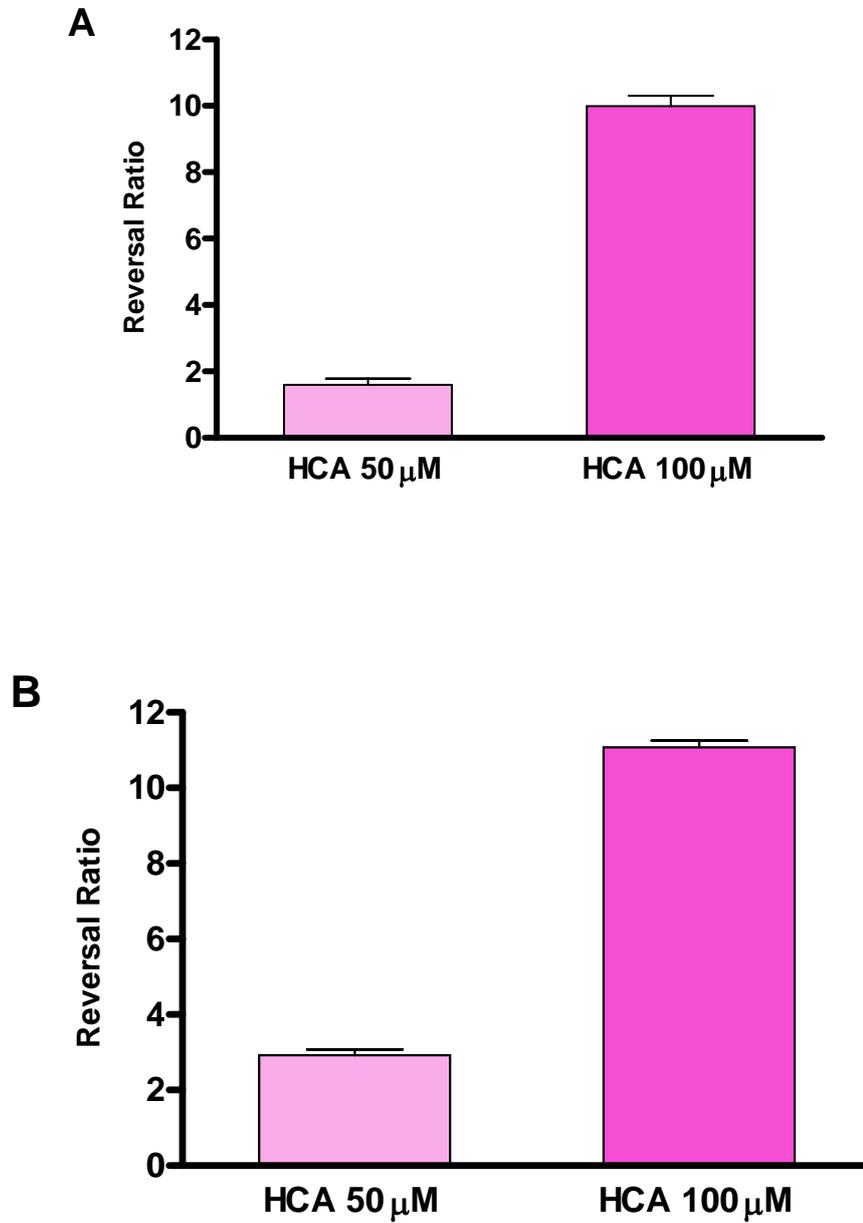
Figure 42. Enhancement of β-caryophyllene (A) and β-caryophyllene oxide (B) cytotoxicity by combination with α-hexylcinnamaldehyde in CCRF/CEM cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
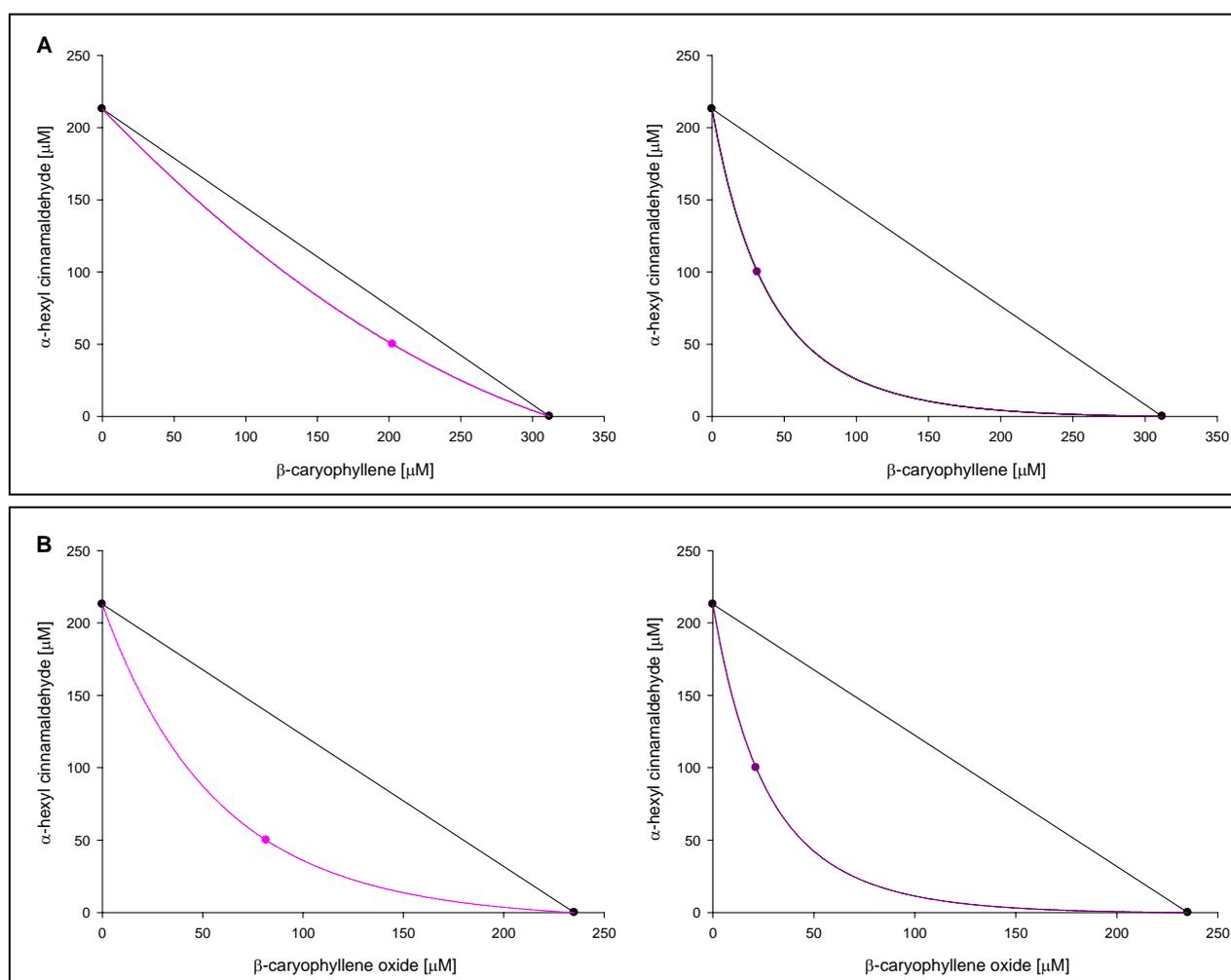
Figure 43. Isobologram analyses in CCRF/CEM cells: IC_{50} concentrations of β-caryophyllene (A) and β-caryophyllene oxide (B) are plotted on x-axis and IC_{50} values of α-hexylcinnamaldehyde on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

Also leukemia cells (CEM/ADR5000) were significantly sensitized by the combination with IC_{10} (100 μM) and IC_{20} (141 μM) of α-hexylcinnamaldehyde (Figure 44). The IC_{50} of doxorubicin was enhanced 4-fold (IC_{50} decrease from 74.28 ± 0.35 to 18.22 ± 0.56 μM, CI 0.65 ± 0.01) and 7-fold (IC_{50} decrease from 74.28 ± 0.35 to 10.08 ± 0.21 μM, CI 0.70 ± 0.01) respect to the doxorubicin alone (Figure 45). According to the combination index, the isobologram analysis showed synergistic effect (Figure 46).
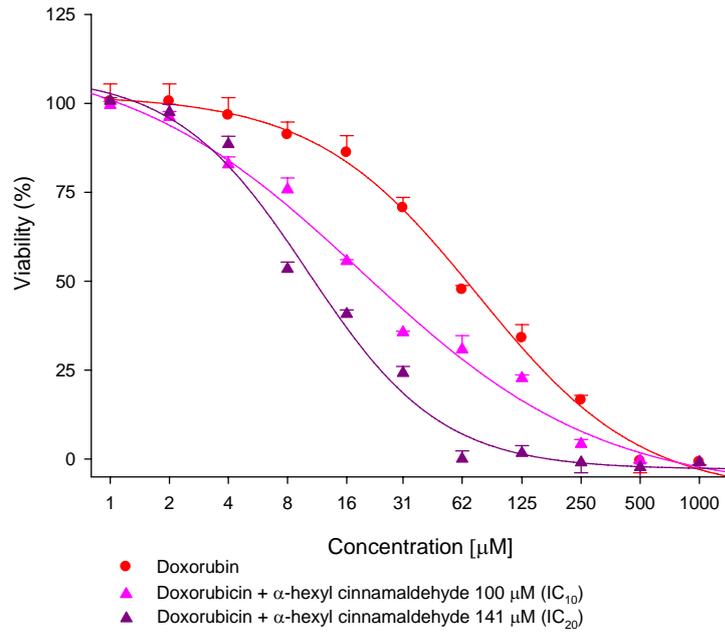
Figure 44.   Doxorubicin dose response curve on viability of CEM/ADR5000 cells with and without α-hexylcinnamaldehyde. Values are expressed as mean ± SEM (n = 9).
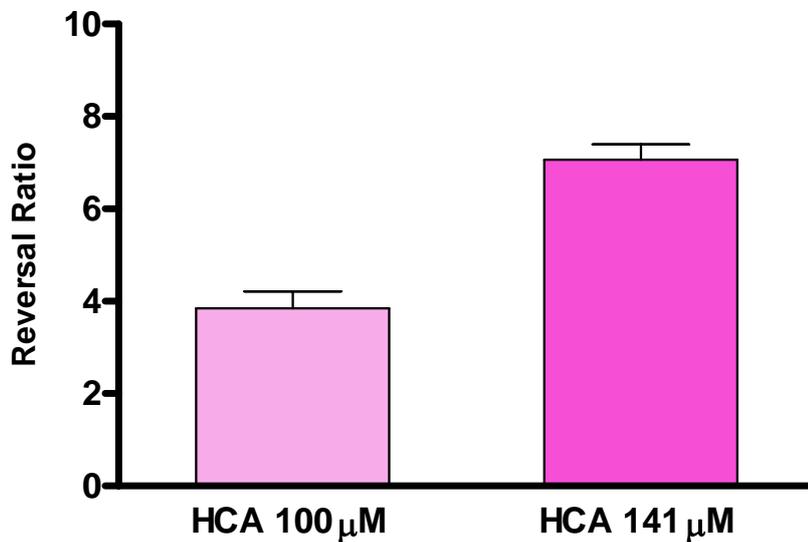
Figure 45.   Enhancement of doxorubicin cytotoxicity by combination with α-hexylcinnamaldehyde in CEM/ADR5000 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
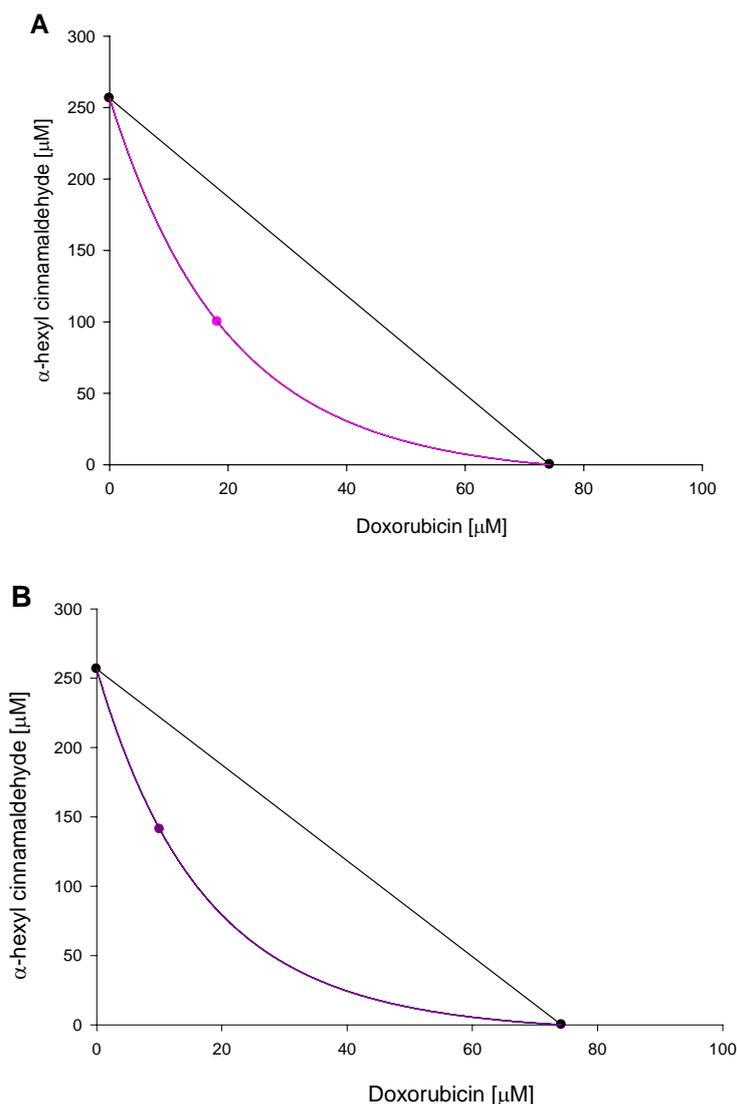
Figure 46. Isobologram analyses in CEM/ADR5000 cells: IC_{50} concentrations of doxorubicin are plotted on x-axis and IC_{50} values of α-hexylcinnamaldehyde on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

In CEM/ADR5000 cells, co-incubation of β-caryophyllene and β-caryophyllene oxide with non-toxic concentrations of α-hexylcinnamaldehyde (IC_{10} = 100 μM and IC_{20} = 141 μM) resulted in a significant ($p < 0.001$) and synergistic increase in their cytotoxicity (Figure 47A and B).

The IC_{50} value of β-caryophyllene decreased 3-fold (from 368.48 ± 2.23 to 126.42 ± 1.12 μM) and 7-fold (from 368.48 ± 2.23 to 51.04 ± 1.81 μM) when combined with IC_{10} and IC_{20} of α-hexylcinnamaldehyde (Figure 48A). The combination index (CI) was 0.75 ± 0.003 and 0.71 ± 0.001 for the lower and highest concentration, respectively.

Regarding β-caryophyllene oxide, the IC_{50} values decreased from 297.98 ± 3.33 to 72.22 ± 0.70 μM (CI 0.65 ± 0.004) and to 37.67 ± 0.07 μM (CI 0.69 ± 0.006) in combination with non toxic concentrations of α-hexylcinnamaldehyde. The cytotoxicity enhancement was equal to 4 and 8-fold, respectively (Figure 48B).

According to the combination index, the isobologram analysis showed synergistic effect (Figure 49).
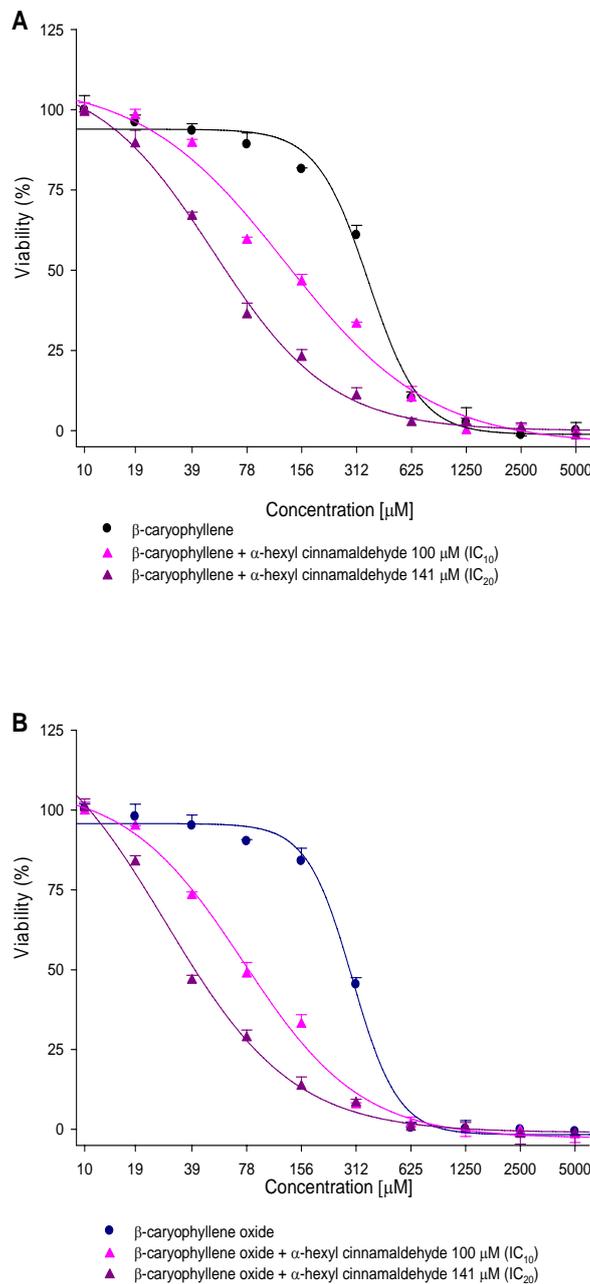
Figure 47. β-caryophyllene (A) and β-caryophyllene oxide (B) dose response curves on viability of CEM/ADR5000 cells with and without α-hexylcinnamaldehyde. Values are expressed as mean $\pm$ SEM (n = 9).
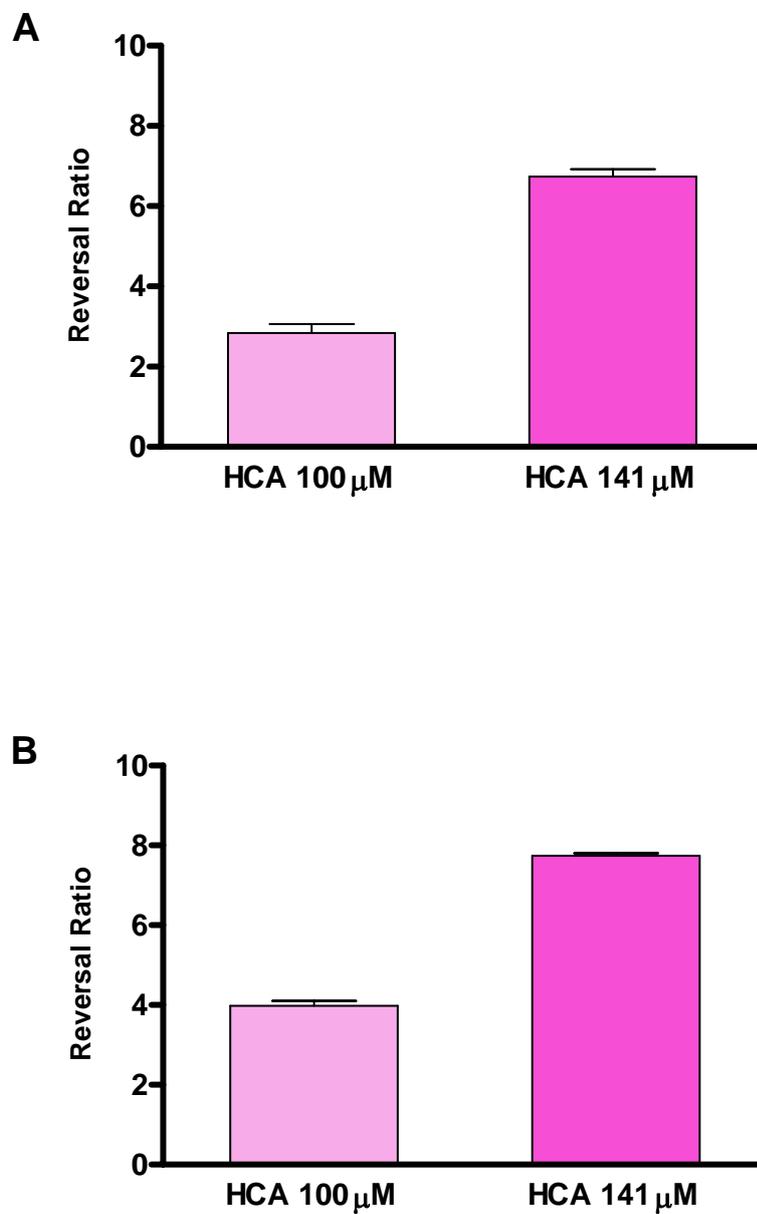
Figure 48. Enhancement of β-caryophyllene (A) and β-caryophyllene oxide (B) cytotoxicity by combination with α-hexylcinnamaldehyde in CEM/ADR5000 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
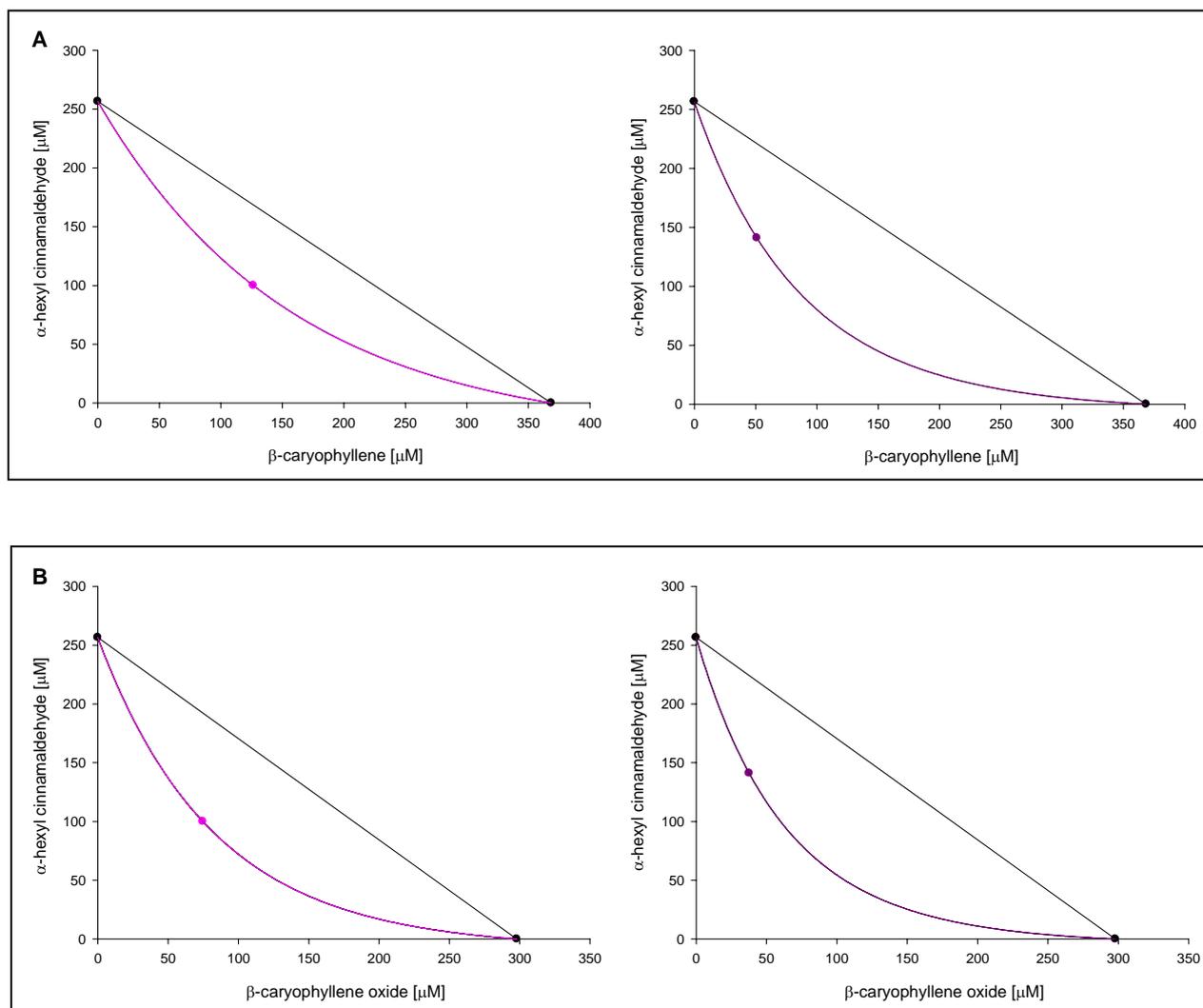
Figure 49. Isobologram analyses in CEM/ADR5000 cells: IC_{50} concentrations of β-caryophyllene (A) and β-caryophyllene oxide (B) are plotted on x-axis and IC_{50} values of α-hexylcinnamaldehyde on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

In Caco-2 cell line, the IC_{50} value of doxorubicin in combination with IC_{10} (100 µM) and IC_{20} (181 µM) of α-hexylcinnamaldehyde statistically decreased of 3-fold (IC_{50} decrease from 5.24 ± 0.66 to 1.86 ± 0.14 µM, CI 0.69 ± 0.01) and 6-fold (IC_{50} decrease from 5.24 ± 0.66 to 0.82 ± 0.05 µM, CI 0.74 ± 0.02 and) respect to the doxorubicin alone (Figure 50 and 51). Regarding the isobologram analysis, it was in agreement with the combination index and showed a synergistic effect (Figure 52).
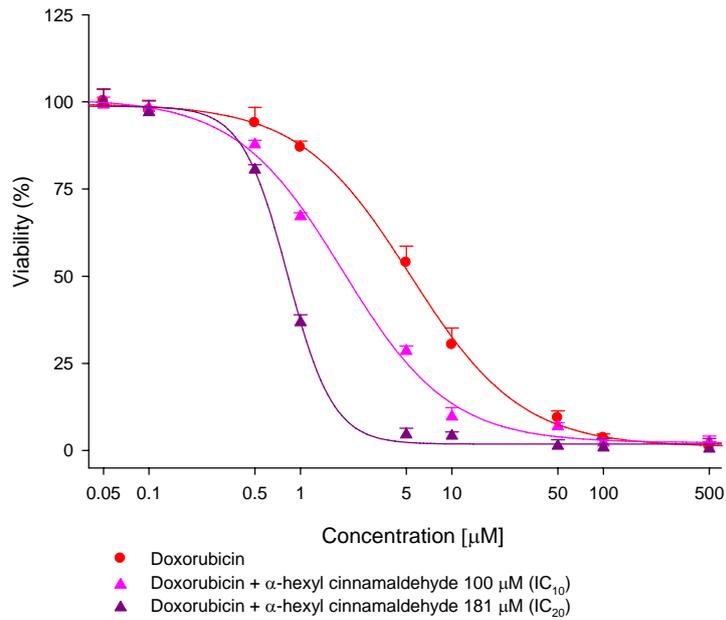
**Figure 50.** Doxorubicin dose response curve on viability of Caco-2 cells with and without α-hexylcinnamaldehyde. Values are expressed as mean ± SEM (n = 9).
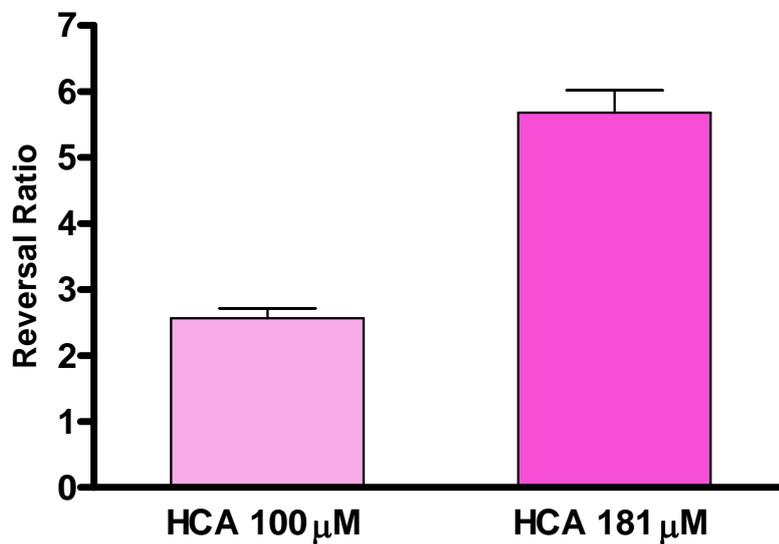
Figure 51. Enhancement of doxorubicin cytotoxicity by combination with α-hexylcinnamaldehyde in Caco-2 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
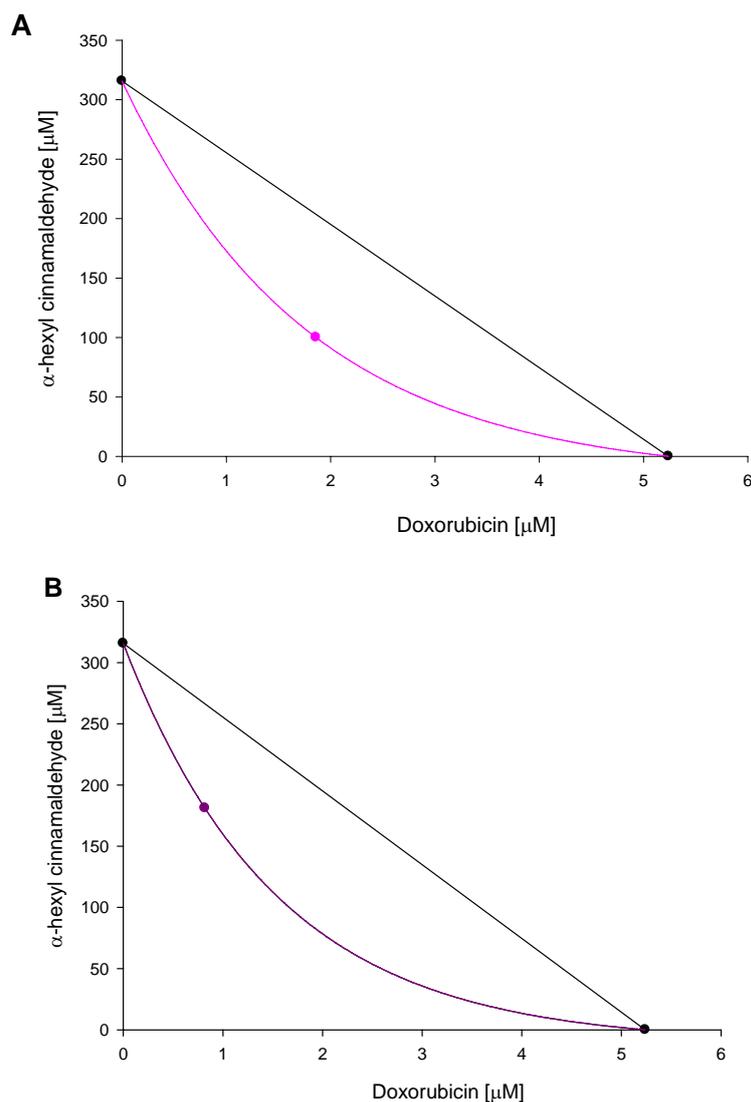
Figure 52. Isobologram analyses in Caco-2 cells: IC_{50} concentrations of doxorubicin are plotted on x-axis and IC_{50} values of α-hexylcinnamaldehyde on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

The co-incubation of β-caryophyllene with non-toxic concentrations of α-hexylcinnamaldehyde (IC_{10} = 100 μM and IC_{20} = 181 μM), in Caco-2 cell line, resulted in a significant ($p < 0.01$ or $p < 0.001$) increase in its cytotoxicity (Figure 53A).

The IC_{50} value of β-caryophyllene decreased from 1103.34 ± 17.32 to 907.53 ± 23.56 μM (CI 1.2 ± 0.01) and from 1103.34 ± 17.32 to 633.34 ± 21.68 μM (CI 1.2 ± 0.01) when combined with both concentrations of α-hexylcinnamaldehyde. The reversal ratio indicated a cytotoxicity enhancement equal to 1 and 2-fold in presence of IC_{10} and IC_{20} of chemosensitizer (Figure 54A). Once again, the isobologram analysis was in agreement with the combination index and it showed antagonistic effect (Figure 55A).

Also the cytotoxicity of β-caryophyllene oxide was statistically (p < 0.001) enhanced by the combination with IC_{10} and IC_{20} of α-hexylcinnamaldehyde (Figure 53B). The IC_{50} value of β-caryophyllene oxide decreased from 332.30 ± 3.97 to 157.99 ± 2.25 µM (RR 2, CI 0.8 ± 0.003) and from 332.30 ± 3.97 to 122.03 ± 1.62 µM (RR 2.7, CI 0.9 ± 0.01) when combined with both concentrations of α-hexylcinnamaldehyde (Figure 54B).

The combination index (CI) was 0.80 ± 0.003 and 0.95 ± 0.001 for the lower and highest concentration, respectively. The isobologram analysis, according to the combination index, showed a synergistic effect (Figure 55B).
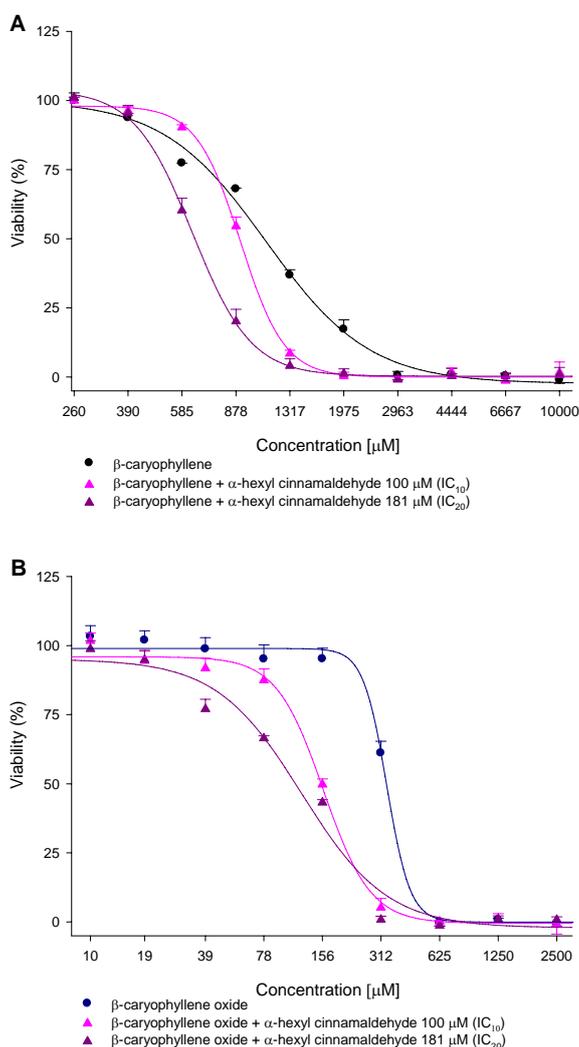
Figure 53. β-caryophyllene (A) and β-caryophyllene oxide (B) dose response curves on viability of Caco-2 cells with and without α-hexylcinnamaldehyde. Values are expressed as mean ± SEM (n = 9).
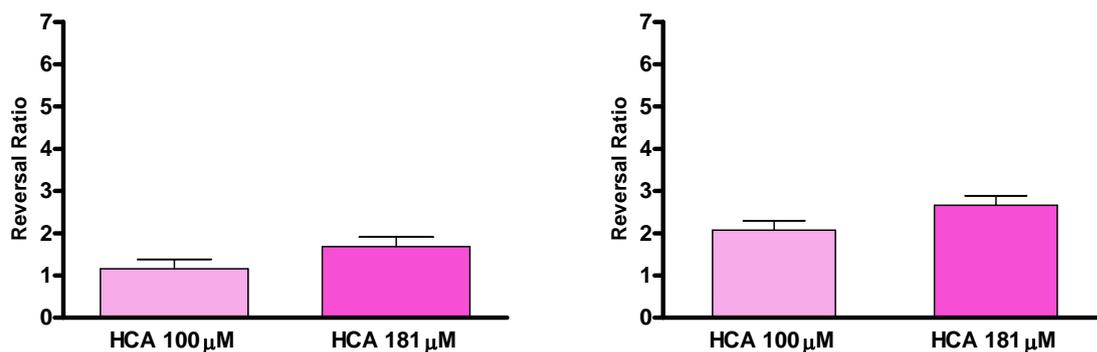
Figure 54. Enhancement of β-caryophyllene (A) and β-caryophyllene oxide (B) cytotoxicity by combination with α-hexylcinnamaldehyde in Caco-2 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
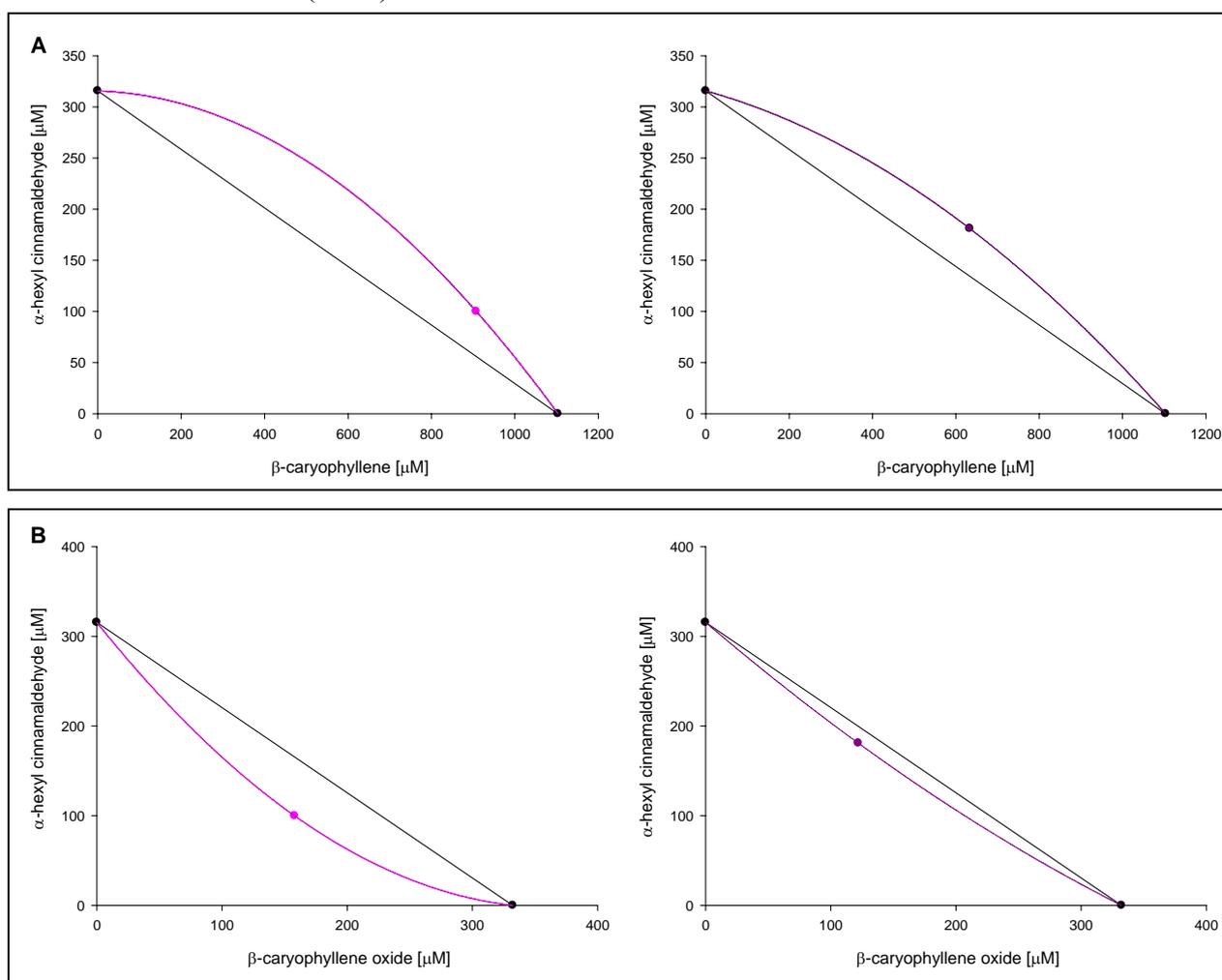
Figure 55. Isobologram analyses in Caco-2 cells: IC_{50} concentrations of β-caryophyllene (A) and β-caryophyllene oxide (B) are plotted on x-axis and IC_{50} values of α-hexylcinnamaldehyde on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

The cytotoxicity and reversal data presented above suggest that α-hexylcinnamaldehyde effectively interfere with at least one mechanism of drug inactivation, especially drug export. To explore this possibility, rhodamine123 retention assay was performed to determine ABC-transporter activity in the cells.

The intracellular fluorescence of Rho123 was significantly increased in a dose-dependent manner, as shown in Figure 56, in Caco-2 cells treated with the test substance. Results were expressed as % inhibition of P-gp relative to the positive control verapamil (100%). The α-hexylcinnamaldehyde was more active than verapamil to inhibite the MDR efflux pumps. At the same concentration of the positive control (20 μM), the fluorescence intensity was 134.6 ± 5.5. These results indicate that α-hexylcinnamaldehyde is inhibitor of ABC-transporters.

In addition, efflux assays were quantified using flow cytometry in CEM/ADR5000 cells. The intracellular fluorescence of Rho123 was significantly increased in a dose-dependent manner in CEM/ADR5000 cells when treated with α-hexylcinnamaldehyde. Similar to Caco-2 cells, the α-hexylcinnamaldehyde was active as a P-gp inhibitor, as shown in Figure 57. At the concentration of 10 μM, the fluorescence intensity for the test compound was 106.8 ± 5.32 %. The figure 58 shows flow cytometry histograms of α-hexylcinnamaldehyde.
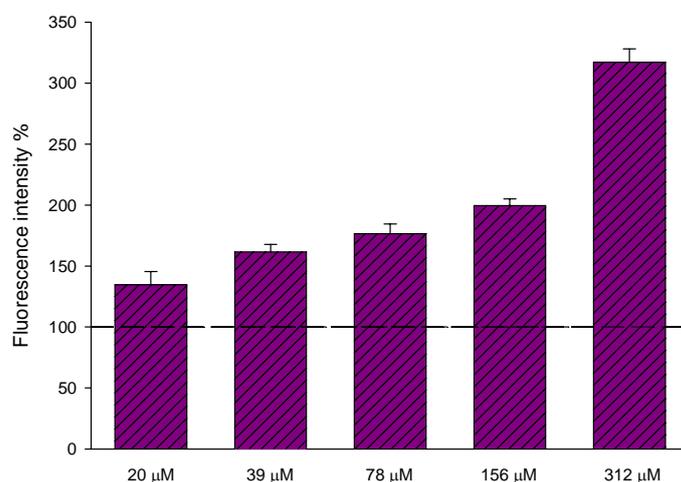
Figure 56.   Effect of α-hexylcinnamaldehyde on Rho 123 retention in Caco-2 cells. Fluorescence intensity was measured by spectrofluorometry. Data are presented as means ± ES of fluorescence intensity % related to verapamil as positive control (dotted line).
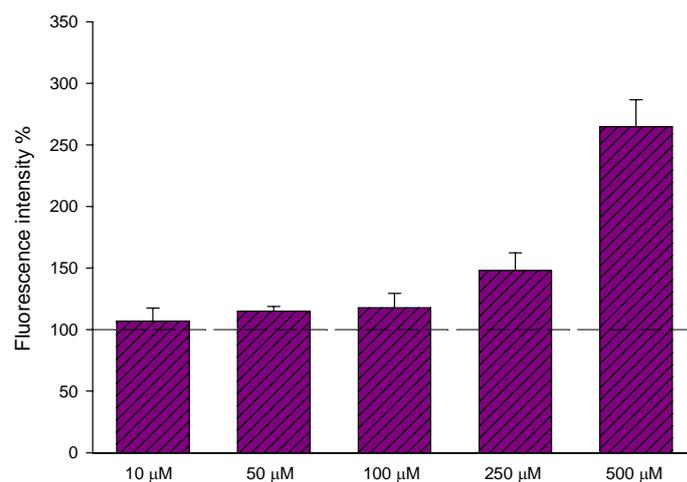
Figure 57. Effect of α-hexylcinnamaldehyde on Rho 123 retention in CEM/ADR5000 cells. Fluorescence intensity was measured using FACS. Data are presented as means ± ES of fluorescence intensity % related to verapamil as positive control (dotted line).
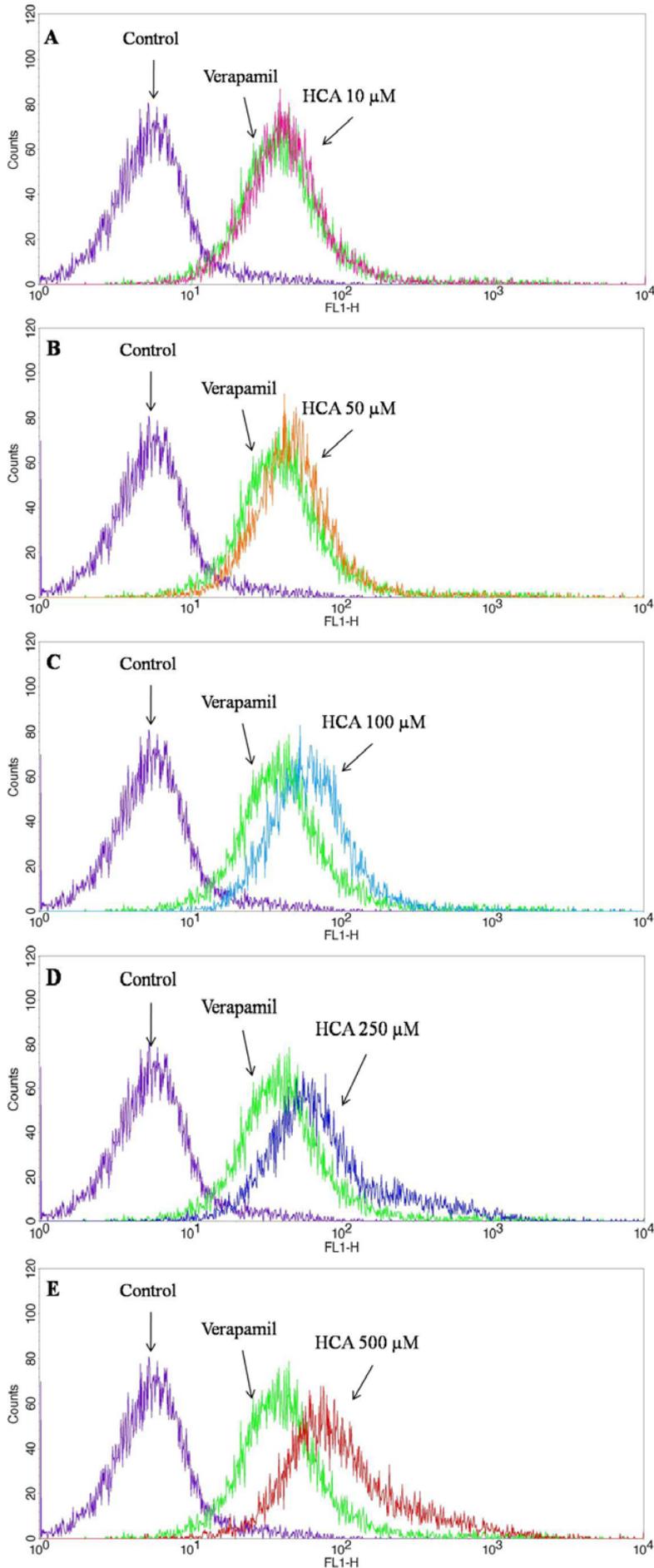
Figure 58. Flow cytometry histograms of α-hexylcinnamaldehyde (A-E). The fluorescence intensity was shifted to the right side indicating that the resistance cell line CEM/ADR5000 retain the Rho 123 as response to treatment with MDR1 inhibitors. Fluorescence intensity was measured using FACS.

β-Caryophyllene

Co-incubation of doxorubicin with very low and non-toxic concentrations of β-caryophyllene (IC_{10} = 185 µM and IC_{20} = 224 µM), in sensitive CCRF/CEM cell line, resulted in a reduction, but not statistically significant, of doxorubicin IC_{50} (from 0.42 ± 0.02 to 0.35 ± 0.005 and to 0.31 ± 0.007 µM, for the lower and the highest concentrations, respectively) (Figure 59).
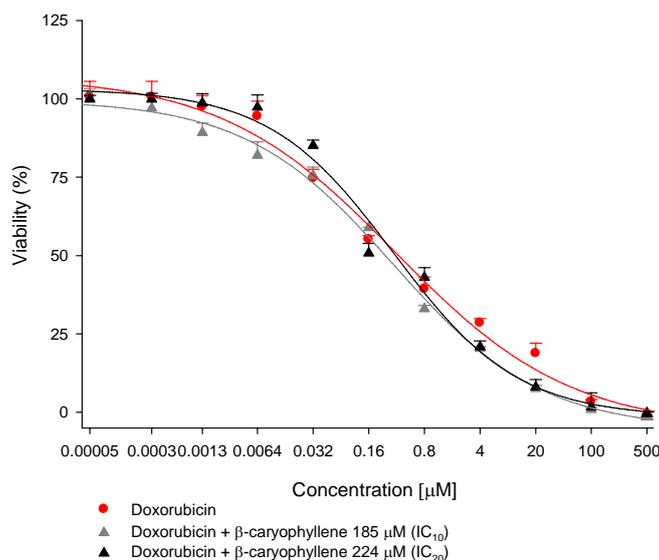
Figure 59. Doxorubicin dose response curve on viability of CCRF/CEM cells with and without β-caryophyllene. Values are expressed as mean ± SEM (n = 9).

Conversely, the combination of α-hexylcinnamaldehyde and β-caryophyllene oxide with IC_{10} and IC_{20} of β-caryophyllene resulted in a significant (p < 0.001) increase in their cytotoxicity (Figure 60A and B).

The IC_{50} value of α-hexylcinnamaldehyde decreased 2.5-fold (from 212.95 ± 6.36 to 83.50 ± 1.81 µM) and 4-fold (from 212.95 ± 6.36 to 51.92 ± 0.22 µM) when combined with non-toxic concentrations of β-caryophyllene (Figure 61A). The combination index (CI) was 0.97 ± 0.03 and 0.93 ± 0.03 for the lower and highest concentrations, respectively; it indicates an additive effect.

Also the IC_{50} value of β-caryophyllene oxide decreased from 235.18 ± 5.18 to 186.45 ± 1.54 and to 136.25 ± 4.93 µM in combination with IC_{10} and IC_{20} of β-caryophyllene (Figure 61B). In this context, the combination index indicates an antagonistic affect, being equal to 1.3 ± 0.03 and 1.3 ± 0.02 for the lower and highest concentrations of β-caryophyllene.

The corresponding isobolograms are reported in Figure 62.
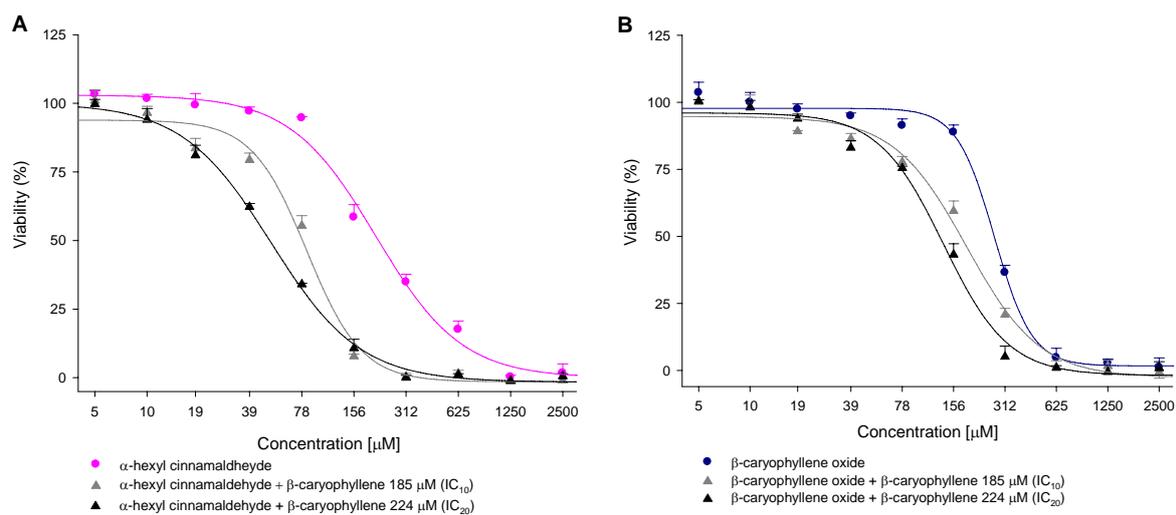
Figure 60. α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) dose response curves on viability of CCRF/CEM cells with and without β-caryophyllene. Values are expressed as mean ± SEM (n = 9).
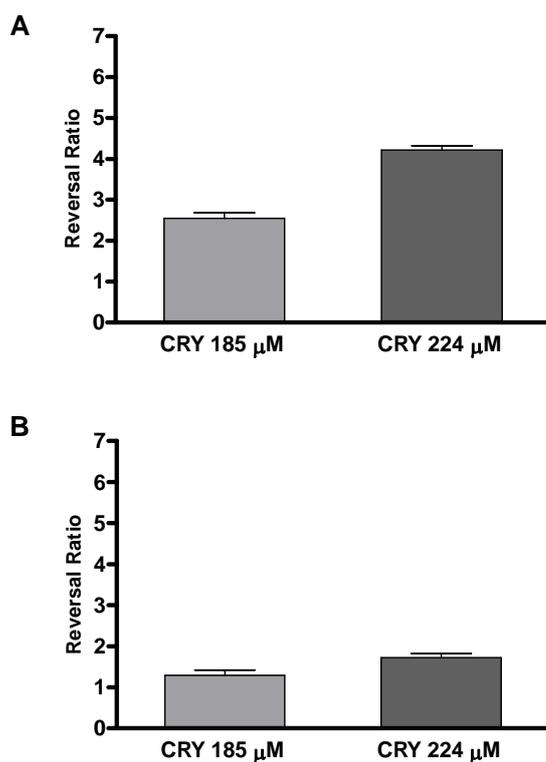

Figure 61. Enhancement of α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) cytotoxicity by combination with β-caryophyllene in CCRF/CEM cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
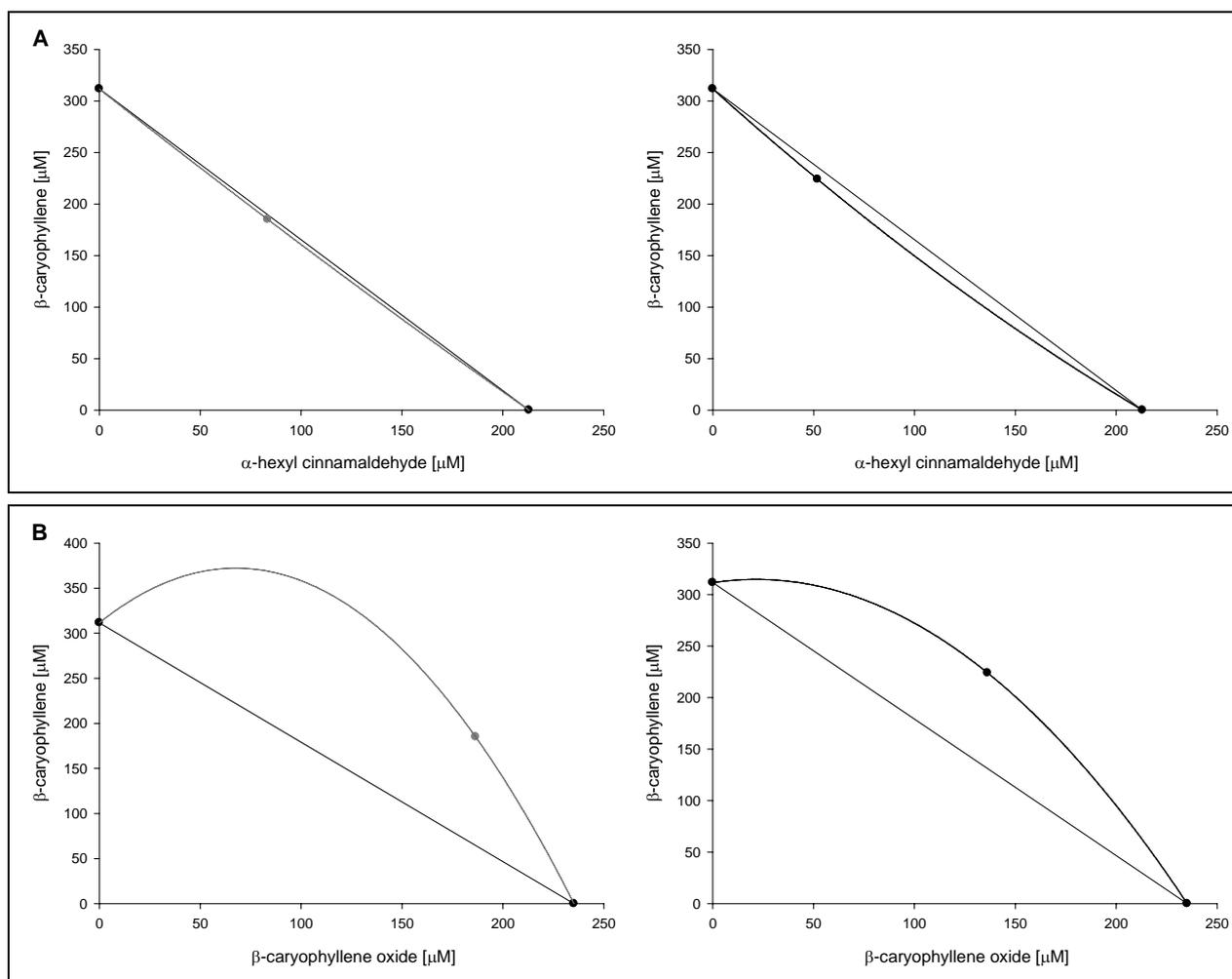
Figure 62. Isobologram analyses in CCRF/CEM cells: IC_{50} concentrations of α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) are plotted on x-axis and IC_{50} values of β-caryophyllene on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

Also leukemia cells (CEM/ADR5000) were significantly (p < 0.001) sensitized by the combination with IC_{10} (180 µM) and IC_{20} (234 µM) of β-caryophyllene (Figure 63). The IC_{50} of doxorubicin was enhanced 1.5-fold (IC_{50} decrease from 74.28 ± 0.35 to 47.23 ± 0.86 µM, CI 1.1 ± 0.02) and 2-fold (IC_{50} decrease from 74.28 ± 0.35 to 35.35 ± 1.33 µM, CI 1.1 ± 0.02) respect to the doxorubicin alone (Figure 64). According to the combination index, the isobologram analysis showed antagonistic effect (Figure 65).
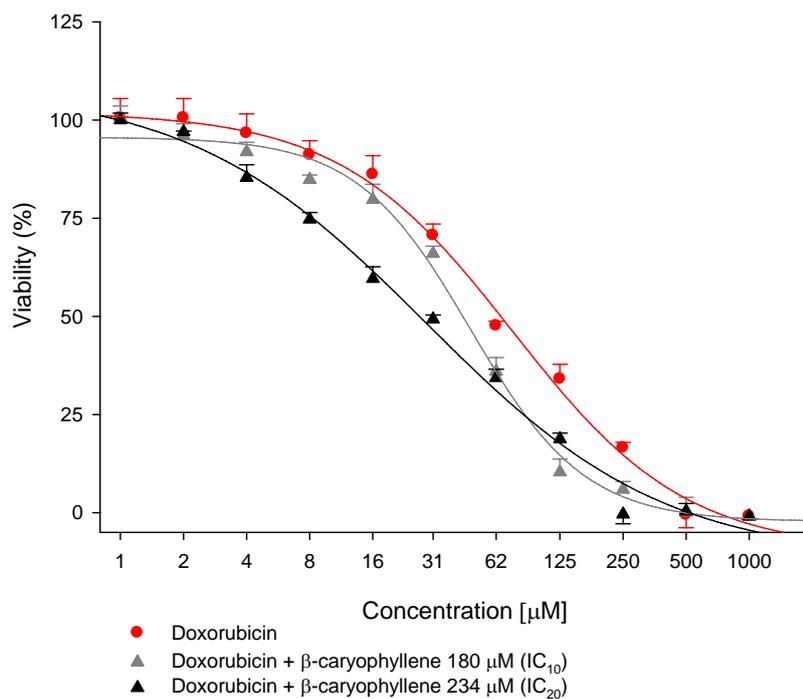
Figure 63. Doxorubicin dose response curve on viability of CEM/ADR5000 cells with and without β-caryophyllene. Values are expressed as mean ± SEM (n = 9).
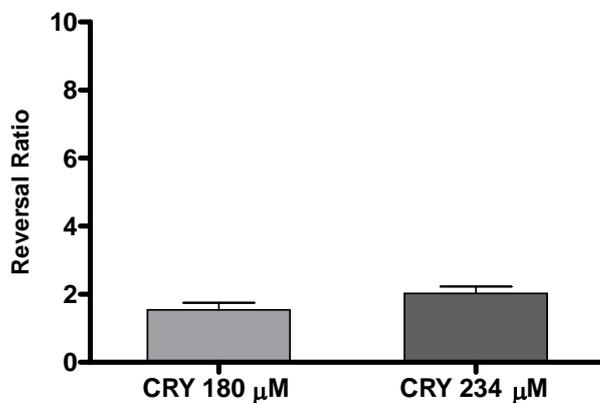
Figure 64. Enhancement of doxorubicin cytotoxicity by combination with β-caryophyllene in CEM/ADR5000 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
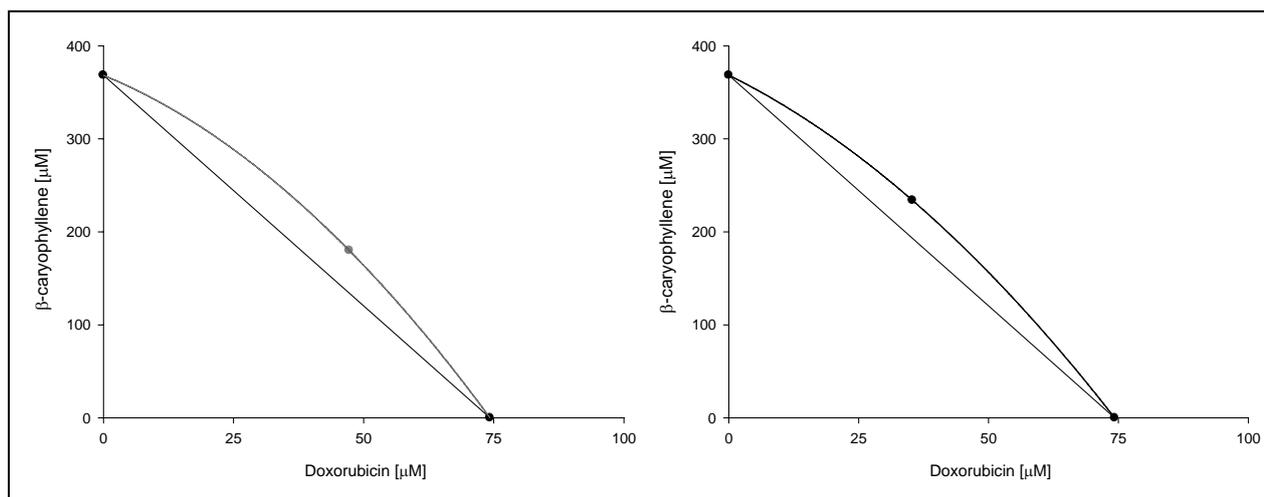
Figure 65. Isobologram analyses in CEM/ADR5000 cells: IC_{50} concentrations of doxorubicin are plotted on x-axis and IC_{50} values of β-caryophyllene on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

In CEM/ADR5000 cells, co-incubation of α-hexylcinnamaldehyde and β-caryophyllene oxide with non-toxic concentrations of β-caryophyllene ($IC_{10} = 180$ µM and $IC_{20} = 234$ µM) resulted in a significant ($p < 0.001$) and synergistic increase in their cytotoxicity (Figure 66A and B).

The IC_{50} value of α-hexylcinnamaldehyde decreased 3-fold (from 256.52 ± 2.27 to 86.31 ± 0.27 µM) and 3.4-fold (from 256.52 ± 2.27 to 73.98 ± 2.11 µM) when combined with EC_{10} and EC_{20} of β-caryophyllene (Figure 67A). The combination index (CI) was 0.84 ± 0.001 and 0.93 ± 0.001 for the lower and highest concentration, respectively.

As regarding β-caryophyllene oxide, the IC_{50} values decreased from 297.98 ± 3.33 to 95.98 ± 2.40 µM (CI 0.84 ± 0.001) and to 80.65 ± 1.71 µM (CI 0.93 ± 0.001) in combination with non toxic concentrations of β-caryophyllene. The cytotoxicity enhancement was equal to 3 and 3.5-fold, respectively (Figure 67B).

According to the combination index, the isobologram analysis showed synergistic effect (Figure 68A and B).
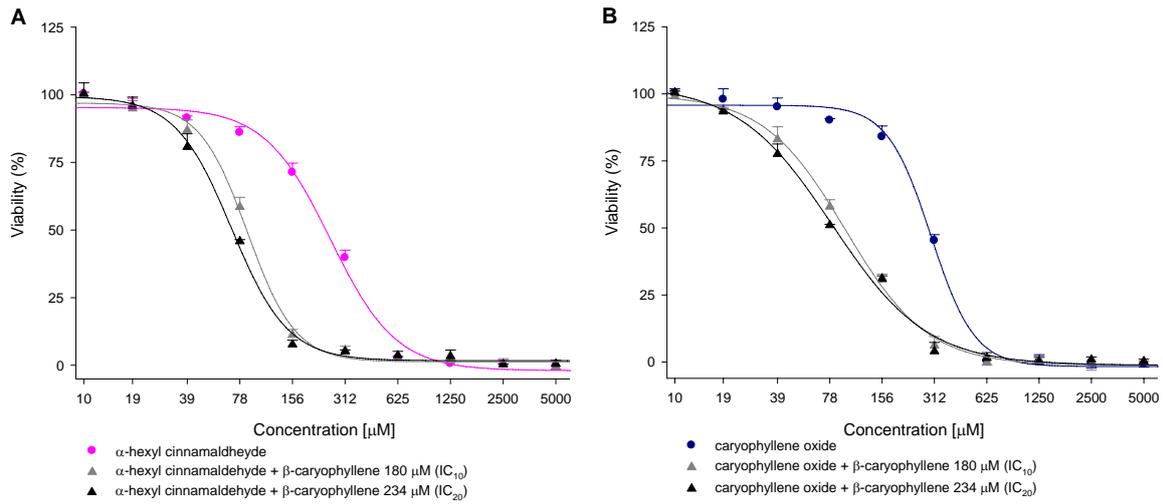
Figure 66. α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) dose response curves on viability of CEM/ADR5000 cells with and without β-caryophyllene. Values are expressed as mean ± SEM (n = 9).
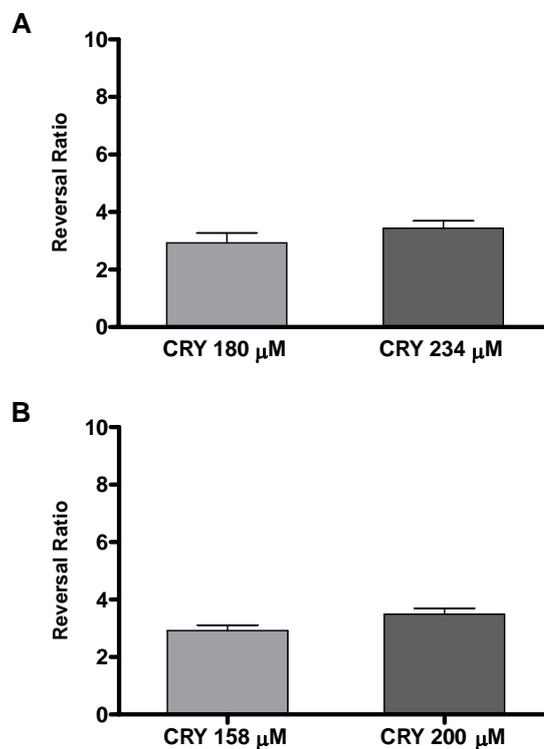

Figure 67. Enhancement of α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) cytotoxicity by combination with β-caryophyllene in CEM/ADR5000 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
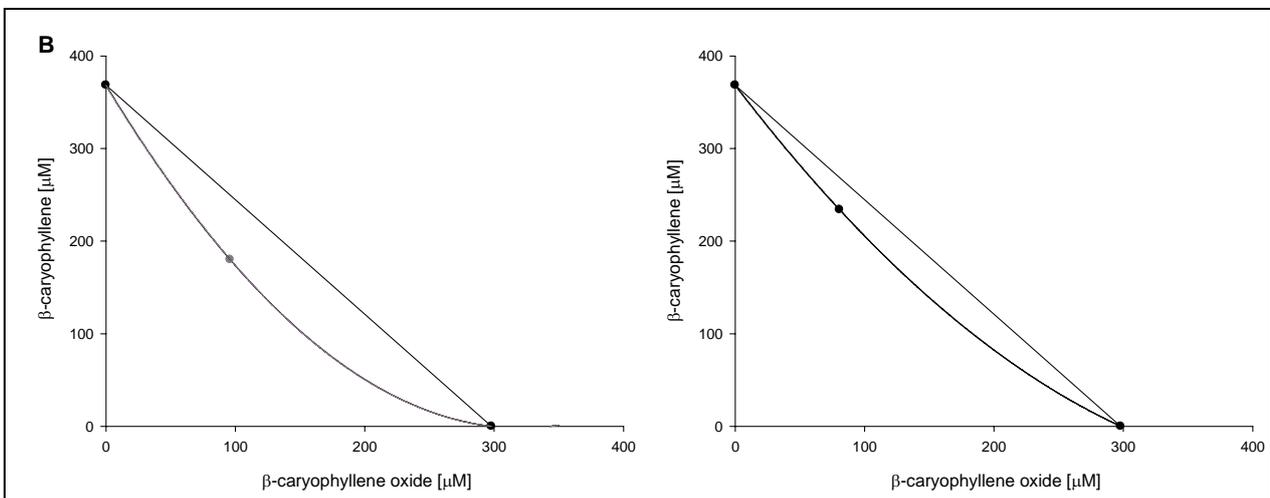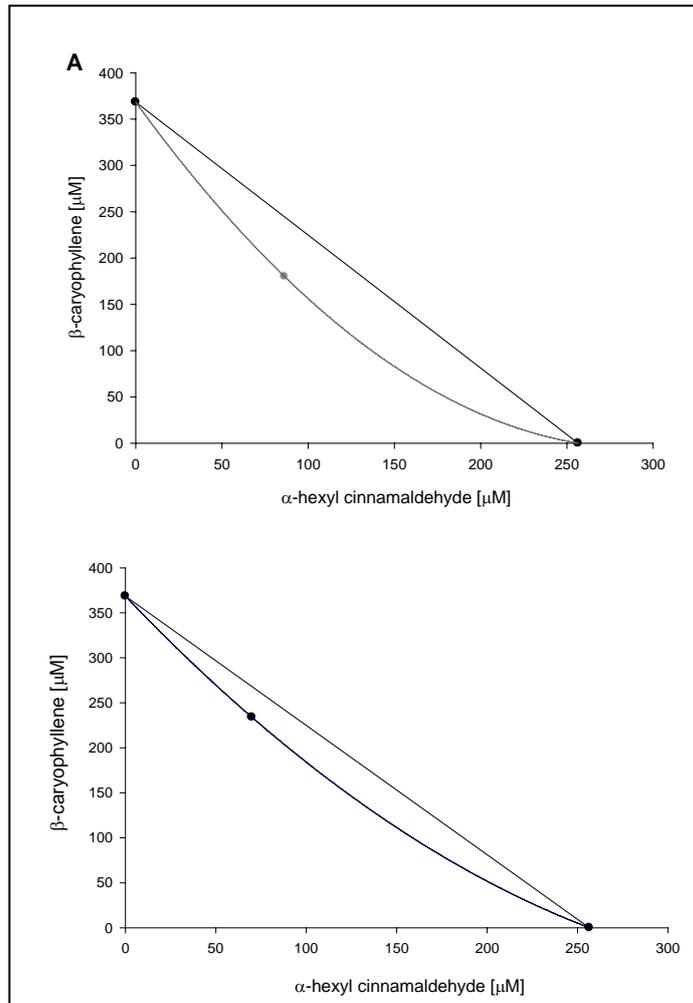
Figure 68. Isobologram analyses in CEM/ADR5000 cells: IC$_{50}$ concentrations of α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) are plotted on x-axis and IC$_{50}$ values of β-caryophyllene on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

In Caco-2 cell line, only the combination with IC_{20} (644 µM) of β-caryophyllene determined a statistically ($p < 0.001$) decrease of the IC_{50} value of doxorubicin (from 5.24 ± 0.66 to 1.15 ± 0.003 µM, CI 0.81 ± 0.03). Also the combination with IC_{10} of β-caryophyllene reduced the IC_{50} value of doxorubicin, but not in a statistically significant manner (from 5.24 ± 0.66 to 3.71 ± 0.05 µM) (Figure 69). The enhancement of cytotoxicity of the antitumoral agent in combination with 644 µM of β-caryophyllene was equal to 4.5-fold (Figure 70). As regarding the isobologram analysis, it was in agreement with the combination index and it showed a synergistic effect (Figure 71).
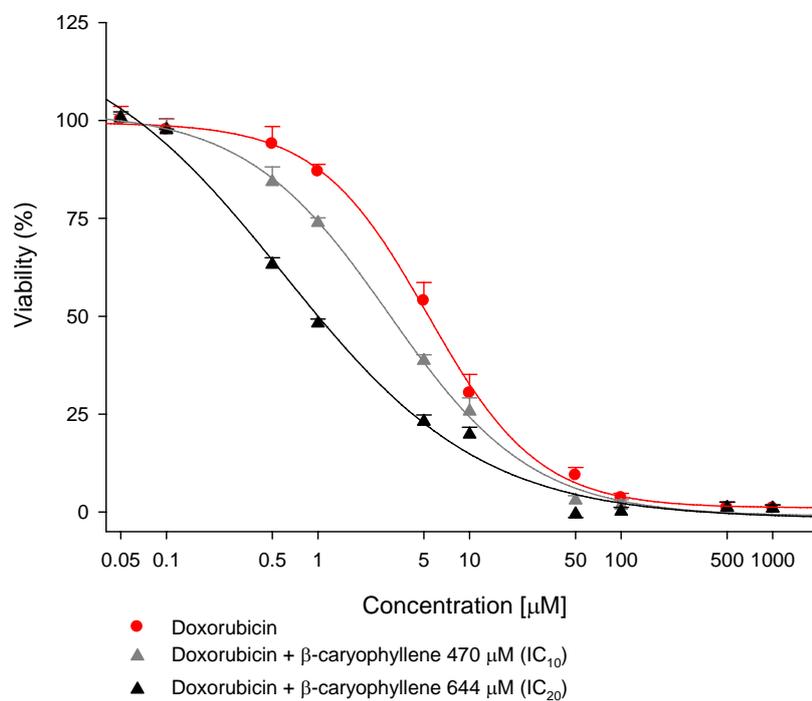

Figure 69. Doxorubicin dose response curve on viability of Caco-2 cells with and without β-caryophyllene. Values are expressed as mean ± SEM (n = 9).
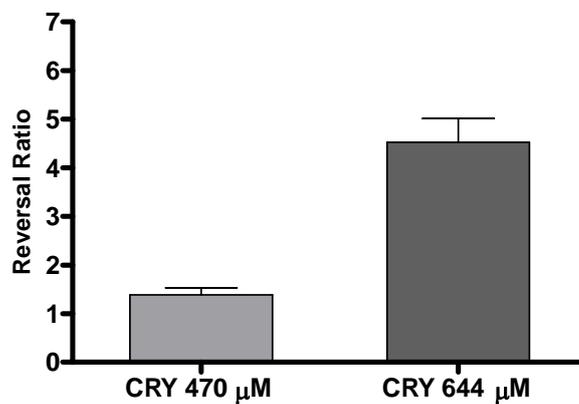
Figure 70. Enhancement of doxorubicin cytotoxicity by combination with β-caryophyllene in Caco-2 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
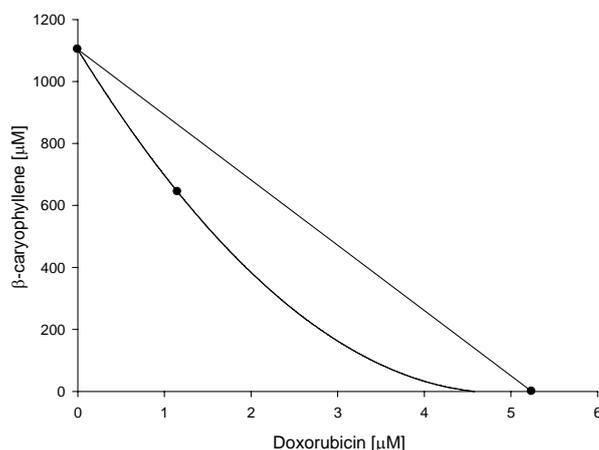

Figure 71. Isobologram analyses in Caco-2 cells: IC_{50} concentrations of doxorubicin is plotted on x-axis and IC_{50} values of β-caryophyllene on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

The co-incubation of α-hexylcinnamaldehyde with non-toxic concentrations of β-caryophyllene (IC_{10} = 470 μM and IC_{20} = 644 μM), in Caco-2 cell line, resulted in a significant (p < 0.001) increase in its cytotoxicity (Figure 72A).

The IC_{50} value of α-hexylcinnamaldehyde decreased from 315.66 ± 4.75 to 200.83 ± 4.09 μM (CI 1.1 ± 0.01) and from 315.66 ± 4.75 to 184.33 ± 3.36 μM (CI 1.2 ± 0.01) when combined with both concentrations of β-caryophyllene. The reversal ratio indicated a cytotoxicity enhancement equal to 1.5 and 1.6-fold in presence of IC_{10} and IC_{20} of chemosensitizer (Figure 73A). In this context, the isobologram analysis was in agreement with the combination index and it showed antagonistic effect (Figure 74A).

Also the cytotoxicity of β-caryophyllene oxide was enhanced by the combination with IC_{10} and IC_{20} of β-caryophyllene, but just in the second case it was statistically significant (p < 0.001) (Figure 72B). The IC_{50} value of β-caryophyllene oxide decreased from 332.30 ± 3.97 to 297.07 ± 4.89 μM (RR 1.1) and from 332.30 ± 3.97 to 246.21 ± 9.78 μM (RR 1.3 , CI 1.4 ± 0.01) when combined with both concentrations of β-caryophyllene (Figure 73B).

The isobologram analysis, according to the combination index, showed an antagonistic effect (Figure 74B).
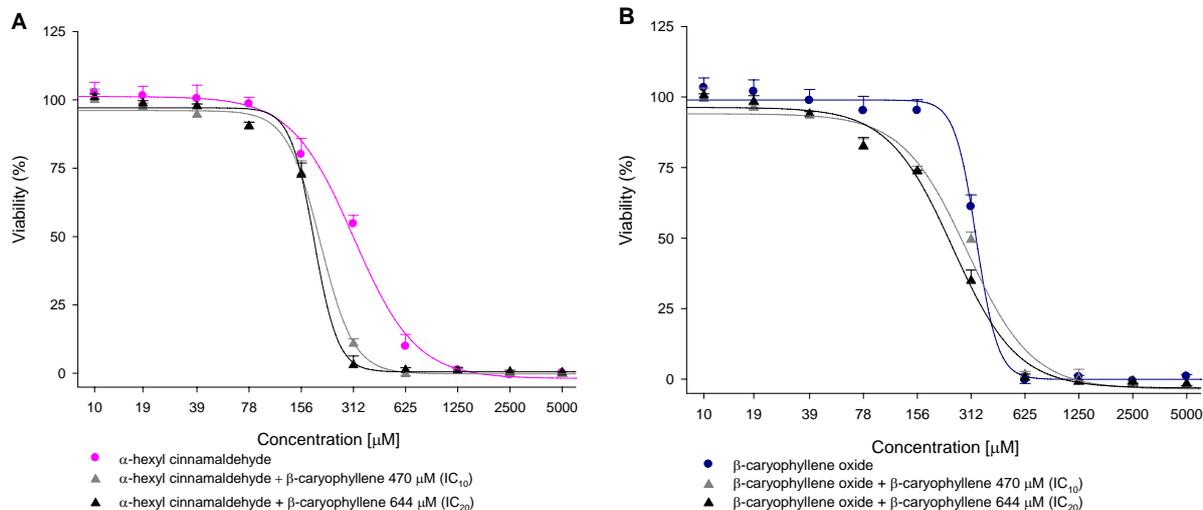
Figure 72. α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) dose response curves on viability of Caco-2 cells with and without β-caryophyllene. Values are expressed as mean ± SEM (n = 9).
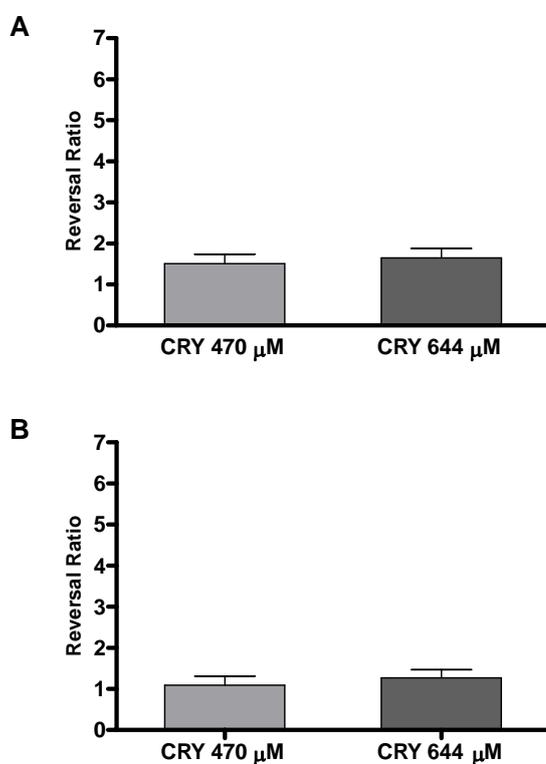

Figure 73. Enhancement of α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) cytotoxicity by combination with β-caryophyllene in Caco-2 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
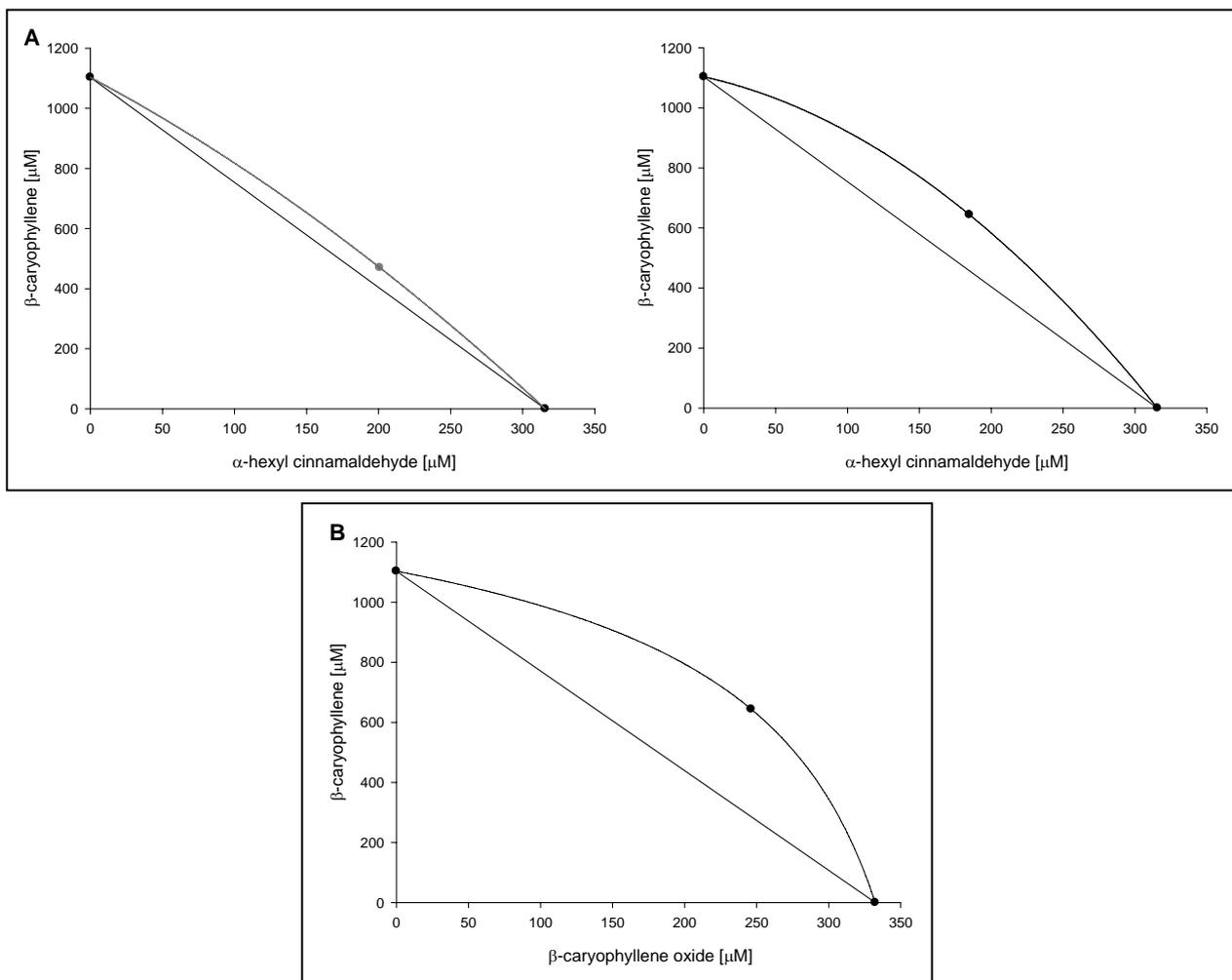
Figure 74. Isobologram analyses in Caco-2 cells: IC$_{50}$ concentrations of α-hexylcinnamaldehyde (A) and β-caryophyllene oxide (B) are plotted on x-axis and IC$_{50}$ values of β-caryophyllene on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

The cytotoxicity and reversal data presented above suggest that β-caryophyllene effectively interfere with at least one mechanism of drug inactivation, especially drug export. To explore this possibility, rhodamine123 retention assay was performed to determine ABC-transporter activity in the cells.

The intracellular fluorescence of Rho123 was significantly increased in a dose-dependent manner, as shown in Figure 75, in Caco-2 cells which were treated with the test substance. Results were expressed as % inhibition of P-gp relative to the positive control verapamil (100%). β-Caryophyllene was able to inhibite the MDR efflux pumps. At the same concentration of the positive control (20 μM), the fluorescence intensity was 99.2 ± 2.4%. These results indicate that the test compound is an inhibitor of ABC-transporters.

In addition, efflux assays were quantified using flow cytometry in CEM/ADR5000 cells. The intracellular fluorescence of Rho123 was significantly increased in CEM/ADR5000 cells when treated with CRY. β-caryophyllene (Figure 76) inhibited the P-gp pump, but with less potency respect to the positive control verapamil. At the concentration of 10 μM, the fluorescence intensity was 79.0 ± 3.4% for β-caryophyllene. The figure 77 shows flow cytometry histograms of β-caryophyllene.
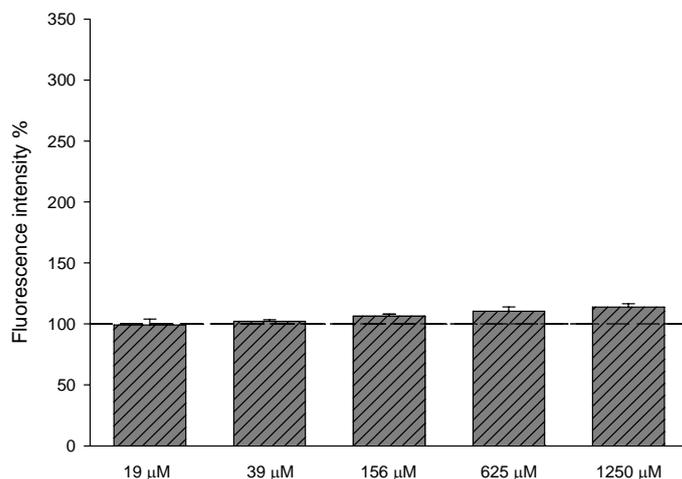

Figure 75.   Effect of β-caryophyllene on Rho 123 retention in Caco-2 cells. Fluorescence intensity was measured by spectrofluorometry. Data are presented as means ± ES of fluorescence intensity % related to verapamil as positive control (dotted line).

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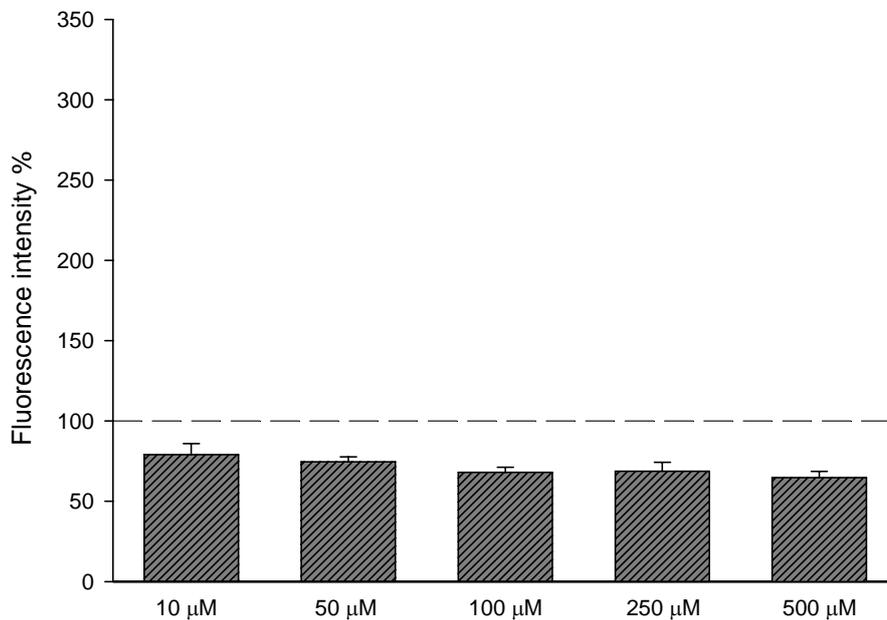
Figure 76. Effect of β-caryophyllene on Rho 123 retention in CEM/ADR5000 cells. Fluorescence intensity was measured using FACS. Data are presented as means ± ES of fluorescence intensity % related to verapamil as positive control (dotted line).
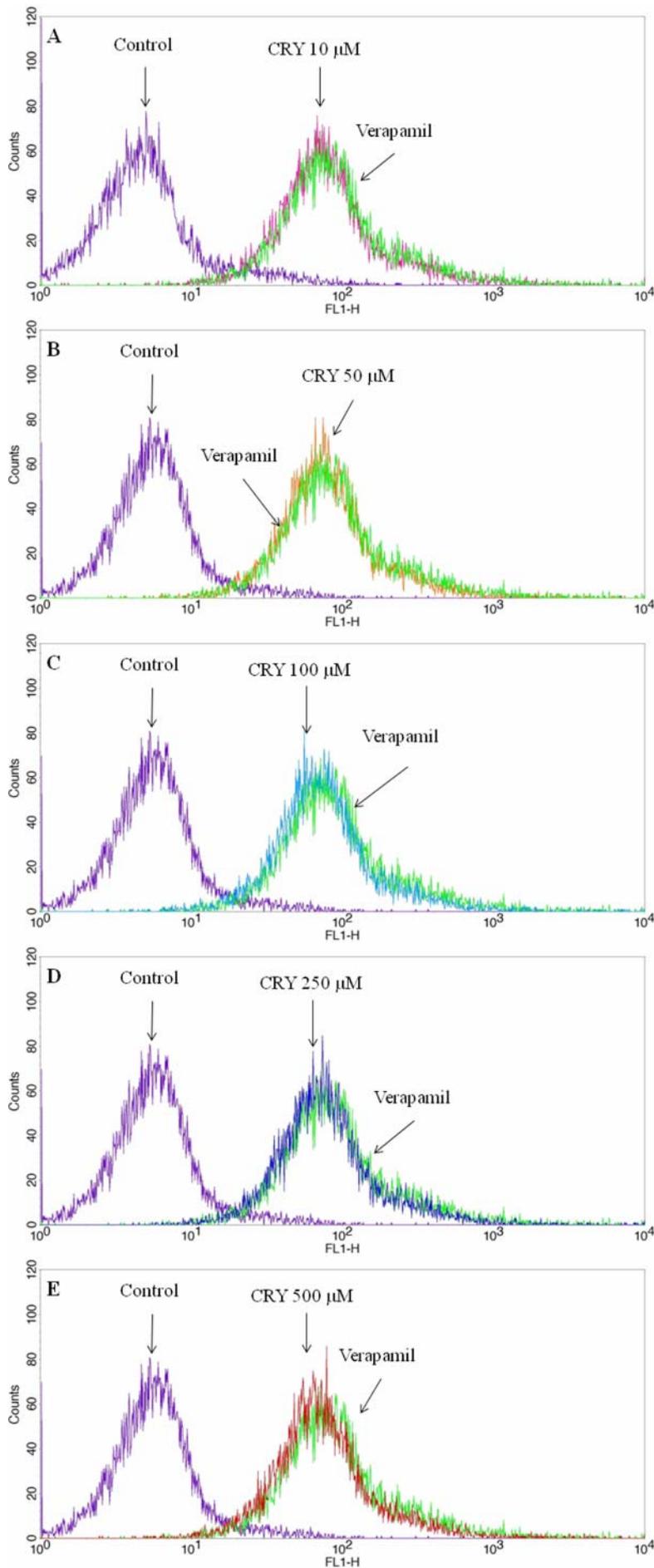
Figure 77. Flow cytometry histograms β-caryophyllene (A-E). The fluorescence intensity was shifted to the right side indicating that the resistance cell line CEM/ADR5000 retain the Rho 123 as response to treatment with MDR1 inhibitors. Fluorescence intensity was measured using FACS.

β-Caryophyllene oxide

Co-incubation of doxorubicin with very low and non-toxic concentrations of β-caryophyllene oxide (IC_{10} = 50 µM and IC_{20} = 100 µM), in sensitive CCRF/CEM cell line, resulted in a significant ($p < 0.001$) increase of doxorubicin cytotoxicity (Figure 78).

The IC_{50} value of doxorubicin decreased 4-fold (from 0.42 ± 0.02 to 0.10 ± 0.01µM) and 6-fold (from 0.42 ± 0.02 to 0.06 ± 0.001 µM) when combined with non-toxic concentrations of β-caryophyllene oxide (Figure 79). The combination index (CI) was equal to 0.49 ± 0.05 and 0.58 ± 0.01 for the lower and highest concentration, respectively, and it indicated synergistic effect. The corresponding isobolograms are reported in Figure 80.
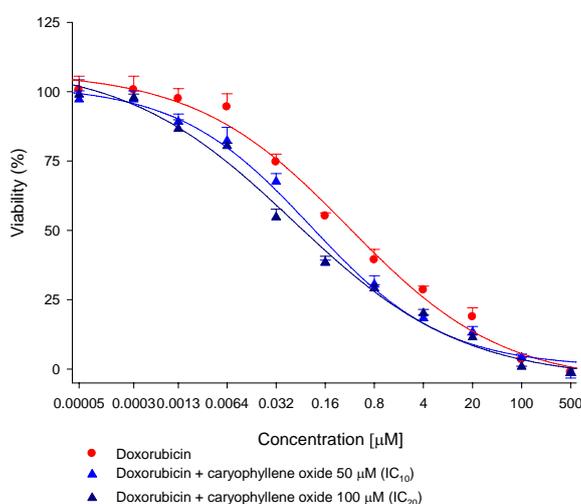
**Figure 78.** Doxorubicin dose response curve on viability of CCRF/CEM cells with and without β-caryophyllene oxide. Values are expressed as mean ± SEM (n = 9).
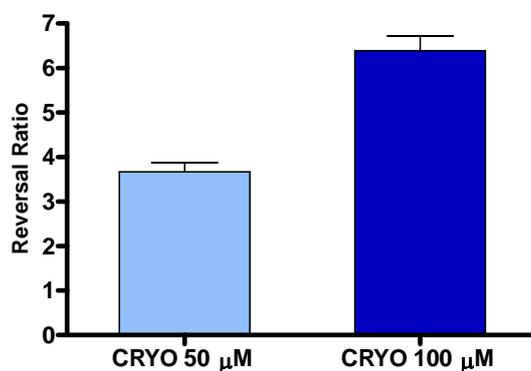

Figure 79. Enhancement of doxorubicin cytotoxicity by combination with β-caryophyllene oxide in CCRF/CEM cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
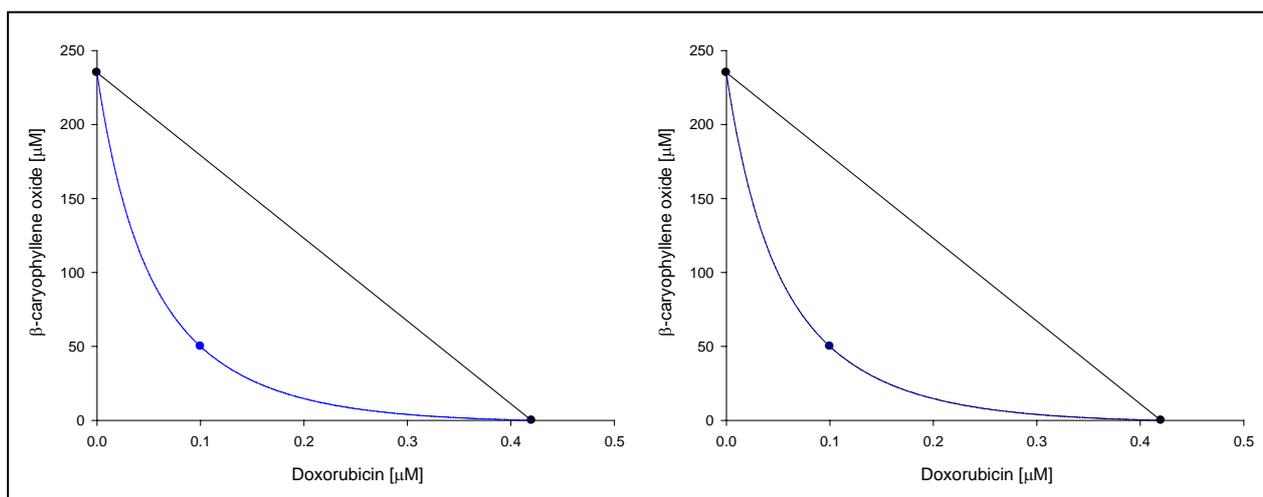
Figure 80. Isobologram analyses in CCRF/CEM cells: IC_{50} concentrations of doxorubicin are plotted on x-axis and IC_{50} values of β-caryophyllene on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

Also, the combination of α-hexylcinnamaldehyde and β-caryophyllene with IC_{10} and IC_{20} of β-caryophyllene oxide resulted in a significant ($p < 0.001$) increase in their cytotoxicity (Figure 81A and B).

The IC_{50} value of α-hexylcinnamaldehyde decreased 3-fold (from 212.95 ± 6.36 to 68.04 ± 1.15 µM) and 5-fold (from 212.95 ± 6.36 to 36.52 ± 2.28 µM) when combined with non-toxic concentrations of β-caryophyllene (Figure 82A). The combination index (CI) was 0.52 ± 0.01 and 0.60 ± 0.01 for the lower and highest concentrations, respectively; CI indicated a synergistic effect, as the isobolograms (Figure 83A).

Furthermore, the IC_{50} value of β-caryophyllene decreased from 311.59 ± 14.36 to 276.17 ± 4.03 and to 253.89 ± 2.91 µM (RR , CI 1.2 ± 0.04) in combination with IC_{10} and IC_{20} of β-caryophyllene oxide (Figure 82B), but only in the second case was statistically significant. In this context, the combination index indicated an antagonistic affect and it was in agreement with the isobologram analysis (Figure 83B).
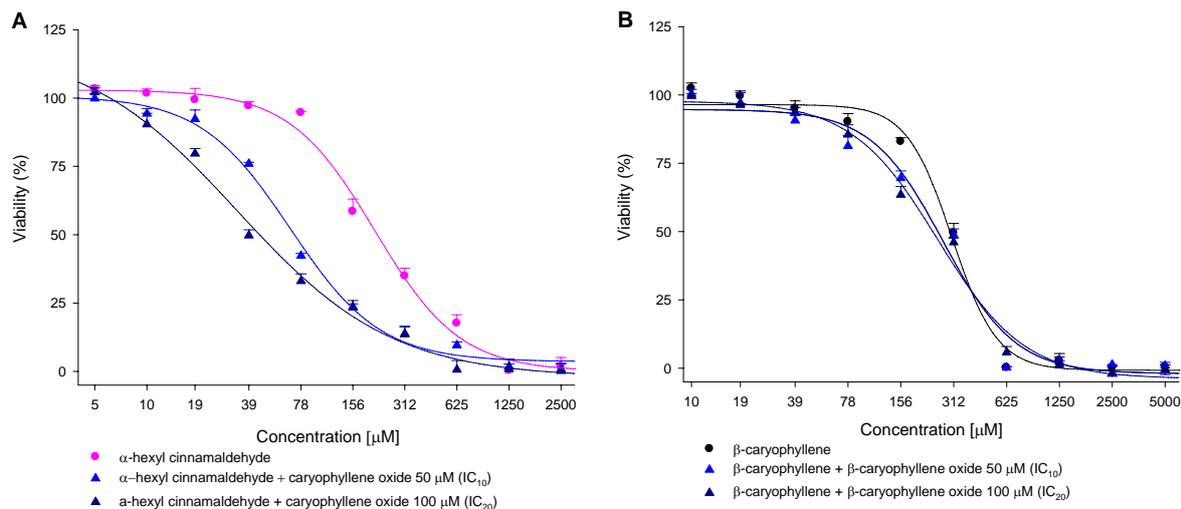
Figure 81. α-hexylcinnamaldehyde (A) and β-caryophyllene (B) dose response curves on viability of CCRF/CEM cells with and without β-caryophyllene oxide. Values are expressed as mean ± SEM (n = 9).
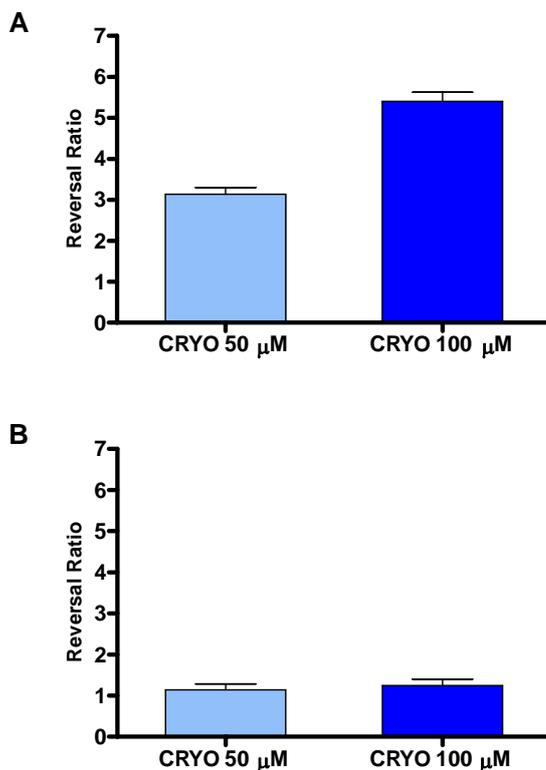
Figure 82. Enhancement of α-hexylcinnamaldehyde (A) and β-caryophyllene (B) cytotoxicity by combination with β-caryophyllene oxide in CCRF/CEM cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
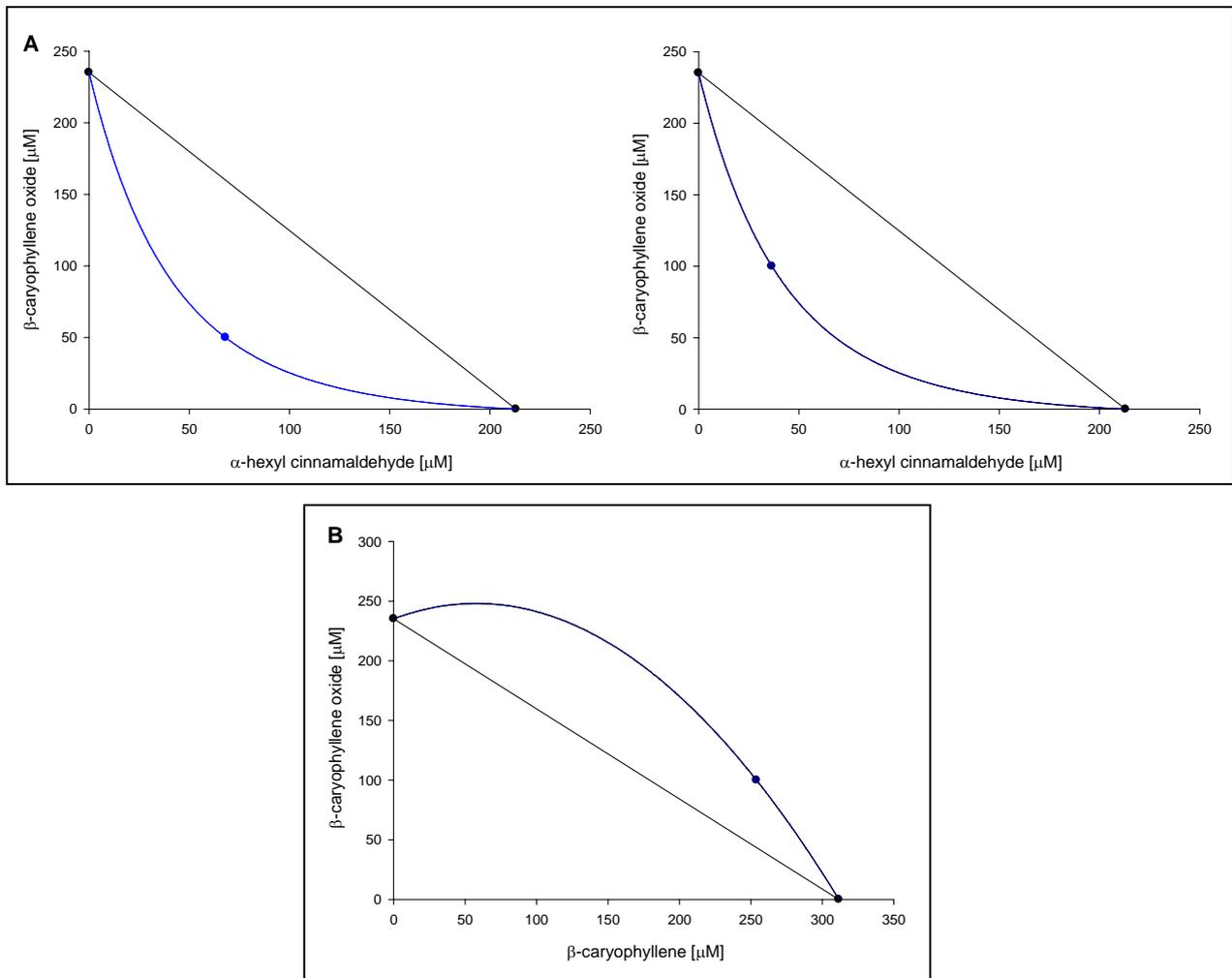
Figure 83. Isobologram analyses in CCRF/CEM cells: IC_{50} concentrations of α-hexylcinnamaldehyde (A) and β-caryophyllene (B) are plotted on x-axis and IC_{50} values of β-caryophyllene oxide on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

Also leukemia cells (CEM/ADR5000) were significantly (p < 0.001) sensitized by the combination with IC_{10} (158 µM) and IC_{20} (200 µM) of β-caryophyllene oxide (Figure 84). The IC_{50} of doxorubicin was enhanced 1.6-fold (IC_{50} decrease from 74.28 ± 0.35 to 45.79 ± 1.12 µM, CI 1.2 ± 0.03) and 2.6-fold (IC_{50} decrease from 74.28 ± 0.35 to 29.44 ± 0.70 µM, CI 1.1 ± 0.02) respect to the doxorubicin alone (Figure 85). According to the combination index, the isobologram analysis showed an antagonistic effect (Figure 86).
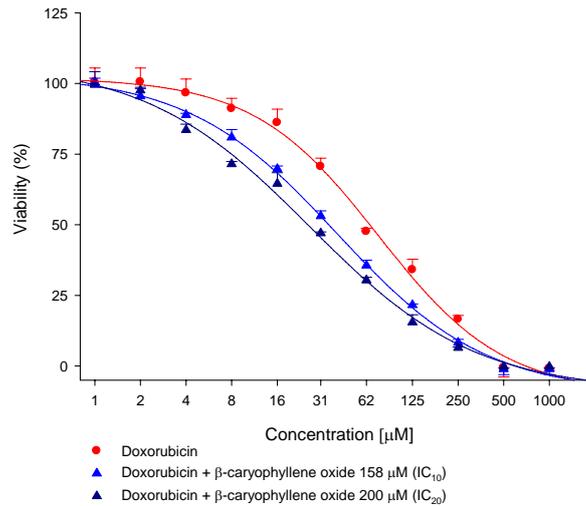
Figure 84. Doxorubicin dose response curve on viability of CEM/ADR5000 cells with and without β-caryophyllene oxide. Values are expressed as mean ± SEM (n = 9).
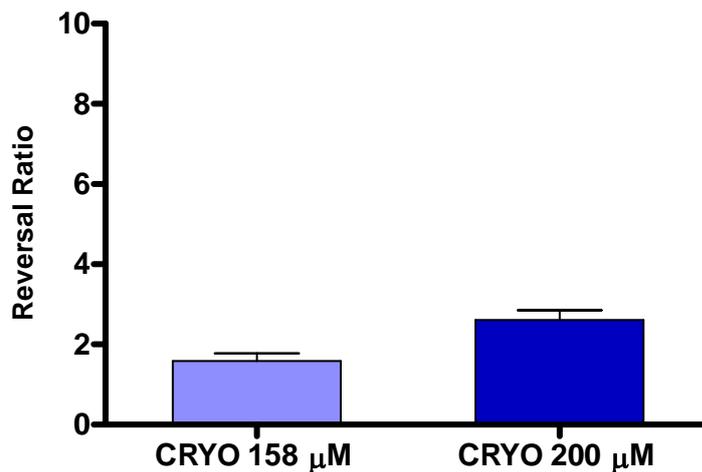

Figure 85. Enhancement of doxorubicin cytotoxicity by combination with β-caryophyllene oxide in CEM/ADR5000 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
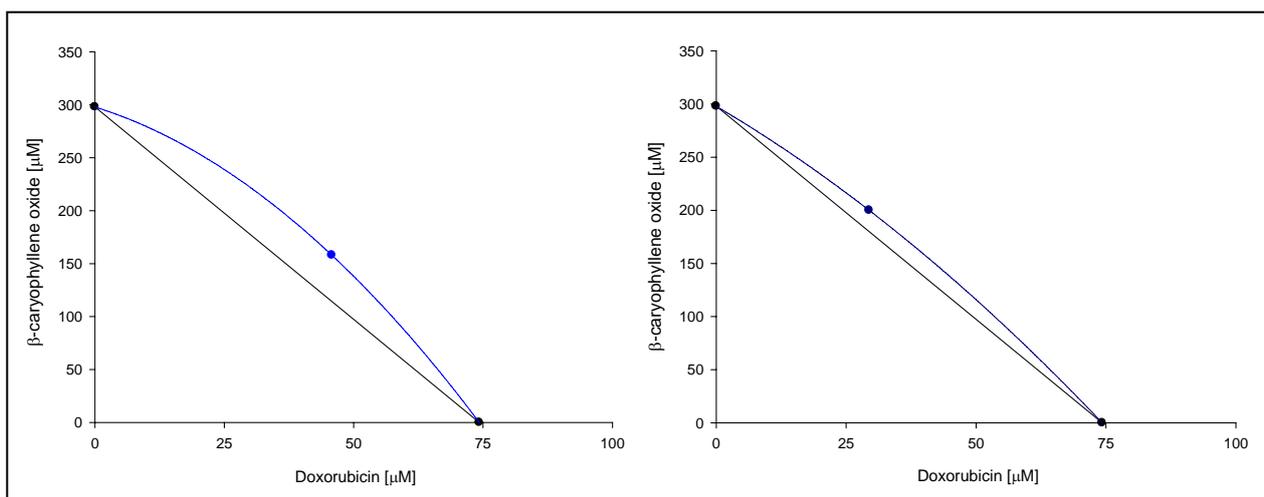
Figure 86.   Isobologram analyses in CEM/ADR5000 cells: IC_{50} concentrations of doxorubicin are plotted on x-axis and IC_{50} values of β-caryophyllene oxide on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

In CEM/ADR5000 cells, co-incubation of α-hexylcinnamaldehyde and β-caryophyllene with non-toxic concentrations of β-caryophyllene oxide (IC_{10} = 158 µM and IC_{20} = 200 µM) resulted in a significant ($p < 0.001$) increase in their cytotoxicity (Figure 87A and B).

The IC_{50} value of α-hexylcinnamaldehyde decreased 2.8-fold (from 256.52 ± 2.27 to 84.80 ± 2.94 µM) and 3.5-fold (from 256.52 ± 2.27 to 73.26 ± 0.06 µM) when combined with IC_{10} and IC_{20} of β-caryophyllene oxide (Figure 88A). The combination index (CI) was 0.90 ± 0.002 and 0.97 ± 0.01 for the lower and highest concentration, respectively.

Regarding as β-caryophyllene, the IC_{50} values decreased from 368.48 ± 2.23 to 255.08 ± 3.05 µM (CI 1.3 ± 0.002) and to 194.13 ± 3.98 µM (CI 1.2 ± 0.001) in combination with non toxic concentrations of β-caryophyllene oxide. The cytotoxicity enhancement was equal to 1.4 and 1.8-fold, respectively (Figure 88B).

According to the combination index, the isobologram analysis showed synergistic effect for the combination between α-hexylcinnamaldehyde and β-caryophyllene oxide, and antagonistic effect for the combination between β-caryophyllene and β-caryophyllene oxide (Figure 89A and B).
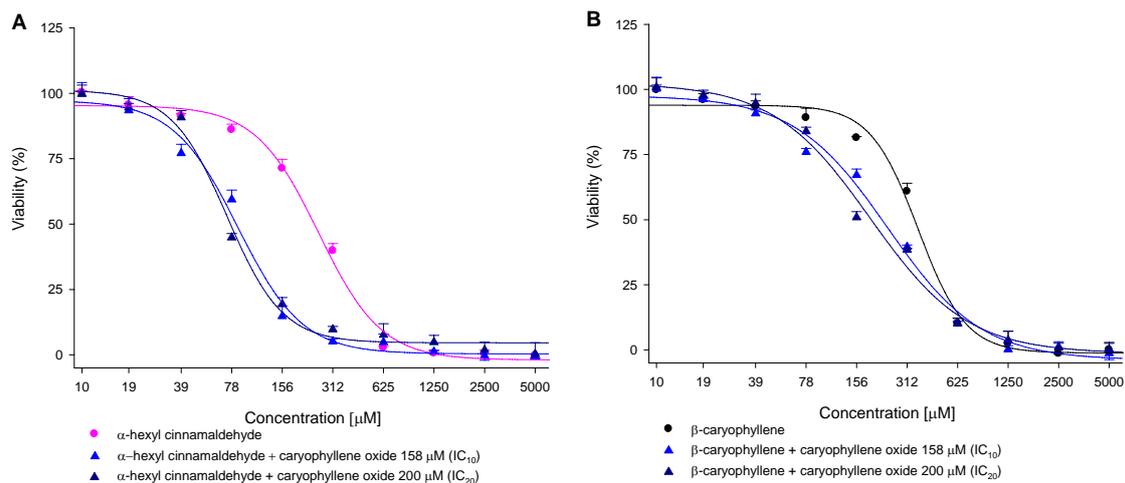
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Figure 87. α-hexylcinnamaldehyde (A) and β-caryophyllene (B) dose response curves on viability of CEM/ADR5000 cells with and without β-caryophyllene oxide. Values are expressed as mean ± SEM (n = 9).
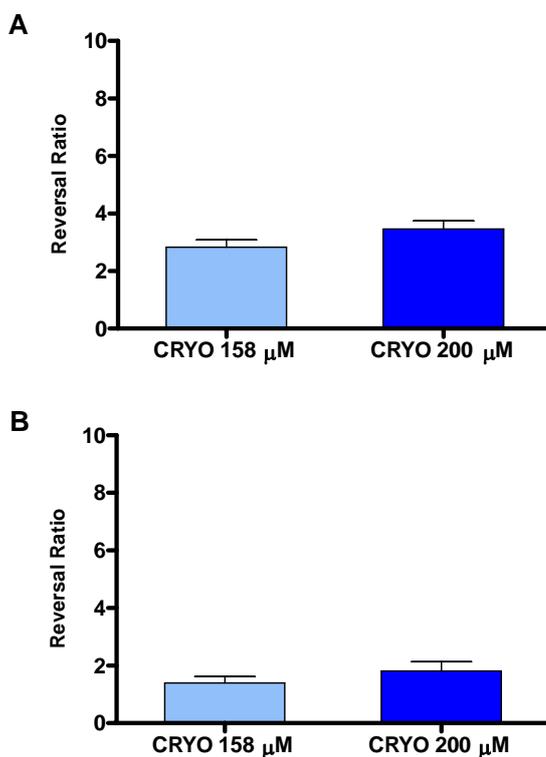
Figure 88. Enhancement of α-hexylcinnamaldehyde (A) and β-caryophyllene (B) cytotoxicity by combination with β-caryophyllene oxide in CEM/ADR5000 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).

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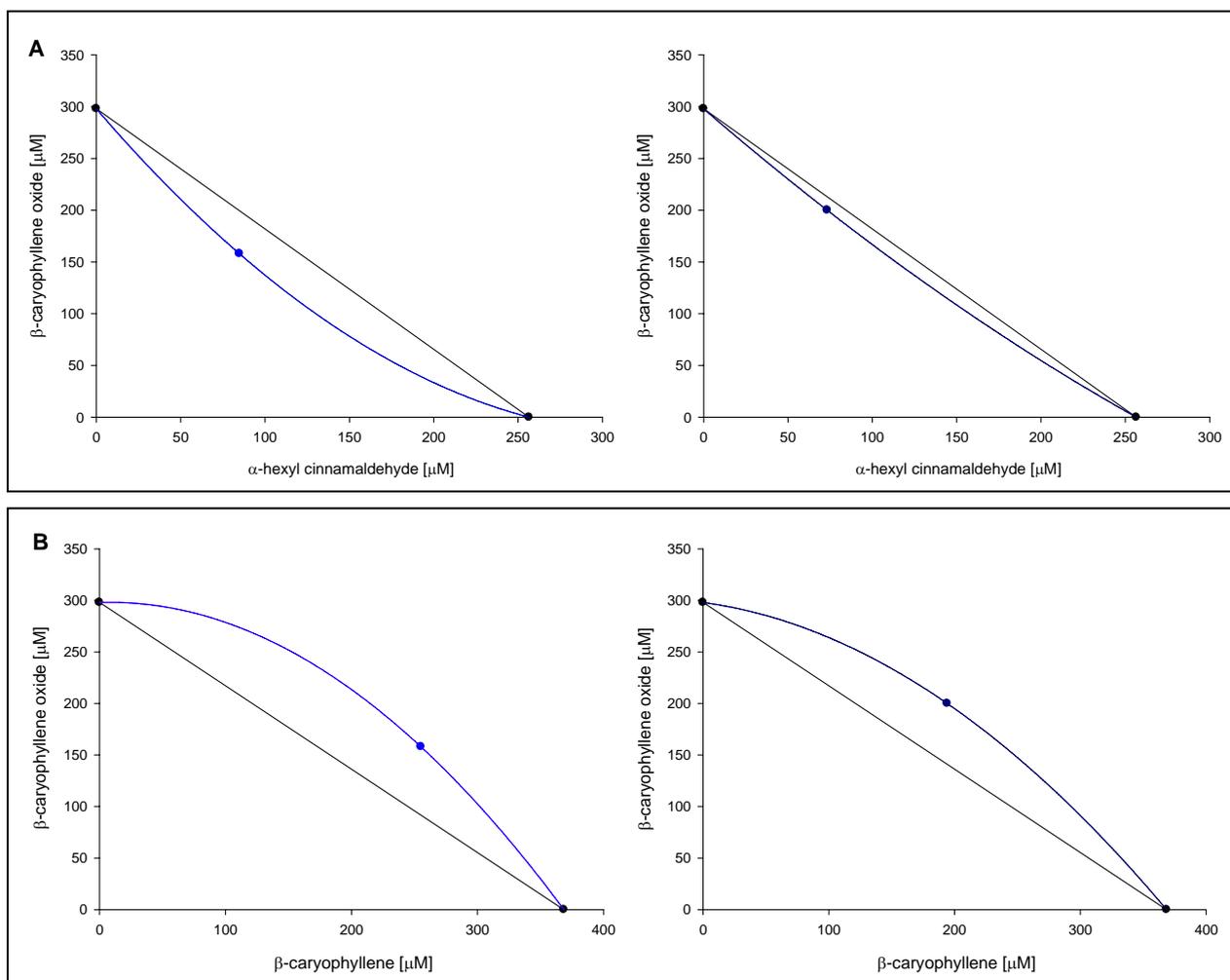
Figure 89. Isobologram analyses in CEM/ADR5000 cells: IC_{50} concentrations of α-hexylcinnamaldehyde (A) and β-caryophyllene (B) are plotted on x-axis and IC_{50} values of β-caryophyllene oxide on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

In Caco-2 cell line, the combination with IC_{10} (100 μM) and IC_{20} (248 μM) of β-caryophyllene oxide determined a statistically (p < 0.01 and p < 0.001) decrease of the IC_{50} value of doxorubicin (Figure 90). The enhancement of the antitumoral agent cytotoxicity was equal to 2-fold (IC_{50} decrease from 5.24 ± 0.66 to 2.57 ± 0.04 μM, CI 0.82 ± 0.06) and 4.7-fold (IC_{50} decrease from 5.24 ± 0.66 to 1.02 ± 0.06 μM, CI 0.96 ± 0.03) in combination with the lower and the highest concentrations of β-caryophyllene oxide, respectively (Figure 91). Regarding as the

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isobologram analysis, it was in agreement with the combination index and it showed a synergistic effect (Figure 92).
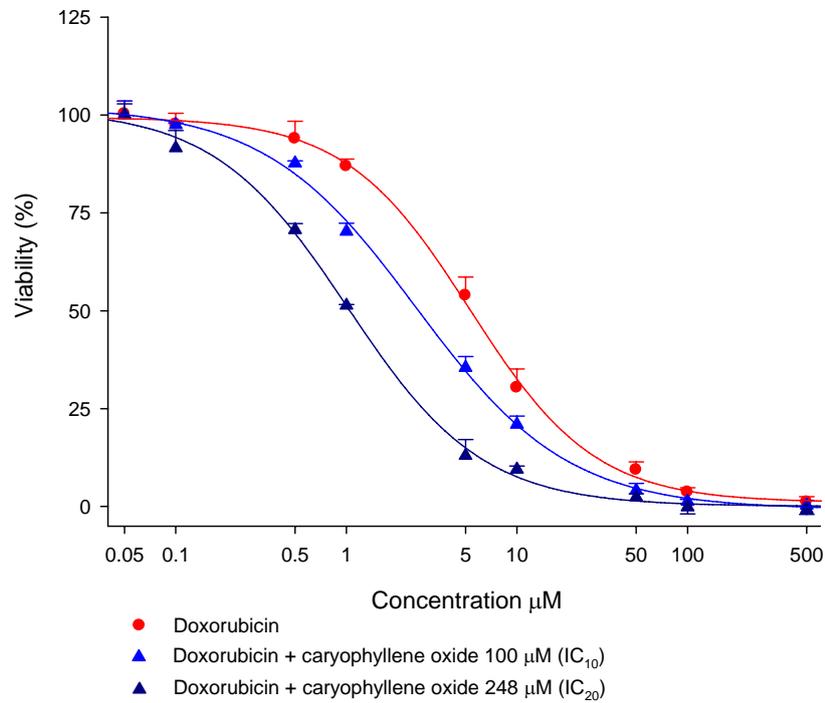
Figure 90. Doxorubicin dose response curve on viability of Caco-2 cells with and without β-caryophyllene oxide. Values are expressed as mean ± SEM (n = 9).
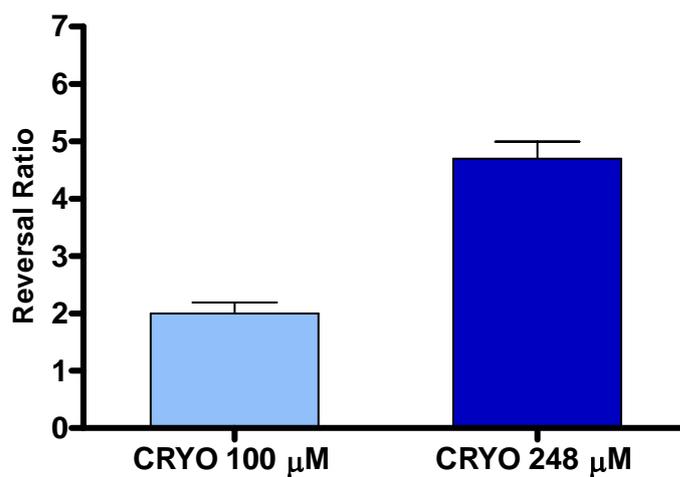
Figure 91. Enhancement of doxorubicin cytotoxicity by combination with β-caryophyllene oxide in Caco-2 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
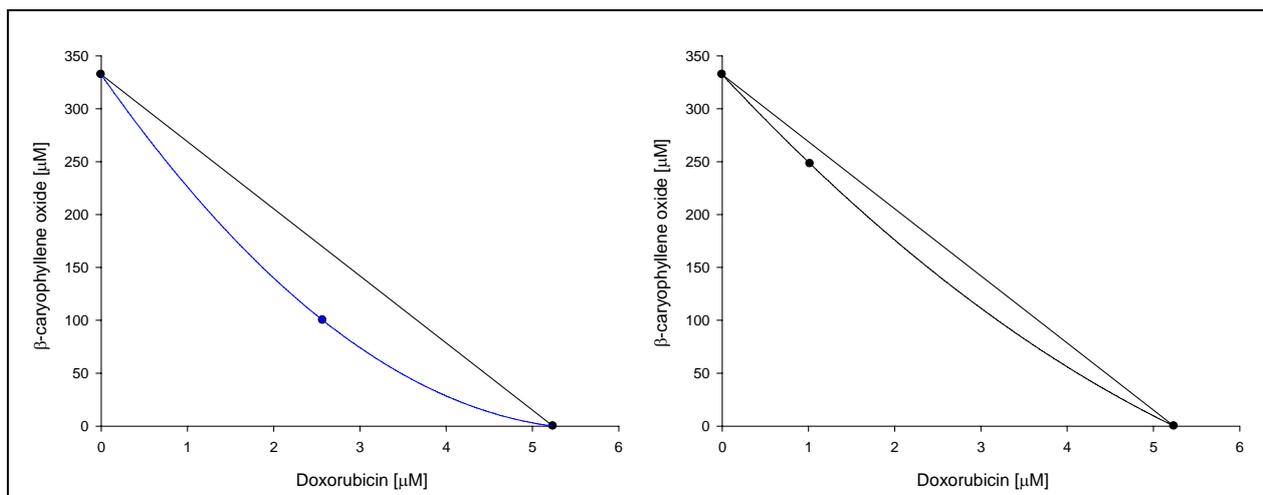
Figure 92. Isobologram analyses in Caco-2 cells: IC$_{50}$ concentrations of doxorubicin is plotted on x-axis and IC$_{50}$ values of β-caryophyllene oxide on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

The co-incubation of α-hexylcinnamaldehyde with non-toxic concentrations of β-caryophyllene oxide (IC$_{10}$ = 100 μM and IC$_{20}$ = 248 μM), in Caco-2 cell line, resulted in a significant (p < 0.001) increase in its cytotoxicity (Figure 93A).

The IC$_{50}$ value of α-hexylcinnamaldehyde decreased from 315.66 ± 4.75 to 189.30 ± 4.10 μM (CI 0.93 ± 0.01) and from 315.66 ± 4.75 to 174.32 ± 4.03 μM (CI 1.3 ± 0.01) when combined with both concentrations of β-caryophyllene oxide. The reversal ratio indicated a cytotoxicity enhancement equal to 1.6 and 1.7-fold in presence of IC$_{10}$ and IC$_{20}$ of chemosensitizer (Figure 94A). In this context, the isobologram analysis was in agreement with the combination index and it showed synergistic effect for the combination with the lower concentration and antagonistic effect with the highest concentration (Figure 95A).

Also the cytotoxicity of β-caryophyllene was enhanced by the combination with IC$_{10}$ and IC$_{20}$ of β-caryophyllene oxide, but just in the second case it was statistically significant (p < 0.001) (Figure 93B). The IC$_{50}$ value of β-caryophyllene decreased from 1103.34 ± 17.32 to 1034.01 ± 18.97 μM (RR 1) and from 1103.34 ± 17.32 to 814.21 ± 15.77 μM (RR 1.3 , CI 1.5 ± 0.01) when combined with both concentrations of β-caryophyllene oxide (Figure 94B).

The isobologram analysis, according to the combination index, showed an antagonistic effect (Figure 95B).
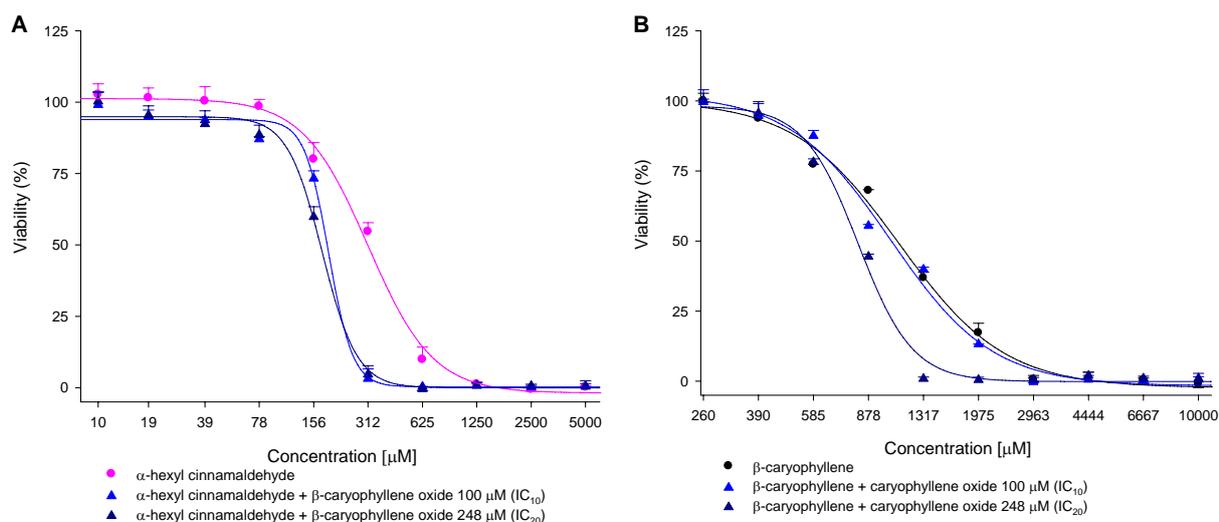
Figure 93. α-hexylcinnamaldehyde (A) and β-caryophyllene (B) dose response curves on viability of Caco-2 cells with and without β-caryophyllene oxide. Values are expressed as mean ± SEM (n = 9).
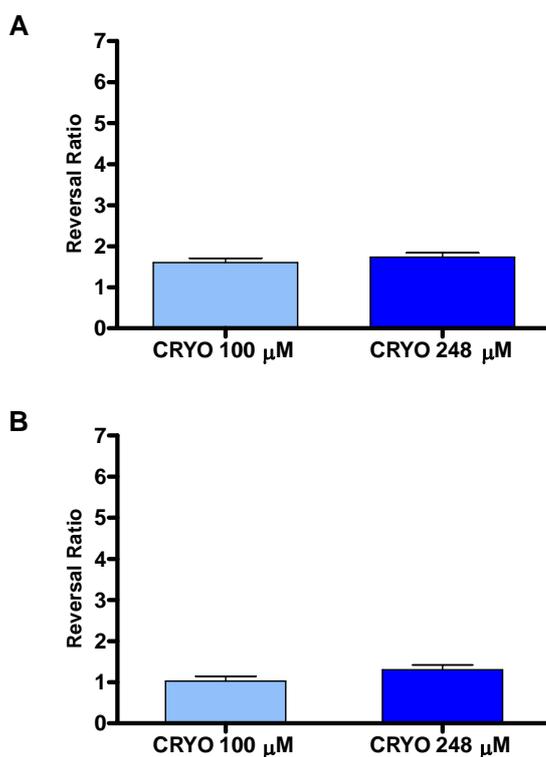
**Figure 94.** Enhancement of α-hexylcinnamaldehyde (A) and β-caryophyllene (B) cytotoxicity by combination with β-caryophyllene oxide in Caco-2 cells. A ratio greater than 1 indicates higher cytotoxicity enhancement factors. Values are expressed as mean ± SEM (n = 3).
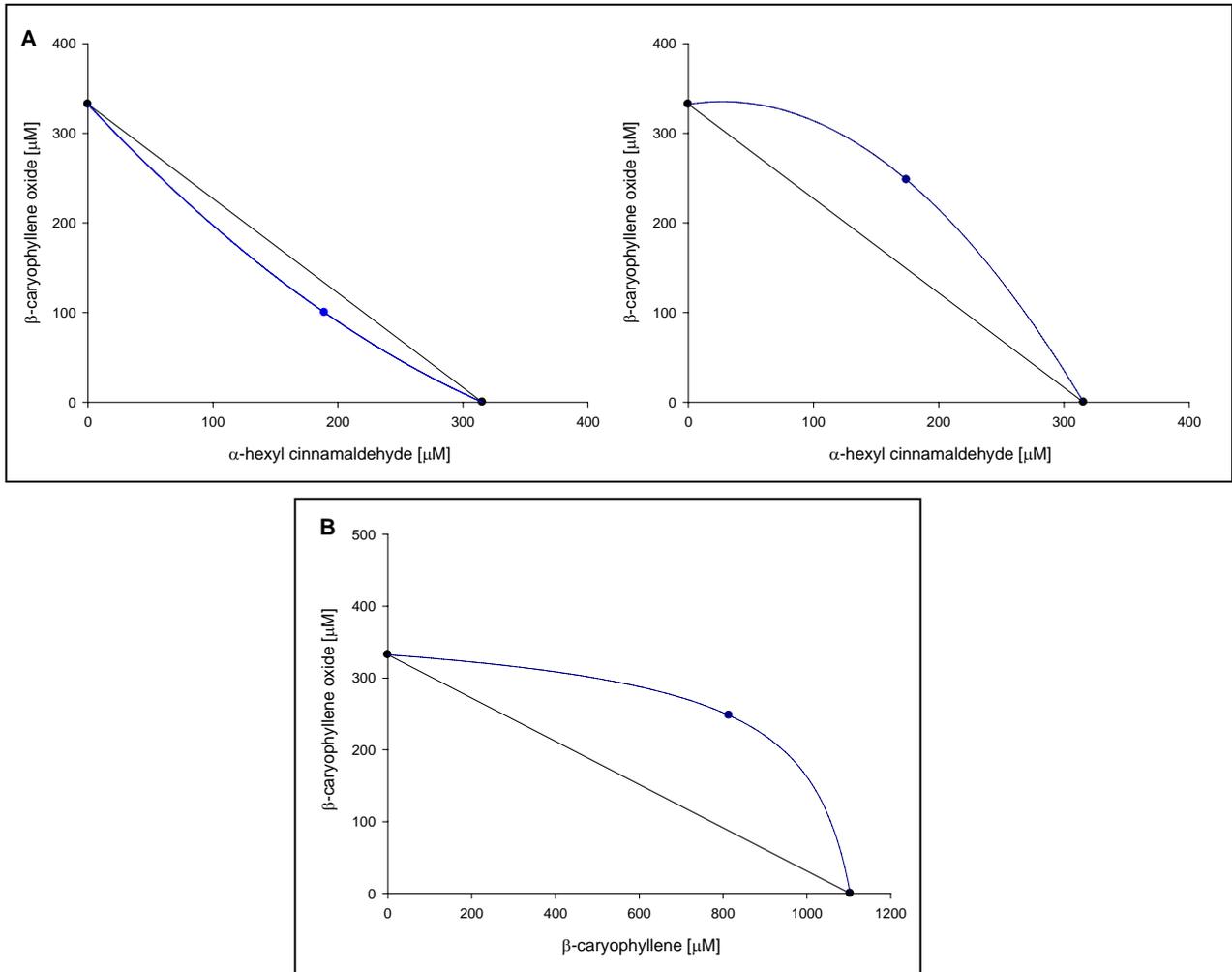
Figure 95. Isobologram analyses in Caco-2 cells: IC$_{50}$ concentrations of α-hexylcinnamaldehyde (A) and β-caryophyllene (B) are plotted on x-axis and IC$_{50}$ values of β-caryophyllene oxide on y-axes. The line connecting these two points is the line of additivity. Points located below the line indicate synergy or above the line antagonism, respectively.

The cytotoxicity and reversal data presented above suggest that β-caryophyllene oxide effectively interfere with at least one mechanism of drug inactivation, especially drug export. To explore this possibility, rhodamine123 retention assay was performed to determine ABC-transporter activity in the cells.

The intracellular fluorescence of Rho123 was significantly increased in a dose-dependent manner, as shown in Figure 96, in Caco-2 cells which were treated with the test substances. Results were expressed as % inhibition of P-gp relative to the positive control verapamil (100%). β-caryophyllene oxide was able to inhibite the MDR efflux pumps. At the same concentration of the positive control (20 μM), the fluorescence intensity was 101.6 ± 3.4%. These results indicate that the test compound is inhibitor of ABC-transporters.

In addition, efflux assays were quantified using flow cytometry in CEM/ADR5000 cells. The intracellular fluorescence of Rho123 was significantly increased in CEM/ADR5000 cells when treated with β-caryophyllene oxide. At the concentration of 10 μM, CRYO (Figure 97) inhibited the P-gp pump with the same potency respect to the positive control verapamil. The fluorescence intensity was 108.4 ± 2.08 % for β-caryophyllene oxide. The figure 98 shows flow cytometry histograms of β-caryophyllene oxide.
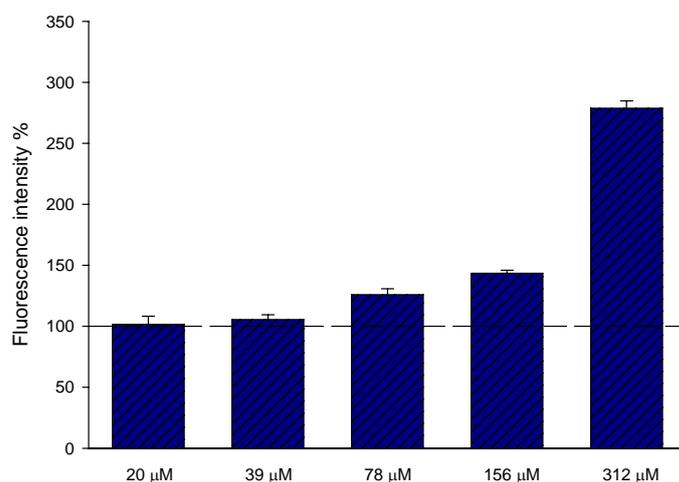
Figure 96. Effect of β-caryophyllene oxide on Rho 123 retention in Caco-2 cells. Fluorescence intensity was measured by spectrofluorometry. Data are presented as means ± ES of fluorescence intensity % related to verapamil as positive control (dotted line).
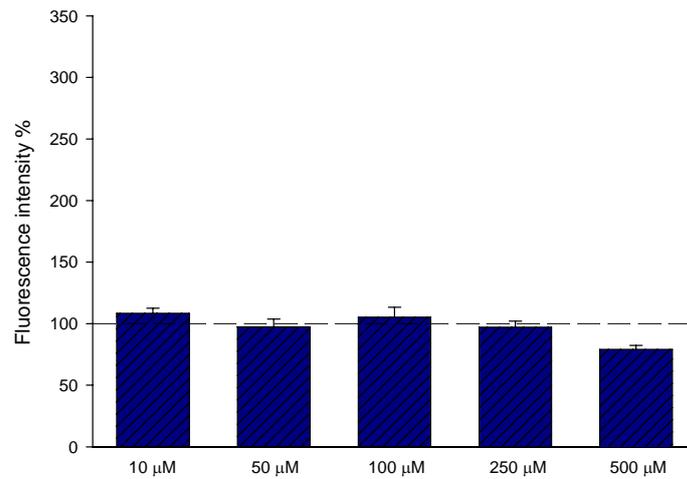
Figure 97. Effect of β-caryophyllene oxide on Rho 123 retention in CEM/ADR5000 cells. Fluorescence intensity was measured using FACS. Data are presented as means ± ES of fluorescence intensity % related to verapamil as positive control (dotted line).
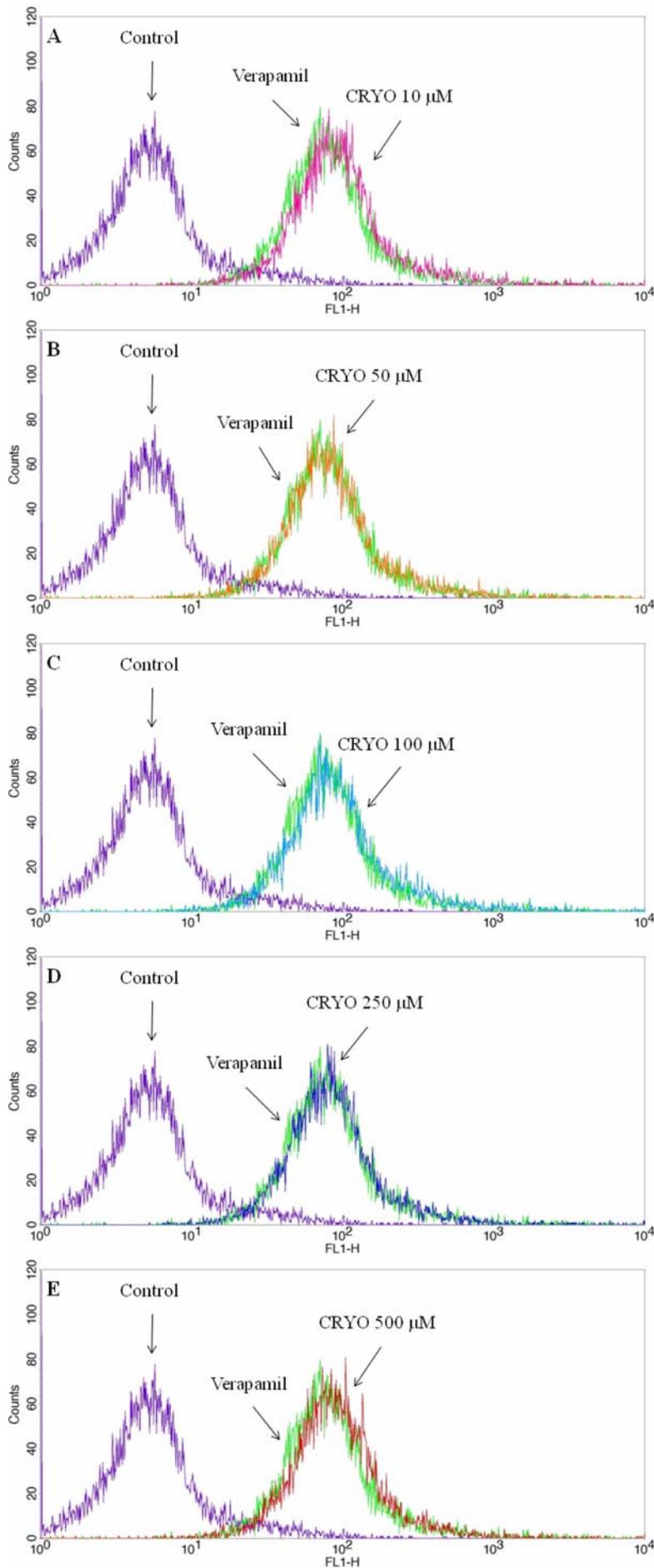
Figure 98.

Flow cytometry histograms of β-caryophyllene oxide (A-E). The fluorescence intensity was shifted to the right side indicating that the resistance cell line CEM/ADR5000 retain the Rho 123 as response to treatment with MDR1 inhibitors. Fluorescence intensity was measured using FACS.

DISCUSSION

α-Hexylcinnamaldehyde

α-Hexylcinnamaldehyde is a synthetic α,β-unsaturated aldehyde naturally-derived and used as ingredient in many personal care (perfumes, creams, shampoos, etc.) and household products, and as additive in food and pharmaceutical industry (Schnuch et al., 2007).

In the present study, we have first tested the potential cytotoxicity of α-hexylcinnamaldehyde in human sensitive (CCRF/CEM) and resistant (CEM/ADR5000 and Caco-2) cancer cell lines. The substance did not cause substantial cell growth inhibition in sensitive and resistant cell lines (IC_{50} values between 212.95 and 315.66 μM). CCRF/CEM and Caco-2 cells were the more and less sensitive lines to HCA-cytotoxicity.

Furthermore, α-hexylcinnamaldehyde was able to synergistically enhance the doxorubicin-cytotoxicity, acting as a chemosensitizer. Particularly, in CCRF/CEM, doxorubicin-cytotoxicity was enhanced 6-fold and 47-fold. These results is notherworty because a reduction in the dose of antitumoral drug, while maintaining the same clinical effect, allows to reduce the toxicity often associated with chemotherapy.

The highest cytotoxicity showed in combination experiments could be caused by a multiple target attack of HCA. In fact, from a chemical point of view, α-hexylcinnamaldehyde is a synthetic α,β-unsaturated aldehyde, characterized by the presence of a carbonyl group containing a polarized carbon–oxygen double bond. The marked difference in the electronegativity between the oxygen and the carbon atoms makes this group able to react with electron-rich biological macromolecules, such as phospholipids, proteins and DNA, while their mediated effects vary from physiological and homeostatic to cytotoxic, mutagenic or carcinogenic (Voulgaridou et al., 2011). Besides, HCA is potentially more reactive than a simple aldehyde, as it also possesses a double bond between carbons 2 and 3 (α and β respectively). The conjugation of the unsaturated function with the carbonyl group makes the α-carbon positively polarized and consequently the preferred site for a nucleophilic attack (Feron et al., 1991). Moreover, α-hexylcinnamaldehyde can influence the membrane stability (Di Sotto et al., unpublished data). When combined with other lipophilic compounds, its effect on the membrane permeability might be potentiated.

The chemosensitizing ability of HCA with doxorubicin could be due to the inhibition ABC-transporters, which reduces the export of cytotoxic drugs through the cells.

The potential inhibition of ABC-transporters was studied in Caco-2 and CEM/ADR5000 cell lines, which represent ideal model to study MDR protein and particularly P-gp (synonym MDR1) for CEM/ADR5000 (Efferth et al., 2003; Gillet et al., 2004). Also, Rho123 was chosen as a fluorescent dye because it is a known Pgp- and MRP-substrate.

α-Hexylcinnamaldehyde inhibited the MDR efflux pumps in both CEM/ADR5000 and Caco-2 cell lines, resulting more potent than the standard verapamil. So we could hypothesizes that HCA is able to inhibit both Pgp and MRP pumps. Wortelboer et al. (2005) reported that compounds containing an α,β-unsaturated moiety can modulate MRP1- and MRP2-mediated transport processes via different mechanisms, including (i) formation of glutathione (GSH) conjugates which can competitively inhibit MRP1 and MRP2, (ii) depletion of GSH, or (iii) direct inhibition of the MRP1 and/or MRP2 mediated transport process through interaction of the parent compound with the MRP molecule. Also cinnamaldehyde, the natural HCA-precursor electrophilic showed to react with thiol groups and GSH, and to inhibit the MRP1-mediated transport of calcein in cells (Wortelboer et al., 2005).

Thereafter, we can suppose that α-hexylcinnamaldehyde could inhibit ABC-transporters activity by interfering with GSH pathway or by direct reacting with Pgp and MRP protein. Although some appear likely to explaining this synergism, further studies are required to understand the true mechanisms involved in the chemosensitizing effect of HCA.

β-Caryophyllene

β-Caryophyllene is a bi-cyclic sesquiterpene with a rare 1,1-dimethylcyclobutane ring fused in a trans configuration to a nine-membered ring containing a 1,5-diene. Several biological activities are ascribed to β-caryophyllene. It has been described as a potential anti-carcinogenic agent, due to its capability to induce the detoxifying enzymes or to improve, *in vitro* and *in vivo*, the natural killer cell activity. β-Caryophyllene has also been reported to increase the anticancer activity of α-humulene, isocaryophyllene and paclitaxel against tumour cell lines. Finally, it exhibited antiproliferative activity in human renal adenocarcinoma and amelanotic melanoma cells (Di Sotto et al., 2010). In the present study, we have first tested the potential of β-caryophyllene cancer cell lines.

β-Caryophyllene did not cause substantial cytotoxicity in both human sensitive (CCRF/CEM) and resistant (CEM/ADR5000 and Caco-2) cancer cells. CCRF/CEM and Caco-2 cells were the more and less sensitive cells to CRY-cytotoxicity, respectively. These results are in agreement with those of Legault et al. (2003), who did not detect cytotoxicity for β-caryophyllene in different cancer cell lines. When tested at low concentrations with doxorubicin, β-caryophyllene potentiated doxorubicin-cytotoxicity, although just in few cases the effect was synergistic. It is important to highlight that a combined effect greater than each drug alone does not necessarily indicate synergism. Sometimes this can be a results of an additive effect or even a slight antagonism (Chou, 2010). In addiction, CRY also resulted able to interfere with ABC-transporters: this effect could be due to its lipophilicity, according to the results obtained with other terpenoids (Wink et al., 2012).

The interaction between β-caryophyllene and P-gp was already reported. Zhang and Lim (2007) highlighted that β-caryophyllene significantly modulate the [^{3}H]digoxin-transport in Caco-2 cell monolayer, without affecting P-gp transporter. This hypothesis was in agreement with Legault et al. (2007). It supposed that β-caryophyllene was accumulated in cancer cell membranes, increasing the membrane permeability; this membrane alteration could facilitate the passage of bioactive compounds through the cytoplasmic membrane. As a consequence, β-caryophyllene could increase the intracellular accumulation of antitumor drugs, potentiating their activity.

We can also suppose that terpenoids target the lipophilic core of proteins, which leads to a disturbance of the interaction of membrane proteins with membrane lipids, and changes the three dimensional conformation of the protein, resulting in modulated protein function (Wink 2008; Eid et al., 2012).

β-Caryophyllene oxide

β-Caryophyllene oxide did not cause a considerable cytotoxicity in all cell tested. Althought these results agree with some published data (Kaneda et al., 1992; Legault et al., 2003), other evidences suggested an antiproliferative activity of β-caryophyllene oxide in different cancer cell lines (Kubo et al., 1996; Sibanda et al., 2004; Jun et al., 2011). These conflicting results may be a consequence of some solubility problems of β-caryophyllene oxide in hydrophilic media (Jun et al., 2011). When tested at low concentrations with doxorubicin, β-caryophyllene oxide potentiated doxorubicin-cytotoxicity, mainly by synergistic effects. These results is notherworty because a reduction in the dose of antitumoral drug, while maintaining the same clinical effect, allows to reduce the toxicity often associated with chemotherapy. CRYO also interfered with ABC-transporters, with a potency similar to verapamil. P-gp modulators act as competitive inhibitors by binding to the membrane protein or by indirect mechanisms related to the expression of the P-gp gene and/or phosphorylation of the transport protein (Wink 2008, 2012). Eid et al. (2013) assumed that lipophilic terpenoids (thymol, menthol, aromadendrene, β-sitosterol-O-glucoside, and β-carotene) probably act as competitive inhibitors of P-gp in cancer cells (Wink, 2008; Wink et al., 2012). However, lipophilic compounds can effectively interact directly with P-gp by forming hydrogen and ionic bonds with aminoacid side chains of the protein, thus interfering with the 3D structure of P-gp (conformation) and inhibiting its activity (Wink, 2008). β-Caryophyllene oxide is a biciclic sesquiterpene with an epoxide group in its molecular structure. Epoxides are reactive functional groups that can bind to amino groups and SH-groups of proteins (Wink and Schimmer, 2010). We therefore postulate, that β-caryophyllene oxide not only could serves as a competitive inhibitor, but might alkylate the transporter protein and thus inhibit it irreversibly. Moreover, most terpenoids are substrates for P-gp and other ABC transporters because of their lipophilicity. If administered as a chemosensitizers in combination with a cytotoxic agent they function as competing inhibitors for binding to the active site of the transporters (Wink et al., 2012).

In conclusion, our results show that low concentrations of CRYO increase the growth inhibition induced by doxorubicin on tumor cell lines. The potentiating effect of β-caryophyllene oxide could be due in part to alteration of membrane permeability, but also could be due to its reactive group epoxide. These data suggest further investigations in order to better establish the mechanism involved in the chemosesitizing effects of β-caryophyllene oxide.

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Chemopreventive agents can modulate all multiple pathways leading to genotoxic damage and, then, to cancer or other mutation-related diseases. Considering that mutations in somatic cells play a key role in cancer initiation and other stages of the carcinogenesis process, the identification of chemopreventive compounds becomes of particular interest. Among chemopreventive compounds, antimutagens are able to interfere with the mutagen by extra- or intracellular mechanisms before DNA-injury (desmutagenic agents) or to prevent fixation and progression of DNA-damage, by the involvement of repair and/or reversion systems (bioantimutagens) (Shamon and Pezzato 1994). In addition to antimutagenicity, some chemopreventive agents can also exert chemosensitizing properties, by increasing the effectiveness of cancer chemotherapy and radiotherapy, when used in combination with chemotherapeutical agents. This approach is useful to prevent the development of multidrug resistance (MDR), which makes cancer cells not-sensitive to a broad range of drugs, with different chemical structures and mechanisms of action. Finally, synergistic interactions between a chemopreventive compound and a chemotherapeutic drug can occur: this is a very desirable goal because it allows to use low dose of anticancer agents, so minimizing the chemotherapy toxicity.

In this study, we evaluated the potential chemopreventive properties of some natural and naturally-derived compounds, particularly the sesquiterpenes β-caryophyllene (CRY) and β-caryophyllene oxide (CRYO), and the aldehyde α-hexylcinnamal (HCA).
We found that the sesquiterpenes tested, especially CRYO, are able to protect the cells from the DNA-injuries of cigarette smoke. The compounds were active in all strains and treatment protocols (pre-, co-, post-): this allows to hypothesize the involvement of multiple mechanisms, among which the inhibition of the smoke-induced oxidative stress. These results suggest further investigations on CRY and CRYO as potential chemopreventive compounds against smoking damage, to use as tobacco ingredients. For instance, the addition of the sesquiterpenes to tobacco or to filter tip could block the cancerogens before they induce DNA-damage, so representing a useful preventive strategy against smoke-toxicity.
A remarkable antimutagenicity was registered for α-hexylcinnamaldehyde against various nitroarenes and in different experimental conditions. Taking into account that nitroarenes are environmental pollutants and food toxicants, identified as carcinogen in rodents (Moller et al., 1989) and possibly carcinogenic to humans (IARC, 1989), the HCA-antimutagenicity suggests further investigations in order to well establish its potential application as a protective additive in

commercial products, particularly cosmetics, households and food. HCA also resulted able to enhance the cytotoxicity of the antitumor drug doxorubicin, and to inhibit the Pgp and MRP pumps in both sensitive and resistant cancer cells. At our knowledge, the evidences for a chemopreventive potential of HCA have not been described up to now. These results suggest a potential application of this compound as a chemosensitizer, both to overcome multidrug resistance, and to reduce the chemotherapy toxicity without affecting its efficacy. Restoring drug sensitivity in multidrug resistant cancer cells, particularly by inhibiting the ABC-transporter function or by modulating the activity of chemotherapics, is a very important goal to overcome cancer.

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1. Di Sotto A, Maffei F, Hrelia P, **Di Giacomo S**, Pagano E, Borrelli F, Mazzanti G, 2014. Genotoxicity assessment of some cosmetic and food additives. Regul Toxicol Pharm 68, 16-22.

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1. Di Sotto A, Mazzanti G, Savickiene N, Staršelskytė R, Baksenskaite V, Di Giacomo S, Vitalone A, 2014. A lectin-rich fraction from aerial parts of *Urtica dioica* L. with antimutagenic activity (Pharmaceutica biology).

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1. **Di Giacomo S**, Di Sotto A, El-Readi MZ, Mazzanti G, Wink M, 2013. Identification of new chemosensitizer agents against multidrug resistant cancer cells. 36° Congresso Nazionale della Società Italiana di Farmacologia. Torino, 23-26 October (communication).

2. Di Sotto A, **Di Giacomo S**, Cocchiola R, Eufemi M, Mazzanti G, 2013. Natural sesquiterpenes inhibit DNA-damage by tobacco smoke. 36° Congresso Nazionale della Società Italiana di Farmacologia. Torino, 23-26 October (poster).

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