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OXIDATIVE STRESS EVALUATION IN HEPATOCELLULAR CARCINOMA - THERAPEUTIC IMPLICATIONS -

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Oxidative Stress Evaluation in Hepatocellular Carcinoma – therapeutic implications

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ABSTRACT

Hepatocellular carcinoma is one of the most frequent cancers worldwide and effective therapy is currently lacking. Several known environmental risk factors for hepatocellular carcinoma development lead to generation of reactive oxygen species promoting oxidative stress. On the other hand, since mitochondria is the main site for reactive oxygen species production, it may have a relevant role in hepatocarcinogenesis. Moreover, neoplastic cells have a higher mitochondrial membrane potential than normal cells, which may be explored in the development of new approaches to treat hepatocellular carcinoma.

The aim of this work is to evaluate the therapeutic efficacy of new compounds targeting the mitochondria, such as Dequalinium, a lypophilic cation, and the natural bioactive compounds, vitamin C (ascorbic acid and dehydroascorbic acid), and epigallocatechin-3-gallate, a green tea polyphenol, both in monotherapy and in association with each other and with conventional anticarcinogenic drugs (5-fluorouracil and doxorubicin) in order to identify which of them may be a useful therapeutic approach in hepatocellular carcinoma. We also intended to clarify the molecular mechanisms involved in the cytotoxicity induced by these new drugs, including the influence of oxidative stress, mitochondrial function and the expression levels of proteins involved in apoptosis mitochondrial pathway.

For this purpose, we use the HUH-7 cells, an hepatocellular carcinoma cell line, maintained in culture in absence and presence of increasing concentrations of Dequalinium, epigallocatechin-3-gallate, ascorbic acid and dehydroascorbic acid, in monotherapy or in combination with each other and with conventional the anticarcinogenic drugs, 5-fluorouracil and doxorubicin, during 96 hours. The antiproliferative effect was assessed by the Alamar Blue assay and cell death by optic microscopy and flow cytometry upon staining cells with

Annexin V and propidium iodide. The expression of the apoptosis-regulating molecules, BAX and BCL-2, was assessed using monoclonal antibodies labelled with fluorescent probes. Oxidative stress was evaluated through the intracellular reactive oxygen species accumulation, peroxides and superoxide anion, using the fluorescent probes DCFH2-DA and DHE, respectively. The mitochondrial function was analysed through the determination of the mitochondrial transmembrane potential using the fluorescent probe JC1. All these parameters were analysed by flow cytometry.

The results obtained suggest that dequalinium, epigallocatechin-3-gallate and vitamin C, as single agents, have an antiproliferative and cytotoxic effect in a dose and time dependent manner. This effect increases when these compounds are used in a daily administration scheme with a lower total dosage. On the other hand, when used in association, a synergistic antiproliferative and cytotoxic effect is observed with dequalinium and epigallocatechin-3-gallate that may be mediated mainly by apoptosis. In opposite when cells are treated with DHA associated with 5-FU an antagonistic effect is observed. When cells are incubated with Dequalinium, mitochondria seems to play an important role in HUH-7 cell death. Besides we observed a cytotoxic effect upon incubation of cells with natural bioactive compounds, a pro-oxidant effect wasn't evident, suggesting other mechanisms involved in cell death.

This study suggests that dequalinium, epigallocatechin-3-gallate and vitamin C may constitute new therapeutic options for hepatocellular carcinoma both in monotherapy and in association. However, as the schedule of drug administration schemes and new drugs associations could interfere with drug efficacy, they should be tested in order to improve the therapeutic potential in hepatocellular carcinoma.

Keywords: hepatocellular carcinoma; apoptosis; oxidative stress; reactive oxygen species; dequalinium; epigallocatechin-3-gallate; vitamin C; ascorbic acid; dehydroascorbic acid.

Abbreviations list:

AA: Ascorbic acid

AV: Annexin V

DCF: Dichlorofluorescein

DCFH2: 2',7'-dichlorodihydrofluorescein

DCFH2-DA: 2',7'-dichlorodihydrofluorescein diacetate

DD: Daily dose

DHA: Dehydroascorbic acid

DHE: Dehydroethidium or Hydroethidine

DOX: Doxorubicin

DQA: Dequalinium

EGCG: Epigallocatechin-3-gallate

Et: Ethidium

FC: Flow cytometry

FITC: Fluorescein isotiocianate

HCC: Hepatocellular carcinoma

JC-1: 5,5’,6,6’-tethrachloro-1,1’3,3’-tethraethylbenzimidazolcarbocyanine iodide

OS: Oxidative stress

PI: Propidium iodide

ROS: Reactive oxygen species

SD: Single dose

5- FU: 5-Fluorouracil

ΔΨm :Mitochondria transmembrane potential

1. INTRODUCTION

Primary hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide (Ferlay, 2008) and effective therapy is currently lacking.

Hepatocarcinogenesis is a multistep process and current evidences indicate that both genetic and epigenetic mechanisms are involved in HCC development. These contribute to alteration of numerous signaling pathways leading to disregulated cell proliferation and resistance to cell death (Figure 1) (Avila *et al*., 2006). On the other hand, oxidative stress has been linked to an increased risk of HCC (Wang *et al.*, 2002).

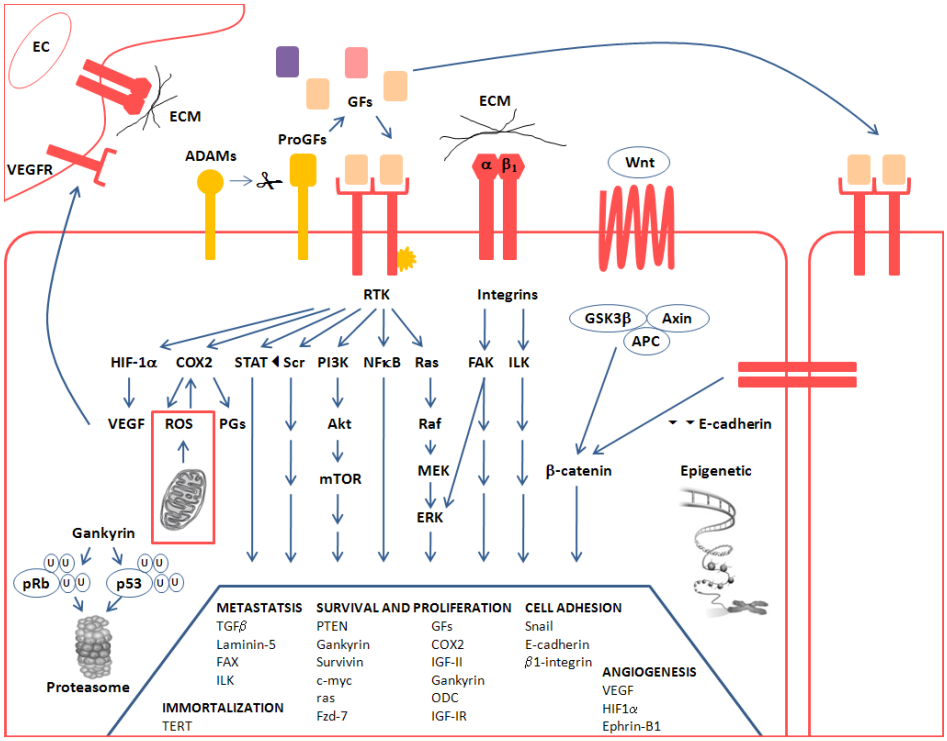


Figure 1. Signalling pathways involved in hepatocarcinogenesis: The figure show several signalling pathways which disregulation could lead to cell proliferation and/or to resistance to cell death in hepatocelular carcinoma. Reactive oxygen species (ROS), produced mainly in mitochondria, may act as cell signalling molecules involved in these altered mechanism (Adapted from Avila *et al.*, 2006).

Reactive oxygen species (ROS) result from cellular metabolism and extracellular processes. The production of superoxide anion ($O_2^{\bullet -}$), the most common radical in biological systems, occurs mostly within the mitochondria. Superoxide anion can be converted to other ROS such as hydrogen peroxide (H_2O_2) according with the reaction:

$$O_2 \xrightarrow{e^-} O_2^{\bullet -} \xrightarrow{e^-,\,2H^+} H_2O_2 \xrightarrow[Fe^{2+}]{e^-,\,-OH^-} {}^{\bullet}OH \xrightarrow{e^-,\,H^+} H_2O$$

Oxidative stress represents a disturbance in the equilibrium status of pro-oxidant/antioxidant reactions in living organisms. ROS are well recognized for playing both deleterious and beneficial roles (Valko *et al.*, 2006). Different levels of oxidative stress cause different outcomes in cells as represented in figures 2 and 3 (Toyokuni, 2007).

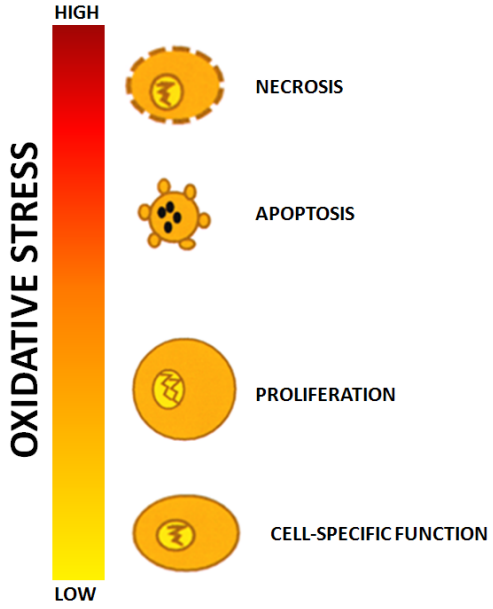


Figure 2. Cellular responses under oxidative stress: Different levels of oxidative stress cause different outcomes in cells (Adapted from Sarmento-Ribeiro, 2000 and Toyokuni, 2007).

In fact, at low/moderate concentrations, ROS have been shown to be key regulators of cellular homoeostasis, in defense against infectious agents, in apoptosis, cell cycle arrest and cellular senescence. On the other hand, ROS also act as secondary messengers (Lowenstein *et al.*, 1994) by activating several signal transduction pathways involved in proliferation, differentiation and apoptosis (Figure 3) (Valko *et al.*, 2007).

However, at high concentration levels, ROS have harmfull effects since they have the potential to interact with cellular components including DNA, lipids and proteins (Esterbauer *et al.*, 1990). As some oxidative DNA lesions are promutagenic, oxidative damage is proposed to play a role in the development of certain cancers (Bartsch, 1996).

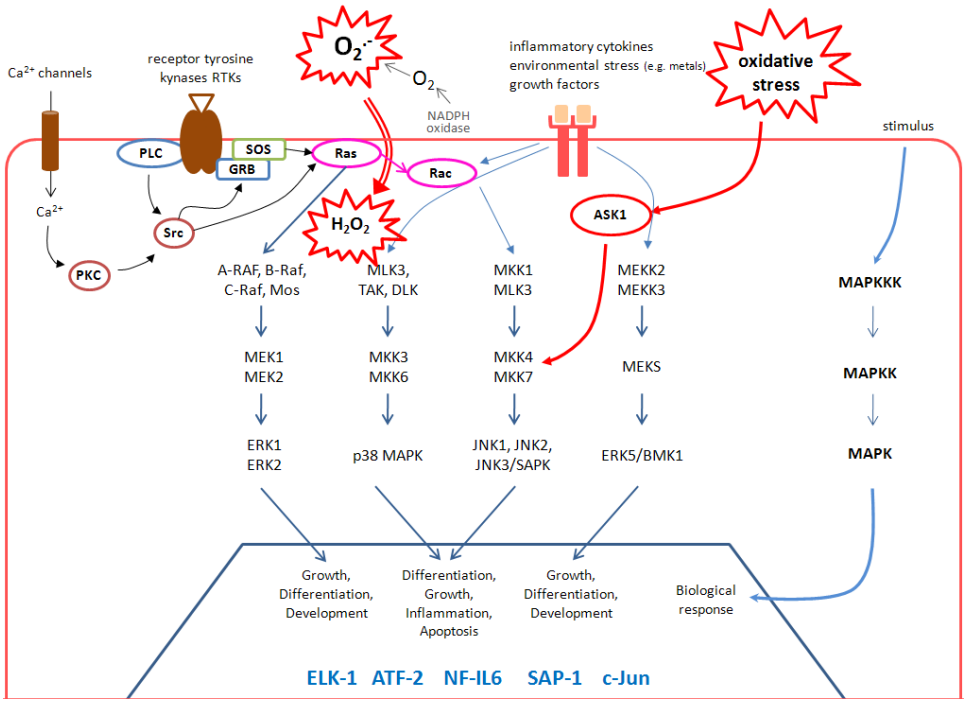
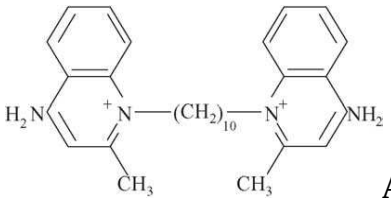


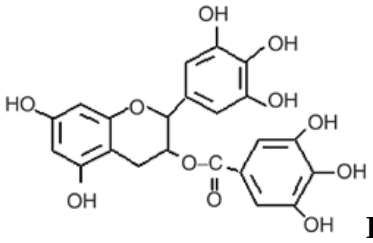
Figure 3. ROS as secondary messengers activate several transduction pathways: The most significant effect of ROS on signalling pathways has been observed in the mitogen-activated protein kinase (MAPK) pathways (Adapted from Valko *et al.*, 2007).

High concentrations of ROS occur when there is an overproduction of ROS or a deficiency in antioxidants systems. Besides the evidences indicating that cancer cells exhibit higher intrinsic oxidative stress levels (Behrend, 2003; Pelicano *et al*, 2004) they aren't enough to cause cellular death (Toyokuni, 2007). Moreover, neoplastic cells have a higher mitochondrial membrane potential than normal cells. Thus, oxidative stress and mitochondria may be used as potential therapeutic targets.

Delocalized lipophylic cations (DLCs) are an example of compounds targeting the mitochondria. They constitute a new class of antitumor membrane-permeable agents which accumulate in mitochondria driven by the negative electric potential across the mitochondrial membrane. DLCs proved to be selectively more toxic in tumor than in normal cells (Modica-Napolitano, 2001 and 2003). Dequalinium (DQA) is a DLC reported to display a potent antitumor activity in different malignancies (Figure 4) (Weissig, 1998).



A



B

Figure 4. Chemical structure of Dequalinium (A) and Epigallocatechin-3-gallate (B). (Adapted from Galeano, 2005 and Yang *et al.*, 2000, respectively).

On the other hand, some natural bioactive compounds such as Epigallocatechin-3-gallate (Figure 4) and Vitamin C (Figure 5) exhibit a pro-oxidant/antioxidant effect depending on their concentration. Epigallocatechin-3-gallate (EGCG), the most abundant green tea polyphenol, may protect normal cells by its antioxidant properties as it can scaveng free

radicals (Ruch *et al*., 1989). However, some results have demonstrated that high concentrations of EGCG can induce oxidative stress only in tumor cells (Yamamoto, 2003). Vitamin C (ascorbic acid, AA, and dehydroascorbic acid, DHA) (Figure 5), at physiological concentrations, act as potent free radical scavenger in plasma. However, at high concentrations, vitamin C shows a pro-oxidant activity that selectively target tumor cells by mediating the production of hydrogen peroxide (Chen *et al*., 2005).

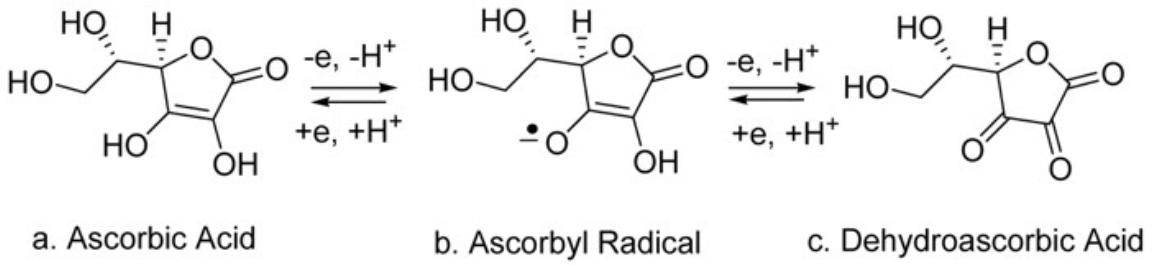


Figure 5. Chemical structures of vitamin C: The figure represents the reduction reactions involved in the formation of the reduced form of vitamin C, the dehidroascorbic acid (DHA), from the oxidative form, the ascorbic acid (AA) (Adapted from Corpe *et al*., 2004).

In sumary, oxidative stress and specifically mitochondria may be involved in hepatoarcinogenesis and may be new potential therapeutic targets. However, the potential therapeutic of oxidative stress modulators and DLCs in HCC is not clarified.

2. AIMS

The aim of this study is to evaluate the therapeutic potential of the new compounds Dequalinium, Epigallocatechin-3-gallate and Vitamin C (AA and DHA) as in monotherapy and/or in association with each other and with conventional anticarcinogenic drugs in a HCC cell line, in order to identify which of them may be a useful therapeutic approach in HCC. It is also our goal to clarify the molecular mechanisms involved in the cytotoxicity induced by these new molecular agents, including the influence of oxidative stress, mitochondria and levels of proteins involved in apoptosis regulation, namely in the mitochondrial pathway.

3. MATERIALS AND METHODS

3.1. Cell culture conditions

The HUH-7 cell line was provided by The European Collection of Cell Cultures (ECACC). Cells were grown in DMEM (Gibco – Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco-Life Technologies) and peniciline/streptomycin (100µg/mL). Cells were seeded at a density of 50000cells/cm^2 and maintained in culture at 37^{o}C in a humidified atmosphere with 5% CO_2.

To evaluate the antiproliferative and cytotoxic effect of DQA, EGCG, AA and DHA (dose-dependent changes), cells were cultured in monotherapy during 96hours, in absence and presence of DQA (Sigma Aldrich, St. Louis, MO, USA) (in concentrations ranging from 0,5µM to 10µM), EGCG (Sigma Aldrich, St. Louis, MO, USA) (25µM to 250µM), DHA (Sigma Aldrich, St. Louis, MO, USA) (0,25mM to 5mM) and AA (Sigma Aldrich, St. Louis, MO, USA) (5mM to 12mM) as in a single dose as in a daily dose administration scheme. Then, cells were incubated during 72 hours with the drugs tested above in combination with each other and with 5-FU or DOX.

3.2. Cell viability evaluation

Following incubation, cell viability was evaluated by a modified Alamar Blue assay (Resazurine, Sigma Aldrich) under the different experimental conditions (Neves *et al*., 2006). Briefly, we added to cells submitted to the different treatments, Alamar Blue 10% (v/v) in DMEM, for 2 hours at 37°C. After, we collected 200µL of supernatant from each well and transferred to 96 well-plates. The absorbance at 570nm and 600nm was measured using a

Mediators PhL luminometer (Mediators Diagnostika, Vienna, Austria) and cell viability was calculated as a percentage of control according to the formula:

$$\frac{[(A_{570}-A_{600})sample]-[(A_{570}-A_{600})blank]}{[(A_{570}-A_{600})control]-[(A_{570}-A_{600})blank]}\times 100$$

3.3. Cell death analysis

Cell death analyis was performed by morphological analysis using optic microscopy and by flow cytometry.

3.3.1. Morphological analysis

After an incubation period of 48 hours in the conditions described in 3.1, cells were trypsined, centrifuged at 300g for 5min and ressuspended in serum in order to obtain a density of 50000cells/μL and then placed on a slide. Then, cells were stained upon incubation for 5min with May-Grünwald solution (0.3% v/v in methanol) (Sigma, St. Louis, MO, USA), diluted in 1:1 ratio with distilled water followed by staining with Giemsa solution (0.75% p/v in glycerol/methanol 1:1) (Sigma, St. Louis, MO, USA) diluted 8x in distilled water for 20 min. After rinsed with distilled water, smears were left to dry at room temperature and cell morphology was analysed by light microscopy using a Leitz Dialux 20 microscope equipped with a photographic chamber.

3.3.2. Flow cytometry analysis

After an incubation period of 48h in the conditions described in 3.1, cells were trypsined, centrifuged at 300g for 5min and resuspended in phosphate buffer (PBS) in order to obtain a density of $1x10^6$ cells/mL. Untreated and treated cells were washed (centrifuged at 300xg during 5min), resuspended in 440µL of Annexin buffer and incubated for 10min at room temperature with 5µl of Annexin V-FITC (Kit from Immunotech SA, Marseille, France) and 2µL of PI. The results were analysed in a FACS Calibur (Becton Dickinson) flow cytometer equipped with an argon ion laser emitting at 488nm. Green fluorescence of Annexin V-FITC was collected with a 525nm band pass filter and red fluorescence of PI with a 610nm band pass filter. The results were expressed in % of viable (V), initial apoptotic (IA), late apoptotic/necrotic (LA/N) and necrotic (N) cells (Aubry *et al.*, 1999; Dourado *et al*., 2007 and Sarmento-Ribeiro *et al*., accepted).

3.4. Evaluation of the mechanisms involved in cytotoxicity induced cell death

3.4.1. Apoptosis-regulating molecules

The expression levels of the apoptosis-regulating molecules, BAX and BCL-2, were assessed by FC using monoclonal antibodies labelled with fluorescent probes. One million of cells were centrifuged and incubated in 100µL cell-permeable solution with 1µg of the antibody anti-BCL-2 labelled with FITC and 1µg of antibody anti-BAX labelled with PE during 15min at room temperature, in dark, according with others (Sarmento-Ribeiro *et al*., accepted) and manufactured protocols. Then, cells were washed with 1mL PBS, centrifuged at 300xg for 5min, ressuspended in the same buffer and analysed in the flow cytometer. The results are presented as Mean Intensity Fluorescence (MIF) arbitrary units and represent the

medium of fluorescence intensity detected in the cells, which is proportional to the proteins concentration in each cell.

For all the assays, negative controls were established with isotype IgG, IgG1 and IgG2b, and submitted to the same procedures.

3.4.2. Mitochondrial function analysis

Mitochondrial function was analysed through the determination of the transmembrane potencial using the dye 5,5',6,6'-tethrachloro-1,1'3,3'-tethraethylbenzimidazolcarbocyanine iodide (JC-1) as described by others (Cossarizza *et al.*, 1993; Almeida *et al*., 2008; Yao *et al.*, 2008 and Abrantes *et al.*, accepted in 2009). This lipophilic cationic probe exists in a monomeric form (M) emitting at 527nm (green fluorescence) and is able to reversibly form aggregates (A), which are associated with a large shift in the emission (590nm, greenish-orange fluorescence) as the mitochondrial membrane becomes more polarized.

After an incubation period of 48hours in the conditions described in 3.1., cells were incubated with 5µg/mL of JC1 during 15min at 37ºC. At the end of the incubation period, the cells were washed twice in PBS, resuspended in a total volume of 500µL and the fluorescent intensity analysed by flow cytometry. The results are expressed as monomer/aggregate ratio.

3.4.3. Evaluation of reactive oxygen species levels

The accumulation of ROS, namely superoxide anion ($O_2^{\cdot-}$) and peroxides (hydrogen peroxide, H_2O_2), was determined using the probes dihydroethidium (DHE, Molecular Probes, Eugene, OR) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH2-DA) (Invitrogen), respectively. Both, DHE and DCFH2-DA, are stable nonfluorescents lipid permeable compounds, being DFCH2-DA previously converted to DCFH2 by intracellular esterases. Then, DHE and DCFH2 are oxidized by intracellular ROS to form the impermeable

fluorescent compounds Et (Ethidium) and DCF (dichlorofluorescein) that emits, respectively, red and green fluorescence, upon excitation at 488nm, that is proportionally to intracellular ROS levels (Halliwell & Witheman, 2004; Zhao *et al*., 2005 and Zielonka *et al*., 2007).

After an incubation period of 48 hours in the conditions described in 3.1., cells were incubated with 2µL DHE or 1µL DCFH2-DA during 30min at 37ºC and then washed with PBS by centrifugation at 300xg during 5min, as previously described with briefly modifications (Almeida *et al*., 2008; Gonçalves, 2008; Sarmento-Ribeiro *et al*., accepted). The fluorescent intensity of DHE and DCF was measured by flow cytometry (FL-2, 620nm band pass filter and FL-1, between 500 and 530nm band pass filter, respectively). The results are expressed as Mean Intensity Fluorescence (MIF) and represent the mean ± SD of two independent experiments.

3.5. Statistical Analysis

All data are reported as mean ±S.D. A one-way ANOVA and unpaired Student's t tests were used to analyze statistical significance. Differences were considered statistically significant at 95% ($p < 0,05$).

4. RESULTS

4.1. Evaluation of therapeutic potential of DQA, EGCG, DHA and AA – dose and time response curves

In order to evalute the therapeutic potential of the new targeted drugs, HUH-7 cells were cultured in absence and in presence of DQA, EGCG, DHA and AA for up to 72h/96h. and the antiproliferative effect was evaluated by the Alamar Blue assay.

Our results show that when used in monotherapy all the tested compounds induced a decrease in cell viability in a dose, time and administration scheme dependent manner (Figures 6 and 7).

As we can observe in Figure 6A, DQA when used in a single dose (SD) administration scheme has an IC50 (half-maximal inhibitory concentration) of 4,7µM at 48h of incubation and 3,4µM at 96h incubation. However, when used in a daily dose (DD) administration scheme, the same cytotoxic effect was obtained with a decrease in 53% of the dose after 96h of treatment.

In the same way, when cells are incubated with EGCG (Figure 6B), we observed an IC50 of 160µM (48h) and 152µM (72h) when used in a SD administration scheme, but when used in a DD administration scheme, the same cytotoxic effect was obtained with a decrease in 39% of the dose after 72h of treatment.

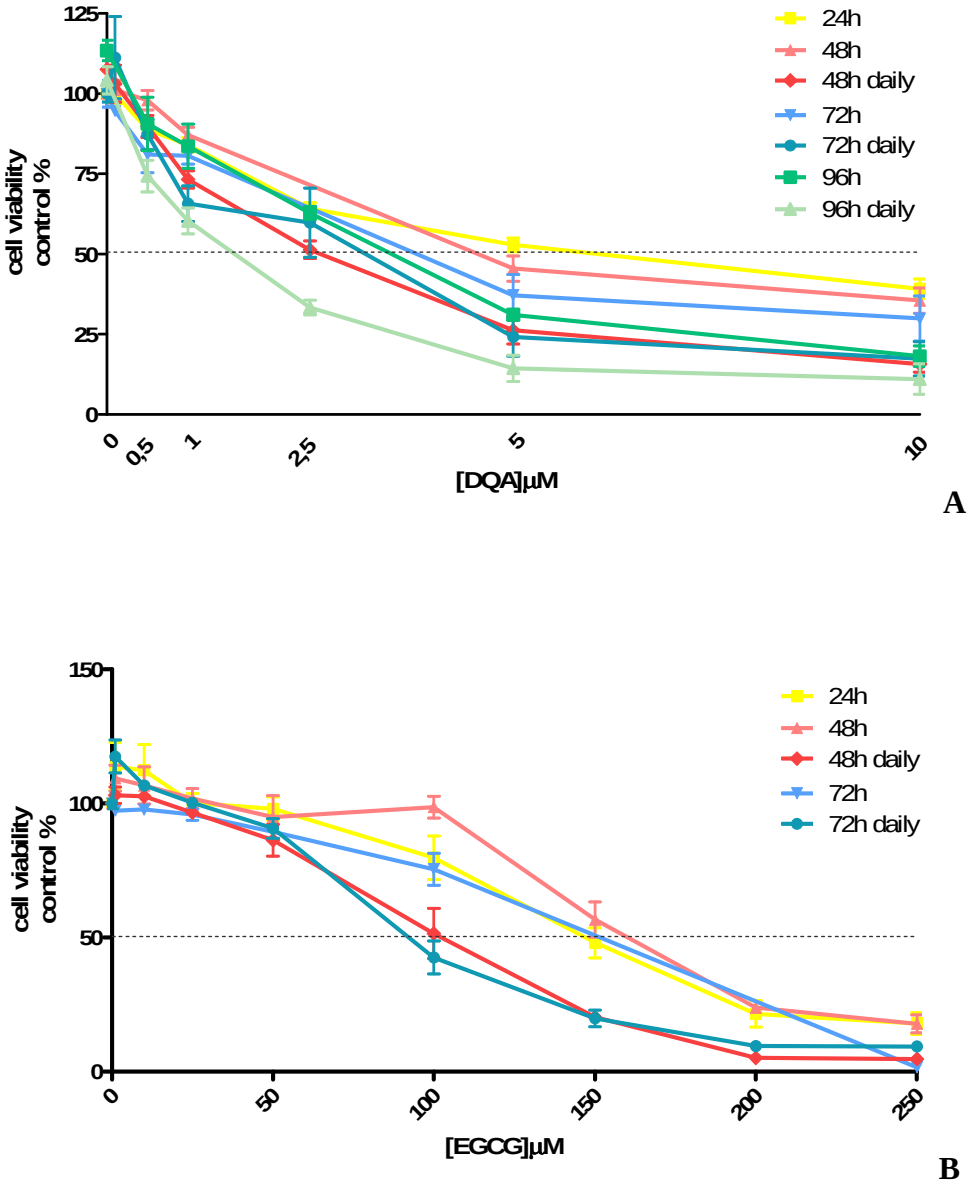


Figure 6. DQA and EGCG dose and time response curves: The effect of DQA (**A**) and EGCG (**B**) in HUH-7 cells viability was evaluated through the determination of cell viability during 96h of incubation with increasing concentrations of DQA and EGCG as represented in figure. Cell viability is expressed in percentage (%) of the control and represents the mean ±SD of 3 independent experiments.

Regarding vitamin C experiments, our results show that despite AA and DHA alone had a modest effect under the tested conditions, we observed a higher antiproliferative effect when HUH-7 cells are treated with DHA (Figure 7). In fact, the reduction of 50% (IC50) in cell viability is obtain earlier and at lower dose in cells treated with DHA. As we can observe in figure 7A, DHA showed an IC50 of 5mM at 96h when used in a SD administration scheme. Nevertheless, this effect increased when this compound was used in a daily administration scheme, allowing obtaining the some effect with a decrease in 20% of the dose at 96h. On the other hand, concerning AA, it wasn't possible to obtain an IC50 when used in a SD administration scheme. However, when AA was administred in a DD scheme, the IC50 at 72h was 12mM (Figure 7B).

In order to evaluate if the drugs tested in monotherapy could have a synergistic antiproliferative and pro-apoptotic effect when used in association with the conventional anticarcinogenic agents, DOX and 5-FU, we performed association experiments (Figure 8).

Our results show that the cytotoxic effect of the drugs in association increased except for the association of DOX with EGCG and with DHA (Figure 8B and 8C) and 5-FU with DQA, with DHA and with AA (Figure 8A, 8C and 8D, respectively). Actually, in the association of DHA with 5-FU, we observe an antagonist effect (Figure 8C).

In fact, we observe an additive synergistic effect in the combination of DQA with DOX (Figure 8A) and EGCG with 5-FU (Figure 8B). A potentiation synergistic effect was observed in the combination of AA with DOX (Figure 8D).

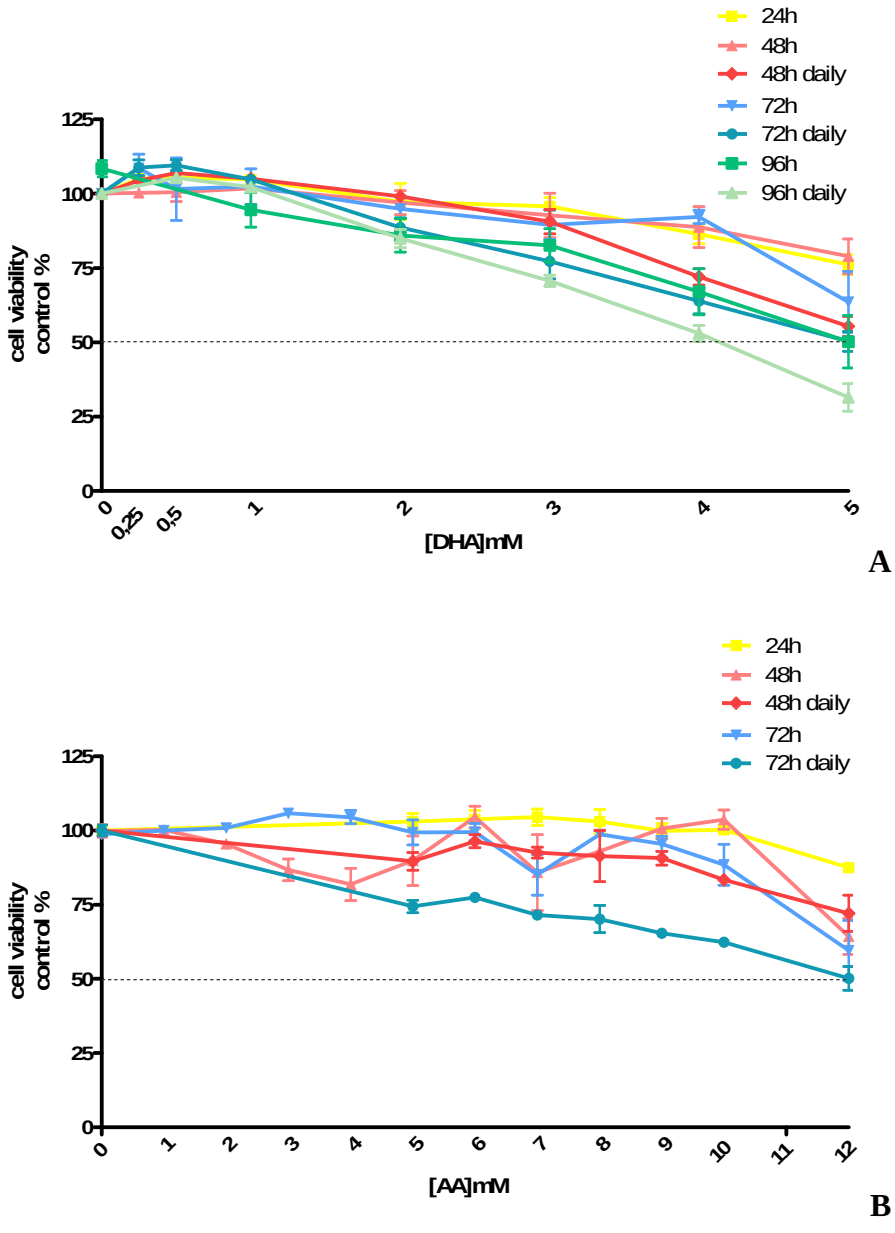


Figure 7. DHA and AA dose and time response curves: The effect of DHA (**A**) and AA (**B**) in HUH-7 cells viability was evaluated through the determination of cell viability up to 96h of incubation with increasing concentrations of DHA and AA. The viability data is expressed in percentage (%) of control and represents the mean ±SD of 3 independent experiments.

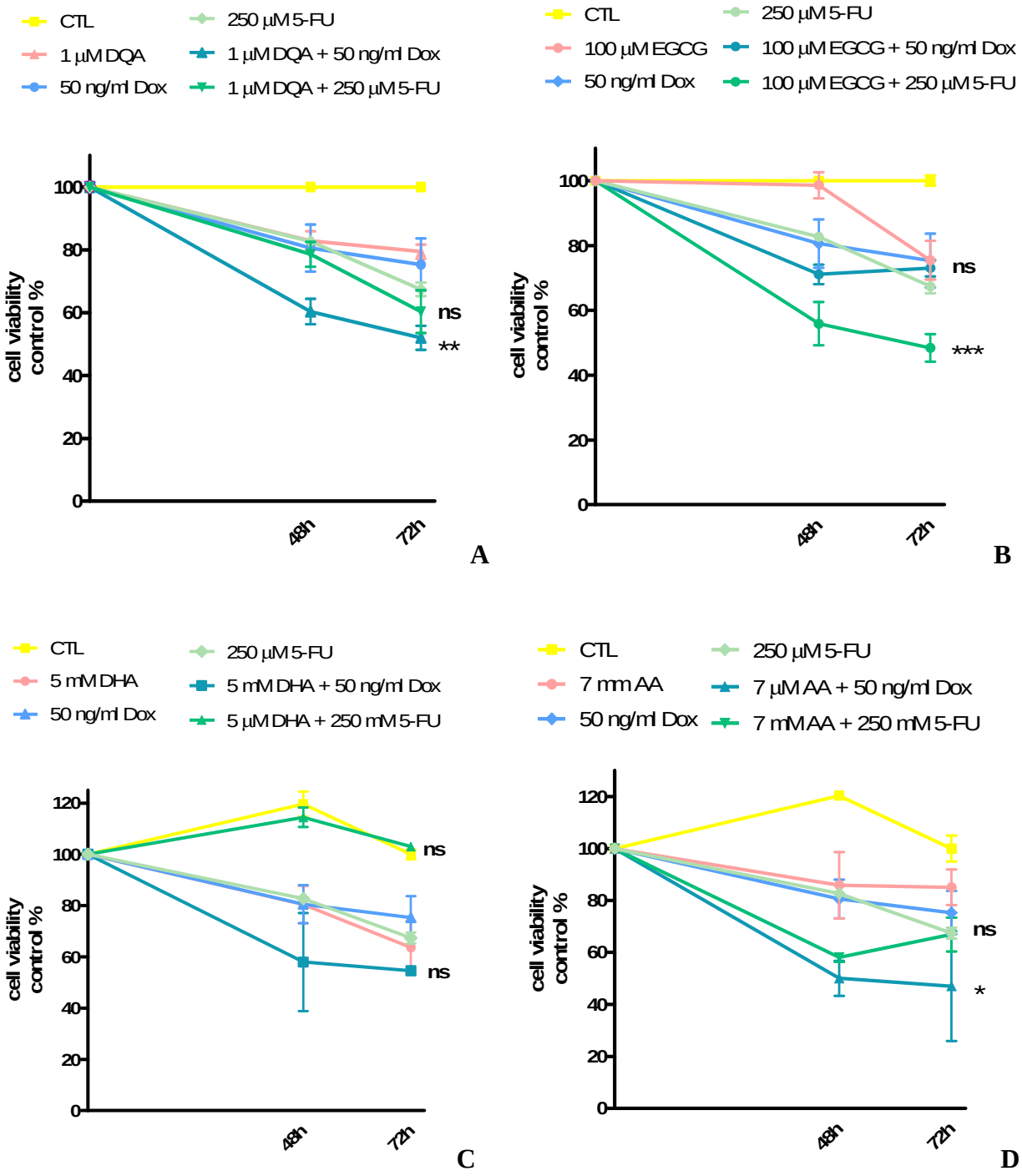


Figure 8. Dose and time response curves of the associations of DOX or 5-FU with DQA (A), EGCG (B), DHA (C) and AA (D): The effect of associations was evaluated through the determination of cell viability at 48h and 72h. All the drugs in the combination schemes are administrated simultaneous. Data is expressed in percentage (%) of control cells and represents the mean ± SD of 3 independent experiments. *p<0,1; **p<0,01; ***p<0,001, results with statistical significance (obtained comparing drugs used in monotherapy with those drugs used in combination after 48h of incubation); ns: no significantly different.

After these encouraging results we tested the associations between the new drugs in study, DQA, EGCG, DHA and AA with each other.

As we can observe in figure 9 an increase in the cytotoxic effect in the combinations involving EGCG with DHA and AA was achieved. In fact, potentiation was observed in the combinations involving EGCG with DHA and with AA (Figure 9B).

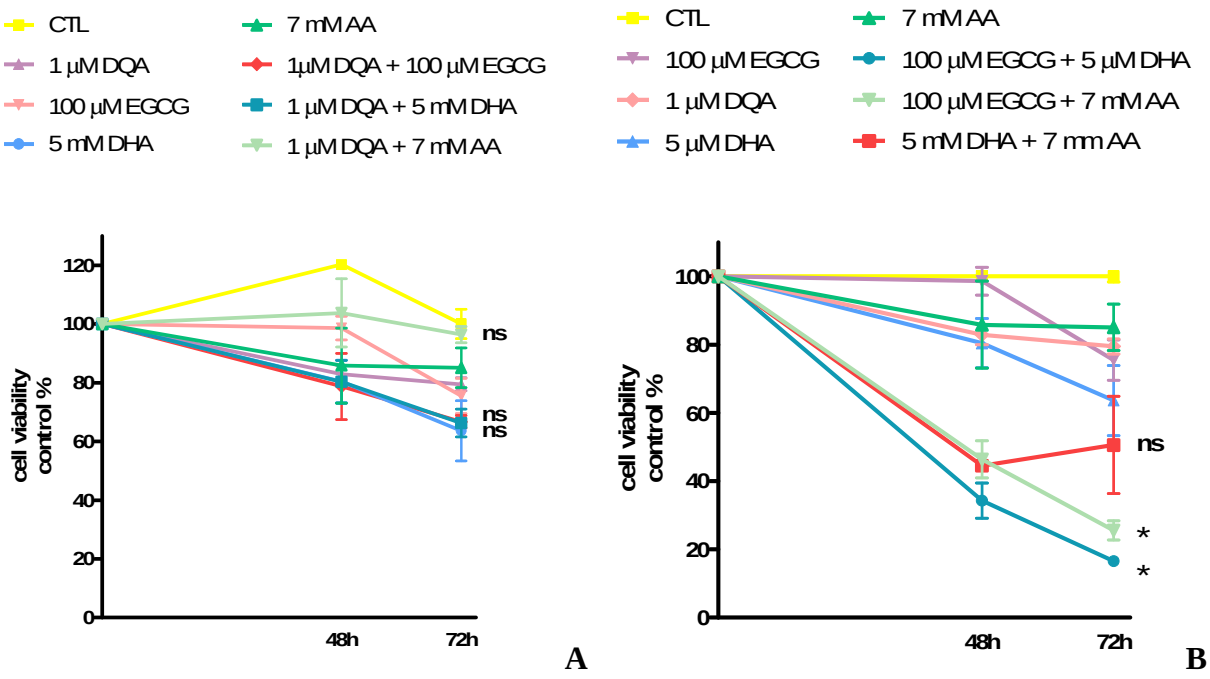


Figure 9. Dose and time response curves of the associations involving DQA (A), EGCG (B), DHA (A and **B) and AA (A** and **B):** The effect of these associations was evaluated through the determination of cell viability at 48h and 72h as described in methods and materials. Data is expressed in percentage (%) of control cells and represents the mean ±SD of 3 independent experiments. Drugs had been administered simultaneously. The difference between experimental groups is statistically significant after 48h of incubation. *p<0,1; **p<0,01; ***p<0,001, statistical difference comparing drugs used in monotherapy with those drugs used in combination; ns: no significantly different.

4.2. Cell death analysis

Since tumor cell death mechanisms can interfere with the therapeutic strategy, we also analysed the cytotoxic effect induced by the referred drugs by studying cell death process through morphological analysis by optical microscopy and by flow cytometry using the AV/PI incorporation.

4.2.1. Evaluation of cell death by optical microscopy - morphological analysis

Figure 10 shows the morphology of cell smears stained with May-Grünwald-Giemsa before (control, 10A) and after treatment with DQA (10B), EGCG (10C), DHA (10D), DOX (10F), 5-FU (10G), EGCG with AA (10K) and AA with DOX (10M). As it can be seen, cells have mostly morphological evidence of cell death by apoptosis, such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation.

Whereas morphological evidence of apoptosis in association with morphological evidences of cell death by necrosis, such as rupture of plasma membrane and extravasation of the intracellular content and intact nuclei were observed in cells smears incubated with AA (10E), DQA with DOX (10H), EGCG with 5-FU (10I) and EGCG with DHA (10J). On the other hand, regarding the cells incubated with DHA with 5-FU, neither morphological evidence of cell death by apoptosis nor by necrosis was found.

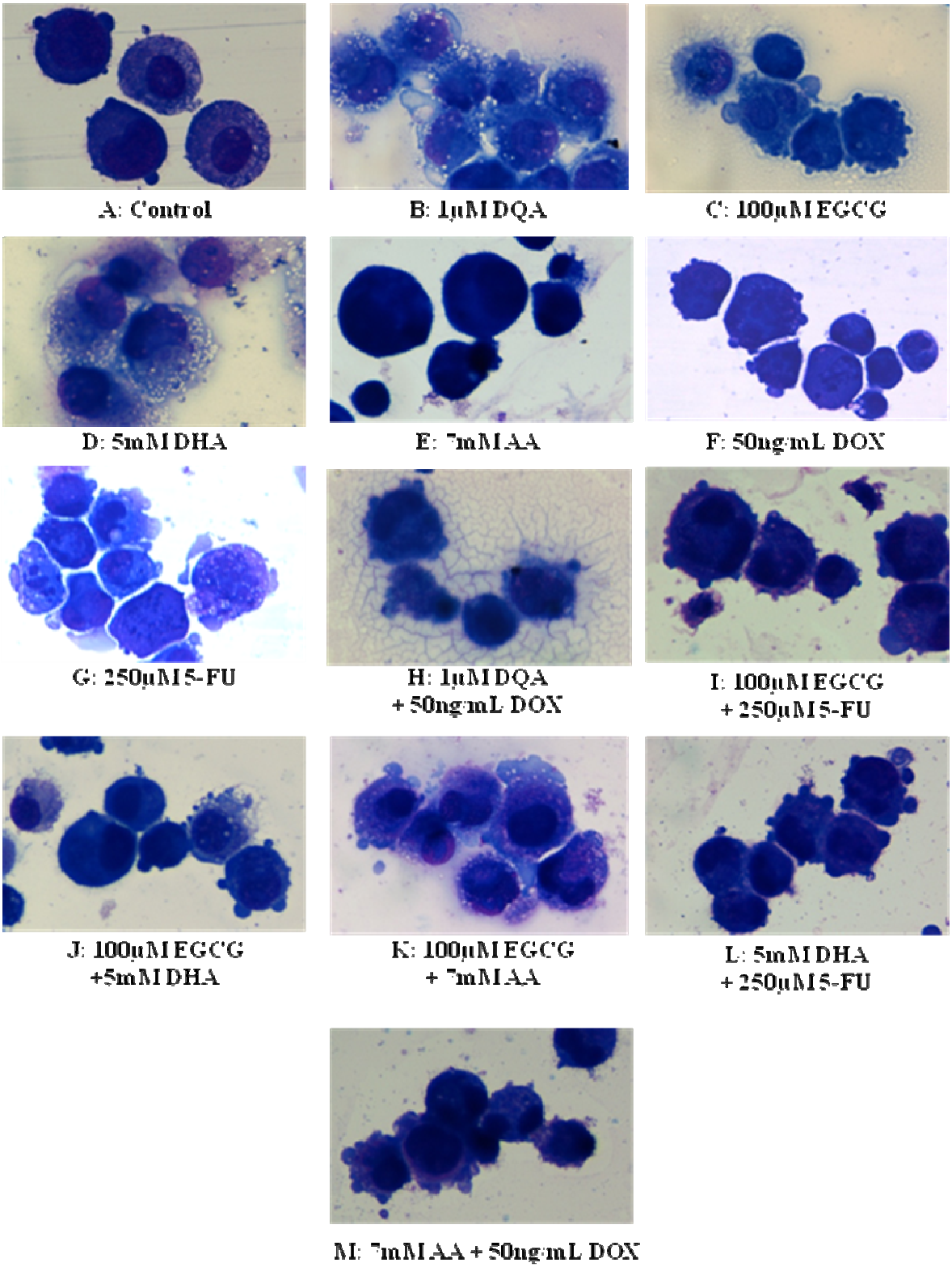


Figure 10. Morphological analysis of HUH-7 cells: Control cells are shown in **A** and cells treated with drugs indicated in the cell smears are shown in **B** to **M**. Cell smears were stained with May-Grünwald-Giemsa as described in material and methods section. Amplification: 1000x

4.2.2 Evaluation of cell death by flow cytometry

In order to confirm our results and evaluate the extent of apoptosis and necrosis, we used a flow cytometry assay based on staining the cells with AV-FITC and PI incorporation.

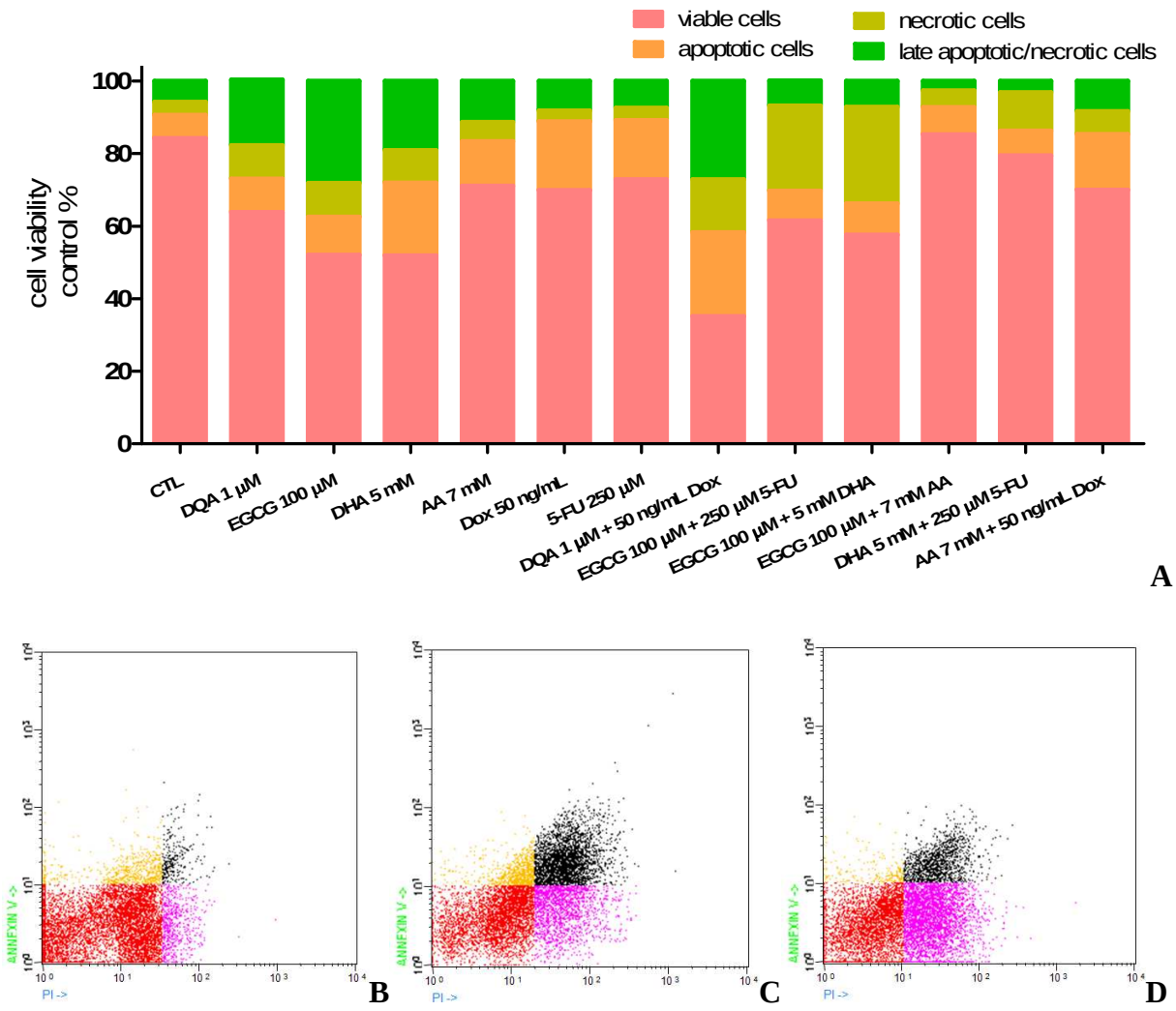


Figure 11. Evaluation of cell viability and death by flow cytometry: HUH-7 cells were incubated in absence (CTL) and in presence of 1µM DQA, 100µM EGCG, 5mM DHA and 7mM AA in monotherapy and in association with each other and with 50ng/mL of DOX and 250µM of 5-FU. Viability and cell death were assessed by FC using Annexin V and propidium iodide staining as described in material and methods. Alive cells are AV/PI negative (pink); early stages of apoptosis are AV positive and PI negative (orange) and cells in late stages of apoptosis are AV/PI positive (dark green). Necrotic cells are AV negative and PI positive (green). Results represented in (**A**) were obtained after 48h of incubation and represent the mean of 2 independent experiments. In (**B**) is represented the dot plot obtained from control cells, in (**C**) the dot plot obtained from cells treated with DHA and in (**D**) the dot plot obtained from cells treated with EGCG with DHA.

As represented in figure 11, in almost all the incubation conditions a decrease in the percentage of viable cells and an increase in the percentage of apoptotic cells was observed These results are in agreement with those obtained in morphological studies, as we observe an increase in the percentage of apoptotic cells and/or in late apoptosis/necrosis in cells treated with drugs, as in monotherapy as in the combinations represented in the Figure 11, except for the combination of EGCG with AA. Besides that, in cells treated with EGCG in combination with 5-FU and with DHA an increase in the percentage of cells in necrosis is detected.

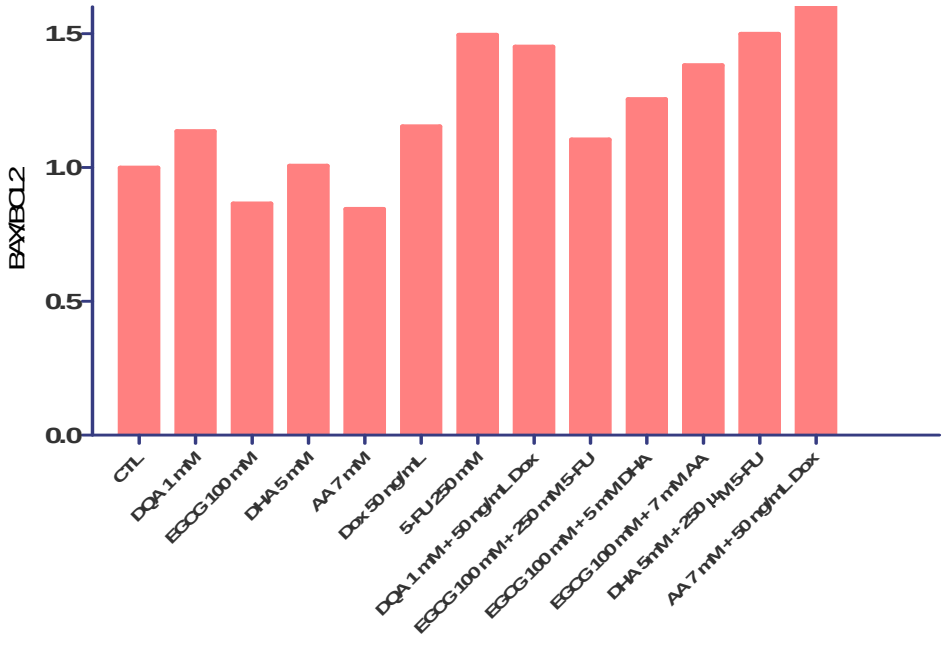
4.3. Evaluation of the mechanisms involved in cytotoxicity induced cell death

4.3.1. Analysis of apoptosis-regulating molecules expression

The anti-apoptotic protein BCL-2 appeared to function by inhibiting the mitochondria depolarization. Conversely, the pro-apoptotic protein BAX induced mitochondria depolarization leading to a decrease in mitochondria membrane potential and, consequently, to apoptosis. The ratio of BCL-2 to BAX has been reported to be correlated with susceptibility to apoptosis in cancer cells (Gross *et al.*, 1999).

Thus, in order to evaluate the role of mitochondrial apoptosis pathway in cytotoxicity induced by the drugs used in the study, we determined the expression levels of BAX and BCL-2 proteins by flow cytometry (Figure 12) as described in material and methods.

As showed in figure 12A, DQA, DOX and 5-FU in monotherapy induced an increase in BAX/BCL2 ratio compared with control cells. On the other hand, a decrease in BAX/BCL-2 ratio was observed in the cells incubated with EGCG and AA in monotherapy, while cells treated with DHA haven’t shown a significative difference when compared with the control’s BAX/BCL-2 ratio. However, when these compounds are combined with each other and with conventional anticarcinogenic agents we detected an increase in the BAX/BCL-2 ratio.



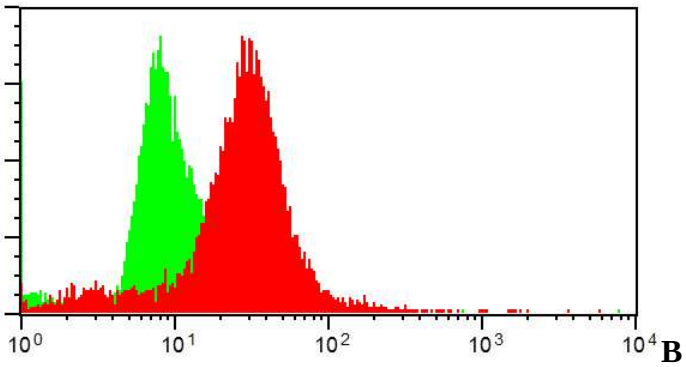


Figure 12. Evaluation of BAX/BCL2 ratio by flow cytometry: HUH-7 cells were incubated in absence (CTL) and in presence of 1µM DQA, 100µM EGCG, 5mM DHA and 7mM AA in monotherapy and in association with each other and with 50ng/mL DOX and 250µM 5-FU. BAX and BCL-2 expression was evaluated as described in material and methods and the ratio BAX/BCL-2 calculated. Results were obtained after 48h of incubation and represent the mean ± SD of 2 independent experiments (**A**). In (**B**) is an example of the histograms obtained, in green is represented the fluorescence intensity of the control cells and in red green the fluorescence intensity of the cells incubated with DQA.

4.3.2. Analysis of mitochondrial dysfunction

To further evaluate the role of mitochondria in the cytotoxicity induced drugs we analysed the mitochondrial transmembrane potential ($\Delta\Psi_m$) by flow cytometry (Figure 13).

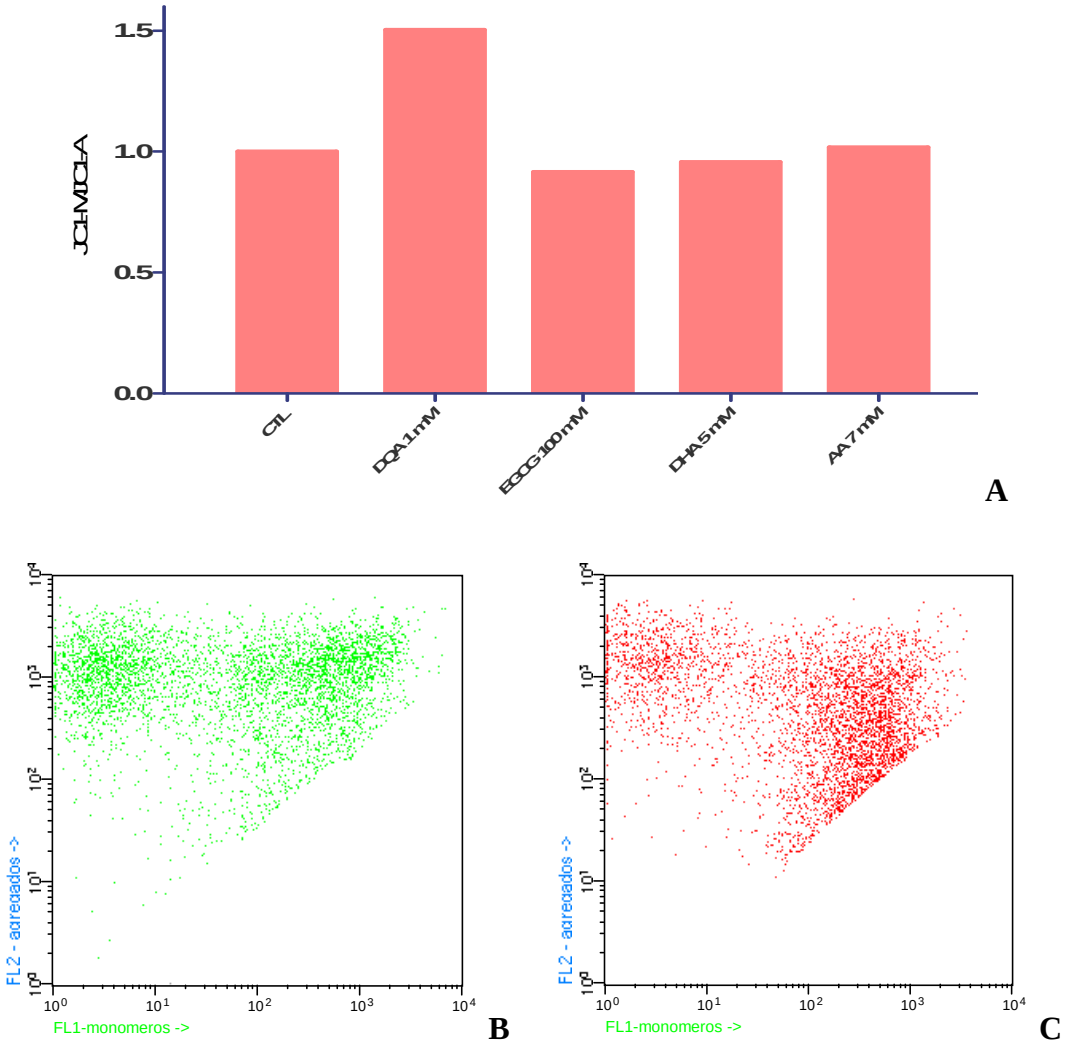


Figure 13. Evaluation of mitochondrial membrane potential by flow cytometry: HUH-7 cells were incubated in the absence (CTL) and in the presence of 1µM DQA, 100µM EGCG, 5mM DHA and 7mM AA in monotherapy. Mitochondrial transmembrane potential ($\Delta\Psi_m$) is evaluated by the ratio between JC1-Monomeres (JC1-M) and JC1-Agregates (JC1-A) as described in material and methods. Results were obtained after 48h of incubation and represent the mean ±SD of 2 independent experiments (**A**). In (**B**) is represented the dot plot obtained from control cells and in (**C**) the dot plot obtained from cells treated with DQA.

As showed in figure 13A, only in cells incubated with DQA we observed an increase in JC1-M/JC1-A ratio (M: monomers; A: aggregates) when compared with control cells. These results are in agreement with apoptotic cell death and with the increase in BAX/BCL-2 ratio observed (Figure 12). In cells treated with EGCG, DHA and AA, in the tested conditions, the mitochondrial membrane potential is similar to untreated cells (control).

4.3.3. Analysis of Reactive Oxygen Species levels

To evaluate the influence of oxidative stress in cytotoxicity induced by drugs in study, the production of reactive oxygen species (ROS) through the intracellular expression of peroxides (hydrogen peroxide, H_2O_2) and superoxide anion ($O_2^{\cdot -}$) was evaluated (Figure 14).

As showed in figure 14A, we observe an increase in the expression of intracellular peroxides only in cells incubated with DQA, as represented by the increase of the fluorescence intensity of the DCF. On the other hand, in cells treated with AA and with DHA a decrease in the expression of intracellular peroxides is detected, while cells treated with EGCG didn´t show any significant difference in DCF fluorescence intensity compared to control cells.

In figure 14B is represented the expression of intracellular superoxide anion. As we can see, in cells treated with all the tested drugs a decrease in the fluorescence intensity of DHE is determined, indicating lower intracellular superoxide anion production compared with untreated cells.

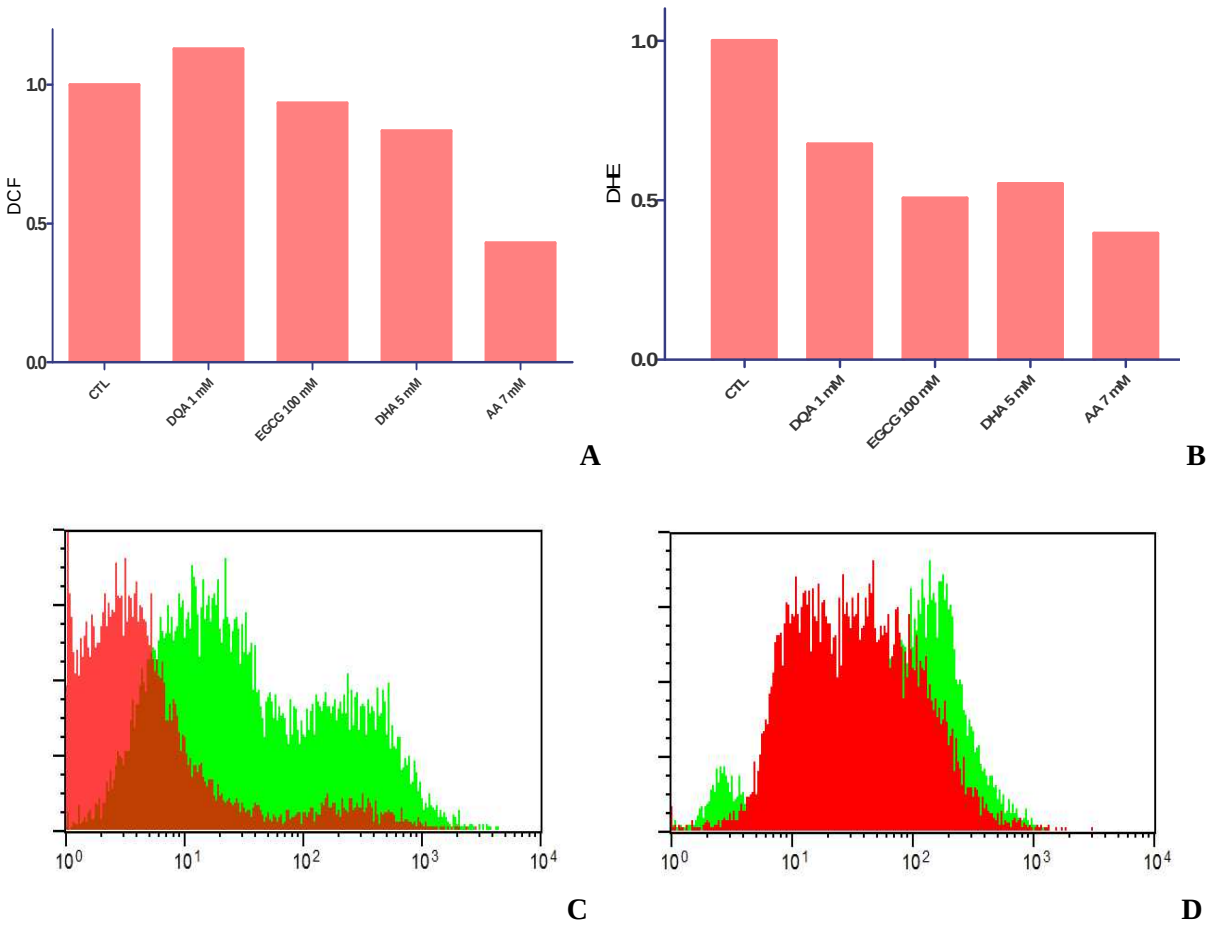


Figure 14. Evaluation of intracellular peroxides and superoxide anion levels in HUH-7 cells by flow cytometry: Cells were incubated in absence (CTL) and in presence of 1µM DQA, 100µM EGCG, 5mM DHA and 7mM AA in monotherapy. Intracellular expression of peroxides (hydrogen peroxide, H_2O_2) (**A**) and superoxide anion ($O_2^{\cdot-}$) (**B**) was evaluated by mean fluorescence intensity (MIF) of DCF and DHE, respectively, as described in material and methods and the variation related to control is calculated. All results were obtained upon incubation of cells during 48h and represent the mean ± SD of 2 independent experiments. In (**C**) is an example of the histogram of control cells (green) and cells incubated with AA (red) and in (**D**) is an example of the histogram of control cells (green) and cells incubated with AA (red).

5. DISCUSSION AND CONCLUSION

There is evidence to suggest that most known environmental risk factors for HCC development lead to generation of reactive oxygen species (ROS). Recently, the role of mitochondria in carcinogenesis has been under numerous investigation, in part because their prominent role in apoptosis, ROS production and other aspects of tumor biology (Olaia, 2007). Furthermore, certain anticancer agents including doxorubicin, bleomycin, and arsenic trioxide kill cancer cells via mechanisms involving ROS generation. (Hileman *et al.*, 2004)

On the other hand, the importance of natural bioactive compounds with antioxidant/pro-oxidant activity is well known. Thus, their synthetic derivatives may be used as potential anticarcinogenic agents.

Therefore, in this study, we evaluated the therapeutic potential of new compounds targeting the mitochondria, such as Dequalinium (DQA), a lypophilic cation, and natural bioactive compounds, such as Epigallocatechin-3-gallate, a green tea polyphenol, and Vitamin C (the reduced form, ascorbic acid, AA, and the oxidative form, dehydroascorbic acid, DHA) as in monotherapy and/or in association with each other and with conventional anticarcinogenic drugs, in order to identify which of them may be a useful therapeutic approach in HCC.

Naturally occurring dietary agents known to produce chemopreventive effects in experimental cancer models have been shown to target signalling intermediates molecules in apoptotic pathways (Aggarwald *et al*., 2006; Martin, 2006). In recent years, because of their low systemic toxicity, vitamins have been evaluated for their anti-tumour activities and have gained importance because of their prophylactic and therapeutic potential role in many

diseases. Antioxidants, such as vitamin C, show protective effects and, under some circumstances, can develop pro-oxidant properties, dependably on its concentration and cell systems (Ratnam, 2006).

Several studies have shown that intracellular redox changes caused by these agents can modulate the expression of genes involved in signal transduction pathways leading to cell cycle progression, cell differentiation, and apoptosis (Aggarwald *et al*., 2006; Martin, 2006). Compelling evidence indicates that dietary bioactive agents may trigger apoptosis through numerous molecular targets. Other inducers of apoptosis include both intra and extracellular stimuli, such as DNA damage, disruption of the cell cycle, hypoxia, detachment of cells from their surrounding tissue, and loss of trophic signaling (Martin, 2006). Some of these compounds seemed to selectively induce apoptosis in cancer cells while sparing normal cells (Martin, 2006).

The therapeutic strategies used in the present to treat cancer such as chemotherapy and ionizing radiation, induce cellular death mostly by apoptosis, through the production of ROS. As apoptosis can be initiated by high doses of natural bioactive compounds and the tumor cells can be selectively targeted by them, these agents may be considered as potential new therapeutic strategies in cancer, namely in hepatocellular carcinoma.

Our results suggest that natural bioactive compounds have antiproliferative effect in monotherapy in a dose, time and compound dependent manner. In fact, the effect of DHA was obtained earlier and in lower doses when compared with AA. On the other hand, this antiproliferative effect increases when these drugs are in association (synergism) with each other or with conventional anticarcinogenic agents.

Moreover the antiproliferative effect, a cytotoxic effect mediated mostly by apoptosis was observed in HUH-7 cells with both forms of Vitamin C, in agreement with described in other cancer cells lines (Yang *et al*., 2003; Gonçalves, 2008). However, in opposite with the

described by others (Yang *et al*.,2003; Gonçalves, 2008) the cytotoxic effect is not accompanied by a pro-oxidative status, since we didn't observe an increase in ROS levels. But, we hadn´t evaluated the antioxidant defenses which could interfere with oxidative stress, and consequently with ROS levels.

Besides the earlier effect of DHA obtained at lower doses compared with AA, we weren't able, actually, to obtain the IC50 of AA when used in a single dose administration scheme which may be explained by the uptake and cellular vitamin C distribution. As described by some authors, DHA enters the cell through the glucose transporters GLUT1 (Agus *et al*., 1999; Reynolds *et al.*, 2007), besides that, tumor cells have an increase in glucose needs which is counterbalanced by the increase number of membrane glucose transporters number (González *et al.*, 2005). On the other hand, AA enters the cell by a co-transporter with sodium in some cell types (González *et al.*, 2005; Wilson, 2005). These facts may explain the selectivity of this vitamin to the neoplastic cells. Once DHA is inside the cell it is converted to AA with formation of ROS. Thus, DHA seems to be a more effective therapeutic strategy enabling lower systemic toxicity since DHA generates less ROS in normal cells and in the exterior of targeted cells (Reynolds, 2007).

The cytotoxic effect induced by vitamin C is mediated by apoptotic cell death since we observed morphological characteristics of apoptosis such as cellular contraction, nuclear fragmentation, blebbing and apoptotic bodies' formation. However, we didn´t observe a decrease in mitochondrial membrane potential and an increase in BAX/BCL2 ratio suggesting that mitochondria may be not involved in vitamin C induced apoptosis.

The exact mechanism by which vitamin C induces injury and decreased cell survival is unclear. However, oxidative stress exerted by ROS formation, such as $OH^{\cdot -}$, H_2O_2 and $O_2^{\cdot -}$, has been proposed as one mechanism that triggers cell death induced by vitamin C (Maramag *et al*., 1997; Chen *et al*., 2005). According to Chen et *al*. (2005), during the processes of

vitamin C interconversion, ROS production occurs. In this sense, we determined the intracellular production of H_2O_2 and $O_2^{\cdot-}$ in HUH-7 cells in the absence and presence of vitamin C. Our results suggest that vitamin C induced a significant decrease in ROS formation (Figure 14) in opposite with other studies (Chen *et al.*, 2005). However, we didn´t measure other ROS, such as $OH^{\cdot-}$. In fact, according with Maramag et *al*. (1997), $OH^{\cdot-}$ formation occurs during Fenton's reaction in which the ascorbyl anion or radical reduce metal ions such as ferric and cupric ions. These ions react with H_2O_2 producing the $OH^{\cdot-}$ radical. Alternatively, the ascorbyl anion and the radical can react with O_2 leading to the production of $O_2^{\cdot-}$ that subsequently can reduce Fe^{3+} and start Haber-Weiss' reaction (Maramag *et al*., 1997). These mechanisms may well be the cause of pro-oxidant effect of vitamin C and explain the cytotoxic effects shown in human hepatocellular carcinoma cells. On the other hand, other mechanisms may be involved namely the modulation of signal transduction and gene expression by vitamin C.

Actually, intracellular redox changes caused by oxidants and antioxidants can modulate genes expression involved in signal transduction pathways leading to cell cycle progression, cell differentiation, and apoptosis (Allen *et al.*, 2000). Catani *et al.*, (2001) show in cells treated with ascorbic acid, at low pharmacologic concentration (1mmol/L), an increase in the expression of apoptotic genes usually induced by UV irradiation and DNA damage, indicating that vitamin C can modulate gene expression. The therapeutic potential of vitamin C in cancer is further supported by its ability to activate the apoptotic program in DNA-damaged cells, independent of the p53 tumor suppressor gene, through an alternative pathway mediated by p73, which, in contrast, is functional in most tumor types (Ikawa *et al.*, 1999).

Besides that, vitamin C at millimolar intracellular concentrations, inhibits the activation of nuclear factor kappa B (NF-kB), by preventing its inhibitor (IkB) degradation

mediated by TNFα in different human cell lines as well as primary cells through independent mechanisms (Bowie & O'Neill, 2000). NFkB is a rapid response transcription factor that induces the transcription of genes involved in inhibition of apoptosis and promotion of cell proliferation, contributing, when overexpressed, directly to malignancy (Inoue *et al.*, 2007). Repression of constitutive activation of NFkB by vitamin C can induce cell cycle arrest and apoptosis in these cells and attenuate tumor progression in different types of cancer.

Then, we evaluated the therapeutic potential of the green tea polyphenol EGCG. EGCG has shown an antiproliferative effect in monotherapy in a dose and time dependent manner. This effect increases when these drugs is used in association (synergism). Besides the antiproliferative effect a cytotoxic effect was observed mediated by late apoptosis and/or necrosis.

Green tea constituents have been characterized as antioxidants that scavenge free radicals to protect normal cells (Ruch *et al*., 1989). However, recent reports have linked green tea polyphenols to ROS production, especially H_2O_2, and subsequent apoptosis in both transformed and nontransformed human bronchial cells (Yang *et al*., 2000) and in myelodysplastic syndrome (Gonçalves, 2008). EGCG is also able to create differencial oxidative environments in normal epithelial *versus* tumor cells by exploiting compromised redox homeostasis in the tumor cells (Yamamoto *et al.*, 2003).

Our results suggest that EGCG had a cytotoxic effect, which is consistent with the decrease of mitochondrial membrane potential and increase in late apoptosis and/or necrosis. Moreover, we observe a decrease in BAX/BCL2 ratio suggesting that mitochondrial isn't involved in drug toxicity. However, we haven't tested other pro-apoptotic mitochondria molecules such as BAD and cytochrome c.

On the other hand, unlike what is suggested by other studies in Myelodysplastic Syndrome (Gonçalves, 2008), EGCG didn't induced an increase in ROS formation in HUH-7 cells, but a decrease in $O_2^{\cdot -}$ was observed. The use of an insufficient dose of EGCG to bring out its pro-oxidative effects may explain these results. Thus, the cytotoxic effect observed must be explained by another mechanism. In 1997, Zhao *et al*. suggested that EGCG induces cell death in acute promyelocytic leukemia cells by inhibition of DNA topoisomerase II activity. Later, it was found that EGCG induces the formation of H_2O_2 in cells of lung cancer, which may contribute to apoptosis and in part to the anti-proliferative effect (Yang *et al*., 2000). In multiple myeloma cells was found that oxidative stress generated by increased ROS production induces mitochondrial membrane potential alteration, caspase 3 activation, release of cytochrome c and SMAC/DIABLO. Then, Quanungo studies (2005) suggest that EGCG induces apoptosis by directly inhibiting BCL-2 proteins family. Furthermore, they show that apoptosis induced by EGCG in leukemia cells involves ROS formation and mitochondrial membrane depolarization, with the involvement of a cooperative mechanism between the extrinsic and intrinsic apoptosis pathways.

The therapeutic efficacy of EGCG has also been associated with activation of tumour suppressor genes silenced by methylation. This effect is related to the ability of EGCG to inhibit DNMT causing DNA demethylation and reactivation of genes silenced by methylation (Fang et *al*., 2003 and 2007). However, it is unclear whether the role of EGCG as epigenetic modulator is also exerted *in vivo*.

Most of the anticarcinogenic therapeutic strategies are aimed to induce malignant cell death in order to eradicate the tumor, thus limiting its growth and spreading. It is well established that the efficacy of conventional antitumor drugs is due to their ability to induce apoptosis (Makin, 2002 and 2003; Brady, 2003). Mitochondria are now known to play a

critical role in initiating apoptotic cell death. Thus, diverse stress stimuli induce mitochondrial changes, which result in the release of apoptogenic factors into the cytoplasm such as cytochrome c, clearly observed in the early phases of apoptosis. This is associated with changes in the mitochondrial ultra-structure, membrane permeability, transmembrane potential, and caspase activation (Adrain & Martin 2001; Pelicano 2004; Körper, 2004). Intriguingly, a wide variety of carcinoma cells exhibit increased accumulation and retention of delocalized lipophylic cations (DLCs) due to a higher negative mitochondrial transmembrane potential in tumor cells than in normal cells (Modica-Napolitano *et al*., 2001 and 2003). This behavior provides an attractive basis for the use of DLCs in selective tumor cell eradication.

In this study, we evaluate the therapeutic efficacy of Dequalinium (DQA), a lipophilic cation (DLC) that crosses the cell membrane and accumulates in the mitochondria of hepatocarcinoma cell lines. Since the neoplastic cells have a higher mitochondrial transmembrane potential than normal cells (which gives them a greater capacity for accumulation and retention of lipophilic cations), DQA may be a new selective therapeutic strategy for hepatocellular carcinoma. Its anti-cancer effect has been described in cells of various neoplasms (Berlin *et al*., 1998, Galeano *et al*., 2005; Sancho *et al*., 2007; Gonçalves *et al.*, 2009; Ribeiro *et al*. 2010). However, most studies with DQA have been focus in epithelial tumor cells (Berlin *et al.*, 1998) and the potential therapeutic in HCC is not clarified.

Our results suggest that DQA showed an antiproliferative and cytoytoxic effect in HUH-7 cells inducing cell death mostly by late apoptosis/necrosis that is consistent with the observed decrease in mitochondrial membrane potential. The increase in the observed JC-1M/JC1-A ratio may indicate the existence of mitochondrial dysfunction due to yet unclear DQA mechanism of may be a consequence DQA induced apoptosis.

In this study we also tried to understand which administration scheme was more effective. With all the agents tested we observed that the same cytotoxic effect can be obtained with lower dosages when a daily dose scheme was used. This scheme mimics the way of administration used with the majority of anti-tumor agents used *in vivo*. These results suggest a clinical benefit of the daily scheme administration because by lowering the drugs concentration we can decrease the toxicity and possibly the side effects.

Finally, we also studied if the association between the new tested drugs and/or with the conventional anticarcinogenic agents, DOX and 5-FU, may have a therapeutic benefit. Our results showed that in the majority of the tested drugs, when used in association, an increase in cytotoxic effect (synergism) is achieved. In fact, a potentiation synergistic effect was observed in the associations of AA with DOX and in the association of EGCG with DHA and with AA. An addition synergism was obtained in the associations of DQA with DOX and EGCG with 5-FU. In our study mitochondria may have a role in drug induced apoptosis.

Thus, these results suggest a clinical benefit of the use of the drugs in combination, because by lowering the drugs dosage we can decrease the secondary toxicity and possibly the side effects. However, in the association of DHA with 5-FU an antagonism effect was observed suggesting that the choice of the optimal schedule of drugs will also be crucial to the success of the therapy. Besides that, it was also observed the existence of an antiproliferative effect without a concomitant cytotoxic effect.

Hence, we can concluded, in agreement with the studies of Chinery *et al.* (1997) in colorectal carcinoma, that chemotherapeutic agents administered in the presence of EGCG and vitamin C, allows doses reduction providing a novel therapeutic approach for hepatocelullar carcinoma.

Overall, our results provide evidence that all these new targeted drugs may be presented as alternative treatments for HCC, improving patients' health condition. However, new drugs associations, as well as new administration schemes, should be tested in order to improve therapeutic efficacy in HCC.

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