# Interaction between Gambogic Acid and Dihydrofolate Reductase and Synergistic Lethal Effects with Methotrexate on Hepatoma Cells

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**Abstract.** *Gambogic acid (GA), a natural xanthone, has a wide spectrum of pharmacological activities, including repression of telomerase expression and induction of apoptosis of cancer cells. GA has also been reported to reduce the steady-state level of thymidylate synthetase mRNA in a gastric carcinoma cell line. Therefore, it has recently emerged as a candidate for use in cancer treatment. Using hepatoma cells with a dihydrofolate reductase (DHFR) gene amplification and cells transfected with an inducible DHFR transgene, we observed a negative relationship between DHFR expression and resistance to GA. Furthermore, DHFR assays in vitro indicated that in the presence of GA, DHFR activity was slightly inhibited and the affinity of the enzyme for dihydrofolate was markedly decreased. Treatment of rat hepatoma and other human and murine cancer cell lines with methotrexate and GA revealed that the two drugs displayed a marked synergistic lethal effect.*

Hepatocellular carcimoma (HCC) is a common cancer, resistant to conventional chemotherapies and, consequently, novel therapeutic approaches are being sought. Methotrexate is a well-known, effective anticancer drug. It is an inhibitor of dihydrofolate reductase (DHFR), the enzyme controlling the limiting reaction of the folate pathway, which is essential for DNA synthesis. The ubiquity of this methotrexate target, the well-documented mode of action of the drug and

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knowledge of the mechanisms of resistance are such that methotrexate may be a candidate for the treatment of hepatocellular carcimoma (HCC). However, effective doses for HCC are highly toxic, presumably due to the conversion of methotrexate to polyglutamylated methotrexate derivatives in the liver. This led us to investigate associations of methotrexate with other drugs, so as to identify combinations allowing for reduction of the methotrexate dose to better-tolerated, but nevertheless effective levels.

Gambogic acid (GA) is a xanthone derivative, abundant in the resin of *Garcinia morella* and *Garcinia hanburyi* with a wide spectrum of pharmacological activities: i) potent inhibition of telomerase, through both repression of gene expression and enzymatic inhibition (1, 2); ii) effects on nucleoporin efficiency (3); iii) inhibition of survivin (4); iv) induction of apoptosis (5, 6) through activation of caspases 3 and 9, BCL-2 associated X protein (BAX) and B-cell lymphoma-2 (BCL-2) (7, 8); v) inhibition of topoisomerase IIa (9); vi) reduction of the phosphorylation of cell division control protein 2 homolog (CDC2)/p34 (10); and vii) down-regulation of the oncogene murine double minute-2 (MDM2) (11). We were unable to find any report of resistance to GA in the literature. Very little is known about its interaction with other anticancer drugs, although one group reported that GA reversed docetaxel resistance (4). GA was also reported to reduce the steady state level of thymidylate synthetase (*TYMS*) mRNA in a gastric carcinoma cell line. This results in a synergistic effect between GA and 5-fluorouracil, a known TYMS inhibitor (12). As both MTX and GA interfere with folate metabolism, we investigated possible synergism between the two drugs on HCC cell lines.

## Materials and Methods

*Cell lines and culture conditions.* Cells of the Fao rat hepatocarcinoma clone are stably differentiated and tumorigenic (13-15). They were routinely cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with

5% fetal calf serum (FCS). Fao10MTX and Fao20MTX cells, respectively resistant to 10 μM and 20 μM methotrexate, were obtained by stepwise selection in an increasing concentrations of methotrexate (16). The methotrexate resistance of these cells is associated with amplification and overexpression of the gene encoding DHFR. FRT11 is the product of Fao cell transfection with the pTet-Off-Rheoswitch plasmid. This construct was made by introduction of the Tet-off cassette (2352-pb *BsrG*1-*Pvu*II fragment from of pTet-Off vector; Clontech Laboratories, Mountain View, CA, USA) between the *Bsiw*1 and *Ale*1 sites of the vector pNEBRR1 (New England Biolabs, Ipswich, MA, USA). FRT11 cells are potential recipients of pTRE2 and pNEBRX1 vectors containing genes of interest under the control of both inducible systems. Only the Tet-Off system was exploited in this work. FRT11pTREdhfr is the product of transfection of FRT11 by pTREdhfr. pTREdhfr was obtained by introducing the *Hind*III-*Hind*II mouse *Dhfr* cDNA fragment from pSV2dhfr between the *Hind*III-*EcoR*V sites of pTRE2. Transfected clones were selected in 50 nM methotrexate under induction conditions (without doxycycline). Selected clones were screened for inducibility, by testing methotrexate resistance and expression of Dhfr with and without doxycycline (0.2 μg/ml). Selected clones were subjected to stepwise selection in increasing concentrations of methotrexate up to 5 μM, under induction conditions (without doxycycline). The resulting selected clones were checked for repression by doxycycline of amplified *Dhfr* transgene expression and sensitivity to methotrexate. All derivatives of the Fao line were cultured in Coon's modified Ham F12 medium supplemented with 5% FCS.

C2 is a de-differentiated non-tumorigenic clone derived from the rat Fao hepatoma line (13-15). Constitutive methotrexate-resistant C2 derivatives were obtained by stepwise selection in increasing concentrations of methotrexate. This resistance is associated with increased expression of DHFR and amplification of the *Dhfr* gene (16). The cells were cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with 5% fetal calf serum (FCS).

The HepG2Tet-Off Advanced cell line (Clontech) is a derivative of the HepG2 human hepatocarcinoma line. It contains the pTet-off vector and was screened on G418. Inducible expression of the transgene by the pTRE vector was not exploited in these experiments. These cells were cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with 10% fetal calf serum (FCS).

BW1J, a subclone of the BW1 line derived from the mouse hepatoma BW7756 (17), was cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with 5% fetal calf serum (FCS).

Mouse melanoma 44OCl2b (18) is a subclone of the pigmented clone 440, isolated from the 816 melanoma (19). These cells were cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with 5% fetal calf serum (FCS).

PCC4aza-1, a mouse embryonal carcinoma stem cell line (20), was cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with 15% fetal calf serum (FCS).

ARIP, a rat exocrine pancreatic cell line derived from a transplantable tumor (21), was obtained from the American Type Culture Collection (ATCC). These cells were cultured in Coon's modified Ham F12 medium without thymidine and hypoxanthine and supplemented with 10% fetal calf serum (FCS).

The 440Cl2b, BW1J and PCC4 aza1 cell lines were kindly provided by Catherine Fougère-Deschatrette (Unité de Génétique de la Différenciation, URA 2578, Centre National de la Recherche Scientifique).

*Reagents*. Methotrexate was dissolved in NaOH at a final concentration of 100 mM. This stock solution was diluted to 10 mM and 100 μM in water. Methotrexate solutions for DHFR assays were prepared according to the manufacturer's instructions (kit CS0340; Sigma Aldrich, Saint Louis, MO, USA). GA (G8171-25MG; Sigma) was dissolved in ethanol at a final stock concentration of 20 mM. A 1/10 dilution of the stock in dimethyl sulfoxide (DMSO) was used to prepare the culture media. For *in vitro* DHFR assays, the 20mM stock and dilutions were prepared in ethanol (<0.1% ethanol in the reaction mixture).

*Determination of GA resistance of cells overexpressing DHFR*. Fao cells and Fao cells with constitutive over-expression of DHFR (Fao10MTX and Fao20MTX) were seeded at a density of $10^4$ cells per 57 cm$^2$ dish in standard medium. The next day, cells were subjected for 24 h to medium containing between 0.25 and 2 μM GA. Resistant colonies were counted on day 14.

Cells transfected with the *Dhfr* cDNA under the control of the Tet-Off system were checked for resistance to GA: a series of dishes was cultured for each 1, 5 and 11 days under induction conditions (without doxycycline). Cells of these series were then harvested, counted, and plated under induction conditions at a density of $10^4$ cells per dish. The next day, the medium was replaced with medium supplemented with different concentrations of GA (from 0.25 to 2 μM). After 24 h of treatment with the drug, the cells were further grown in standard medium for 14 days, fixed, stained and the colonies of resistant cells counted. Controls under repressed conditions (with 0.2 μg/ml doxycycline) were established using the same protocol.

*Combined treatment of cells*. For the test of resistance of Fao cells to 24-hour drug treatments, cells were plated at a density of $10^3$ cells per dish in standard medium and the medium was replaced the following day with medium containing either 1 μM methotrexate or 0.5 μM GA or both. The next day, the media were again changed to media containing 0.5 μM GA or 1 μM methotrexate or to standard medium, as appropriate. The day after, all media were changed to standard medium. Colonies were fixed, stained and counted on day 14.

For the test of resistance of Fao cells and other cell lines to long-term treatment with the drugs, cells were plated at a density of $10^3$ cells per dish in standard medium and the medium was changed the day after to media containing both drugs at different concentrations. Methotrexate concentrations were between 2 and 30 nM except for HepG2 cells (100-400 nM). GA concentrations were 0.1 to 0.8 μM except for PCC4 cells (0.02-0.1 μM), 440cl2b cells (0.0025-0.01 μM) and ARIP cells (0.01-0.05 μM). Colonies were fixed, stained and counted on day 14.

*Cell growth rate determination*. The growth rate of C2 rat hepatoma cells was determined by inoculating 57 cm$^2$ dishes with $10^5$ cells and harvesting and counting cells every day. The cell growth rate was calculated as the cell doubling time in the log-linear part of the curve. Variations in cell growth rate were also assessed by culturing 200 cells per dish in medium without methotrexate, and recording colony numbers and sizes on day 14, after fixation and staining.

*DHFR assay*. DHFR assays were performed using a kit from Sigma (code CS0340). The CellLytic™ M extraction Reagent (C2978; Sigma) was used to prepare cell extracts. Extracts of Fao cells resistant to high levels of methotrexate and exhibiting strong DHFR expression were used for these experiments. The cells were cultured without methotrexate. DHFR activity was assayed in the presence of both methotrexate and GA as follows: a complete reaction mixture was prepared lacking only the substrate, DHF, but containing the enzyme. Methotrexate or GA was added and the mixture and incubated for two minutes before addition of the other drug. The reaction was then started, by adding DHF to the mixture.

## Results

*Preliminary experiment: lethal synergistic effect of methotrexate and GA on hepatoma cells*. The preliminary experiments involved testing the resistance of Fao rat hepatoma cells to chronic low-dose treatment with methotrexate, GA or a mixture of the two drugs. Two days after inoculation of a series of dishes ($10^3$ cells per 10 cm-diameter dishe) in standard medium, the medium was changed to medium supplemented with either 15 nm methotrexate or 0.5 μM GA or a mixture of the two drugs. The cells were further incubated under the same conditions for 14 days. Cells were then fixed, stained and the number of resistant colonies was recorded. On treatment with 15 nm methotrexate or 0.5 μM GA, cell survival was respectively 60% and 85%, but in the mixture of the two drugs, 85% of the cells were killed. These preliminary findings suggested a synergistic effect of methotrexate and GA.

*GA resistance of cells overexpressing DHFR as a consequence of Dhfr gene amplification*. We used two Fao cell lines, one resistant to 10 μM methotrexate and the other to 20 μM methotrexate, both of which constitutively and strongly express DHFR due to amplification of the *Dhfr* gene (16). We tested the resistance of these cells to 24 h of treatment with each of five concentrations of GA (0.25 to 2 μM). These methotrexate-resistant cells were markedly less resistant to GA than the parental Fao cells (Figure 1A).

We then tested Fao cells transfected with a *Dhfr* cDNA, in which *Dhfr* expression is under the control of the Tet-Off inducible system. Transfected cells resistant to 5 μM methotrexate were obtained by stepwise selection in increasing concentrations of the drug. Resistance to methotrexate emerged 24 h after removing doxycycline, the repressor of the *Dhfr* transgene (data not shown). In contrast, resistance of the transfected cells to increasing concentration of GA decreased as the period of induction of *Dhfr* transgene was prolonged up to 12 days (Figure 1B).

There was a negative correlation between GA resistance and DHFR activity in Fao cells and in their methotrexate-resistant derivatives with constitutive or inducible *Dhfr* overexpression ($R^2$=0.88; Figure 1C).

*Effects of GA on DHFR activity in vitro*. We compared the effects of GA and methotrexate on DHFR activity in Fao cell extracts *in vitro*. GA inhibited the enzymatic activity weakly, and only at much higher concentrations than those required for methotrexate to inhibit the enzyme (Figure 2A). The enzymatic activity was also tested in the presence of both drugs: the result depended on the order of addition of methotrexate and GA to the reaction mixture containing the enzyme. When the enzyme was incubated with methotrexate first, the inhibition induced by combined treatment (93% inhibition) was the sum of inhibitions recorded with each drug tested independently (90% inhibition with methotrexate and 45% with GA). When GA was added to the reaction mixture first, inhibition by the combined treatment (82%) was more modest and lower than the inhibition induced by methotrexate-alone (Figure 2B).

To analyze the interaction between GA and DHFR, the DHFR reaction was run to completion at GA concentrations (0.6 to 6 μM) which do not inhibit enzymatic activity. The plateau corresponding to the completion of the reaction was higher in the presence than in the absence of GA and this effect was strictly dependent on the concentration of the drug (Figure 3A). This indicated that in the presence of GA, the reaction stopped before exhaustion of at least one of the enzyme substrates, either DHF or NADPH. To further investigate this effect, the reaction was run in the presence of GA to reach the plateau and then the mixture was reloaded successively with DHF and then NADPH, or with NADPH and then DHF (Figure 3B).

Re-initialization of the reaction was observed only after the addition of DHF and not of NADPH. Note that in the control experiment, reloading with both substrates was necessary for re-initialization of the reaction (Figure 3C).

*Methotrexate and GA combined treatments of Fao cells*. Two series of experiments were performed to examine methotrexate and GA synergism in altering the DHFR system and thereby killing cancer cells. The first series consisted of short treatments (24 h which is approximately one cell cycle) with the drugs. Three different protocols were used (Figure 4A): (I) GA at 0.5 μM for 24 h and then methotrexate at 10 nM for 24 h, (II) both drugs at the same time for 24 h, and (III) MTX for 24 h and then GA for 24 h. To minimize the pleiotropic effects of GA, we used concentrations of the drug that had no effect on cell survival. The four controls included 24-h treatment with either GA or methotrexate-alone on day 1 or day 2. When cells were treated with methotrexate before GA, there was no additional toxicity of GA (42% of cells surviving in both cases). Pre-treatment with GA led to greater lethality than treatment with methotrexate-alone (28% of cells surviving *versus* 60%; Figure 4B). When GA and methotrexate were applied at the same time, the proportion of cells surviving was slightly lower than that following methotrexate treatment alone (35% *versus* 42%).
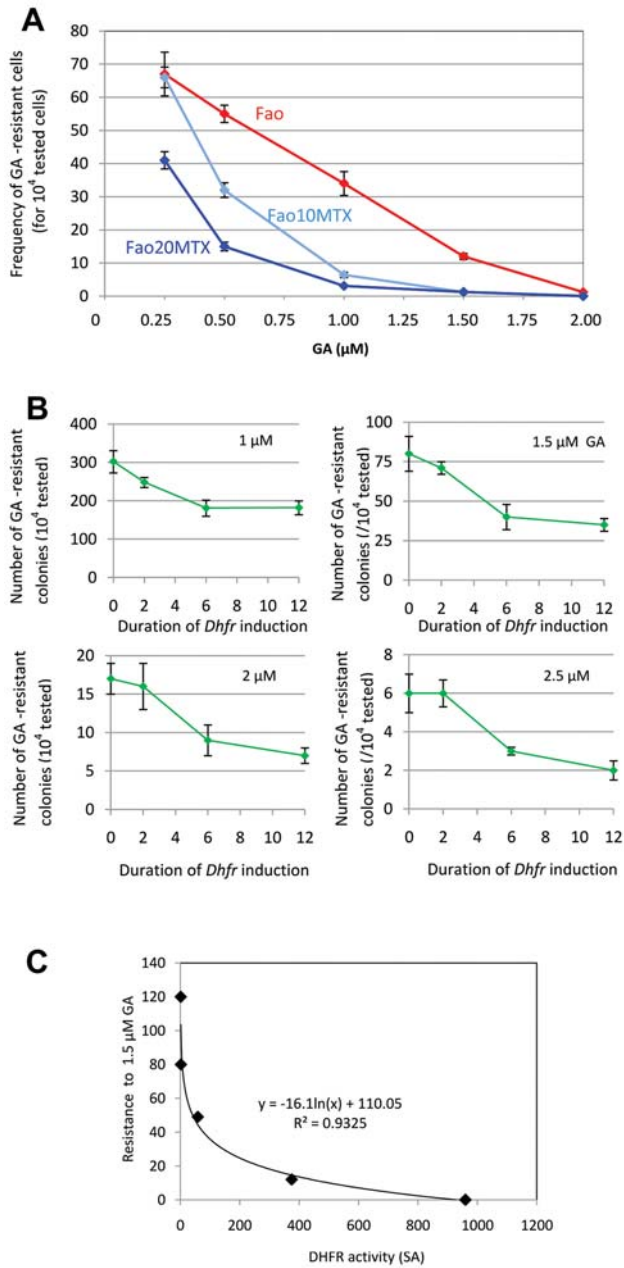
Figure 1. *Resistance to gambogic acid (GA) of Fao cells selected in high concentrations of methotrexate (MTX) and constitutively overexpressing dihydrofolate reductase activity (DHFR). A: Fao cells (red) and Fao cells resistant to 10 μM (light blue) and 20 μM MTX (dark blue), were tested for resistance to 24 h of treatment with different concentrations of GA. Data (mean of triplicates) are the numbers of resistant cells per $10^4$ inoculated cells. B: Resistance to GA of Fao cells overexpressing the inducible Dhfr transgene. The Dhfr transgene was induced for 2, 6 or 12 days and resistance to GA (1 μM, 1.5 μM, 2 μM and 2.5 μM for 24 h) was then tested. Data (mean of triplicates) are numbers of resistant colonies per $10^4$ cells tested. C: Resistance to GA (1.5 μM) vs. DHFR activity in Fao cells and their derivatives (Fao cells constitutively resistant to 10 μM and 20 μM MTX and Fao cells over-expressing the inducible Dhfr transgene under conditions of induction and non-induction of the transgene).*

The second series of experiments involved chronic low-dose treatment with both drugs for 14 days. Again, three concentrations of GA were used (0.1 μM, 0.25 μM, and 0.5 μM) that had very little effect on cell survival (92% cell survival for 0.5 μM). Four concentrations of methotrexate were used: at 5 nM and 10 nM, cells were not affected; at 15 nM and 20 nM, 65% and 30%, respectively, of the cells survived the treatment. Combined treatment with the two drugs markedly enhanced cell death and this effect increased with both GA and methotrexate concentration (Figure 4C and 4D). At 0.25 μM AG and 10 nM methotrexate, only about 30% of the cells survived.

*Synergistic lethal effects of GA and methotrexate on other cell types (Figure 5).* The following tumor cell lines were tested for sensitivity to the cooperative lethal effects of GA and methotrexate: the human HepG2 and mouse BW1J hepatocarcinoma cell lines, a mouse embryonal carcinoma stem cell line (PCC4 aza1), a mouse melanoma line (440Cl2b) and a rat exocrine pancreatic cell line (ARIP). The range of drug concentrations was selected according to the sensitivity of the cell lines to each molecule. The two drugs had marked synergistic lethal effects on the two hepatocarcinoma cell lines (HepG2 and BW1J), similar to the effects observed for Fao. This effect was also observed for the tumoral cell lines PCC4, 440cl2B and ARIP.

*GA resistance and cell growth rate of C2 and methotrexate-resistant C2 cell derivatives.* C2 is a de-differentiated non-tumorigenic variant Fao clone which grows more rapidly than the Fao clone of origin (generation time of about 15 hours *versus* 22 hours). The growth of C2 derivatives resistant to 0.1 and 0.5 μM methotrexate (as a consequence of *Dhfr* gene amplification and overexpression of the enzyme) was similar to that of control C2 cells. These C2 cells, resistant to 0.1 and 0.5 μM methotrexate, were less resistant to GA than were the C2 controls (Figure 6C), as described above for Fao cells. In contrast, for cells resistant to 2 μM and 20 μM methotrexate, with still higher DHFR expression, the population doubling time was longer (Figure 6A and 6B) and these C2 cells exhibited GA resistance that was higher than that of the C2 cells of origin (Figure 6C). This type of effect of DHFR overexpression on both cell growth rate and GA resistance was not observed in slower-growing Fao cells (Figure 6B).

## Discussion

Our analysis of the effects of GA on DHFR activity *in vitro* led to two observations. Firstly GA is a weak inhibitor of DHFR activity. Secondly, in the presence of GA, the reaction catalyzed by DHFR stopped before completion. This effect was suppressed when the reaction mixture was supplemented with DHF but not when supplemented by NADPH. This
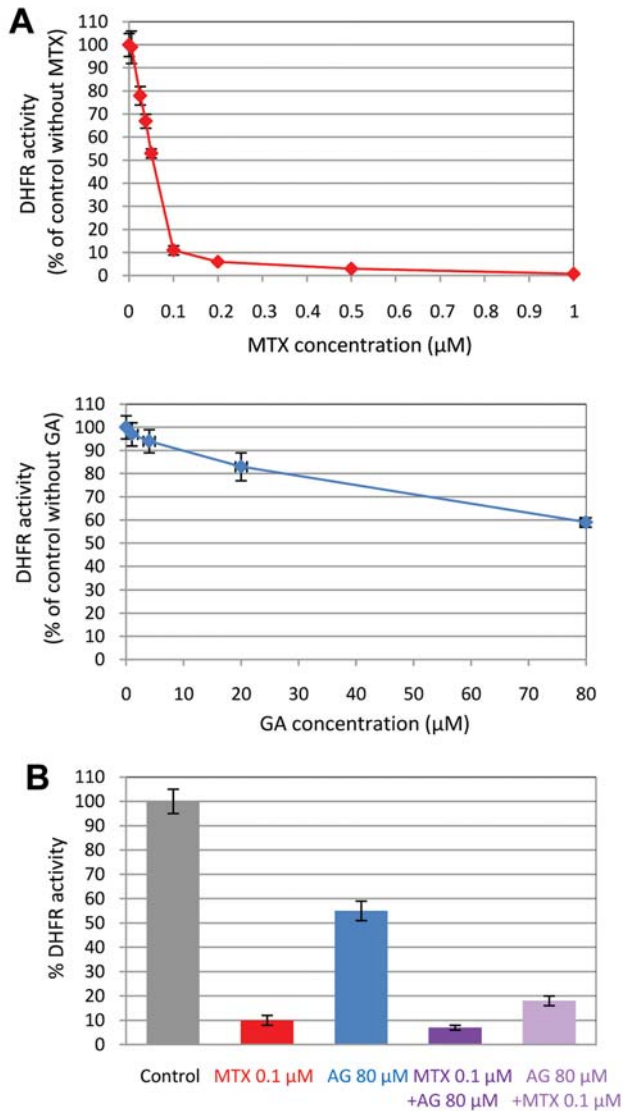
Figure 2. *Effects of methotrexate (MTX) and gambogic acid (GA) on dihydrofolate reductase (DHFR) activity in vitro. A: Inhibition of DHFR activity by MTX and GA. DHFR activity in Fao extracts was measured in the presence of different concentrations of MTX (red) and GA (blue). Reported values are percentages of the activity without any drugs. B: Effects of combined MTX and GA on DHFR activity. The enzymatic reaction was performed under five different conditions: without any drug, in the presence of 0.1 μM MTX, in the presence of 80 μM GA, and in the presence of both drugs (both MTX added before GA and the reverse). The reaction was started by addition of DHF (see Materials and Methods). Reported values are percentages of the DHFR activity recorded without any drugs.*



Figure 3. *Gambogic acid (GA) dose-effect on completion of the dihydrofolate reductase (DHFR) reaction. A: GA-mediated premature arrest of DHFR activity. B: Effect of addition of DHF and NADPH on GA-mediated premature arrest of DHFR activity. In red: The reaction was performed in the presence of 4 μM GA and when the plateau was reached, NADPH was added to the reaction mixture to restore a concentration equivalent to the initial conditions. This did not re-initialize the reaction. DHF was then added to restore the concentration to that of the initial conditions and the reaction restarted. In blue: The same experiment was performed starting with supplementation with DHF. Again, re-initialization of the reaction was observed only with DHF supplementation. C: Control: the same experiments were performed without GA. The reaction only re-started after simultaneous addition of the two substrates.*

suggests that GA reduces the affinity of DHFR for DHF, and that its weak inhibition of the enzymatic activity is a side-effect. Although the details of the molecular interaction between GA and DHFR need to be elucidated, it is possible that these two effects of GA on DHFR are mediated by
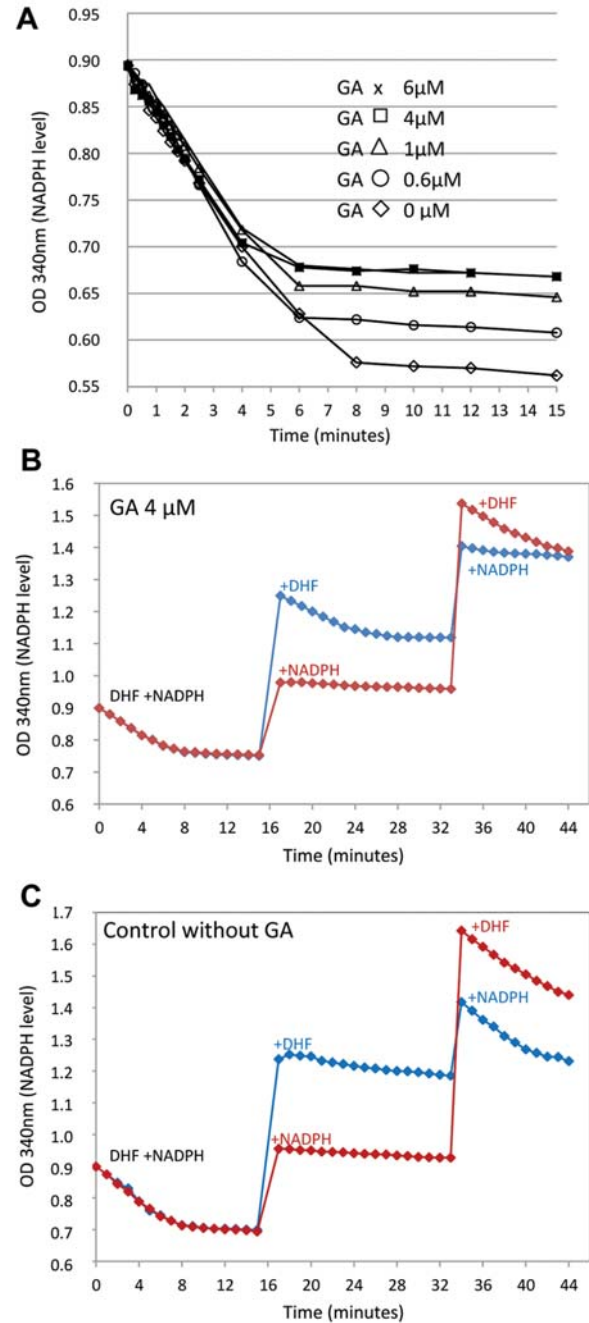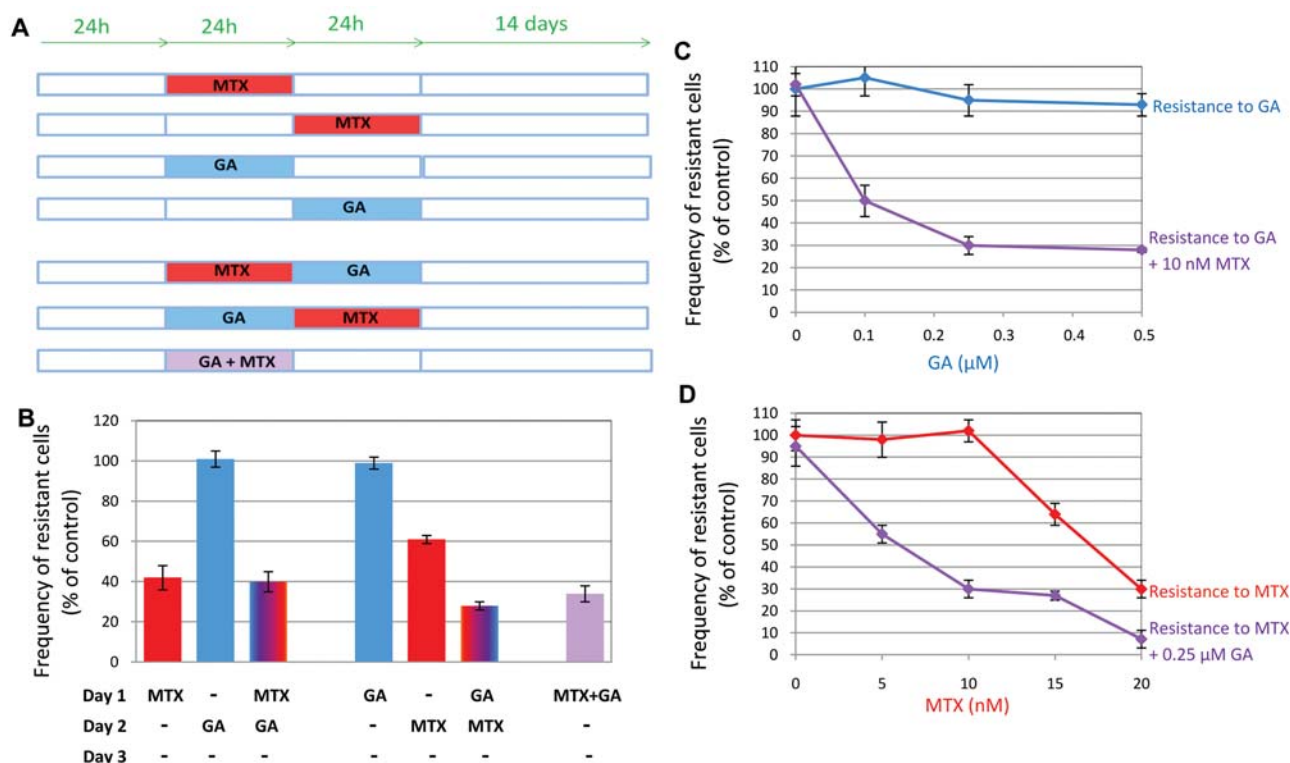
Figure 4. *Effect of treatment with combined methotrexate (MTX) and gambogic acid (GA) on Fao cell death. A: Protocols for 24-h treatments (0.5 μM GA and 1 μM MTX). B: Results of 24-h treatments; abscissa: treatments. Note that 0.5 μM GA-alone had no effect on cell survival. The higher frequency of cells resistant to MTX-alone after treatment on day 2 than on day 1 is due to cell growth during the 24-h delay. C: Long-term treatments with 10nM MTX and different concentrations of GA. Blue: Frequency of cells resistant to GA used alone. Purple: Resistance to different concentrations of GA + 10 nM MTX. D: Long-term treatments with 0.25 μM GA and different concentrations of MTX. Red: Frequency of cells resistant to MTX-alone. Purple: resistance to different concentrations of MTX + 0.25 μM GA. The reported values are percentages of the number of colonies growing in samples without drug treatment.*

DHFR binding to GA in a way that is different from the interaction between methotrexate and the enzyme. This is consistent with the very different chemical structures of methotrexate (a DHF analog) and GA. It is also consistent with the observation that eosin B, another xanthone, is a non-active site inhibitor of bi-functional TYMS-DHFR (22). Furthermore, it would explain the results of the DHFR assays in the presence of combinations of methotrexate and GA: when GA was added first and methotrexate second, to the reaction mixture, the inhibition was slightly lower than that with either drug alone. This is consistent with GA reducing the affinity of DHFR for methotrexate. Accordingly, in cells with inducible expression of exogenous *Dhfr*, GA sensitivity depended on the duration of transgene induction.

C2 cells and the constitutive methotrexate-resistant C2 derivatives exhibited the same inverse correlation between methotrexate resistance and GA resistance as did the parental Fao cells. However, at very high levels of methotrexate resistance and *Dhfr* expression, the cells exhibited a substantially slower growth rate and a restored resistance to

GA. A possible interpretation is that due to the very slow cell growth, the balance was restored between DHF over-metabolism by DHFR and the use of THF in DNA incorporation.

The inhibition of DHFR activity by methotrexate, and the GA-mediated decrease of DHF affinity for the enzyme suggested synergism between the two drugs, for depletion of the reaction product, THF, in dividing cells, resulting in a potent lethal effect. This was tested using two series of experiments with short (24 h) and long (14 days) treatments of Fao hepatoma cells with the two drugs. Interestingly, the consequences depended on the sequence of application of methotrexate and GA. Cell survival following treatment with methotrexate on day 1 and then with GA on day 2 (60%) was similar to that following treatment for two days with methotrexate-alone (62%). In contrast, treatment with GA (on day 1) and then with methotrexate (on day 2) led to the proportion of cells surviving dropping to 28%. When cells were treated concomitantly on day 1 with the two drugs, only 35% of the cells survived. These results are consistent with our model
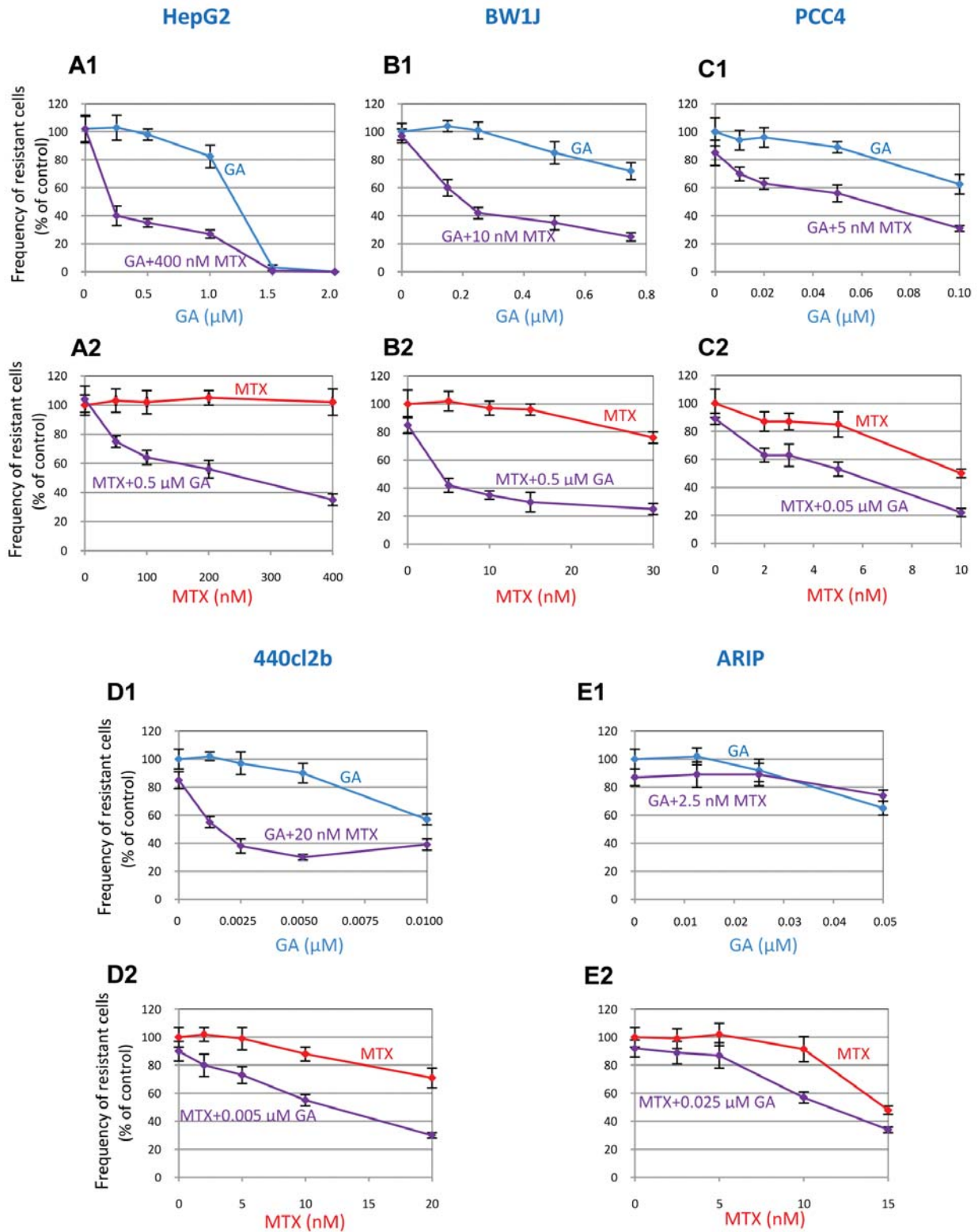
Figure 5. *Effect of long-term treatment with methotrexate (MTX) and gambogic acid (GA) in different cell lines. A: HepG2Tet-off; B: BW1J; C: PCC4aza-1; D: 440cl2b; E: ARIP. For each cell line: series 1: frequency of resistant cells under a constant MTX concentration and different GA concentrations; series 2: frequency of resistant cells under a constant GA concentration and different MTX concentrations. Reported values are percentages of the number of colonies in the medium without drugs.*
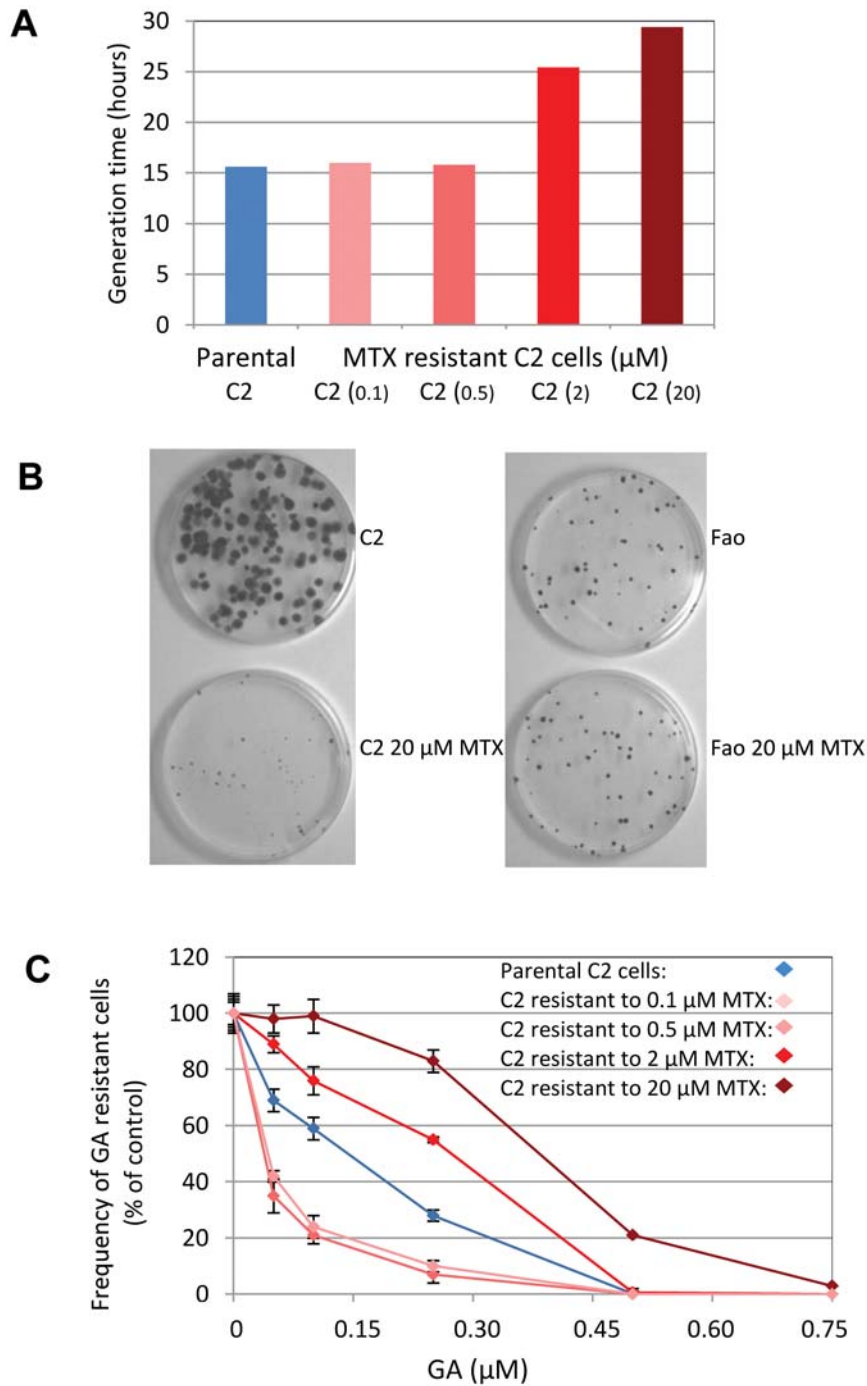
Figure 6. *Gambogic resistance (GA) and growth rate of C2 and Fao cells. A: Population doubling time of C2 cells and methotrexate (MTX)-resistant derivatives. B: Colony sizes of C2 and Fao lines and their MTX resistant derivatives (in medium without methotrexate) on day 14. The number of growing colonies of the C2 and C2 derivative lines was identical. C: C2 cells and C2 cell derivatives selected in increasing concentrations of MTX were tested for resistance to GA.*

of the mode of action of the two drugs: pre-treatment with methotrexate increases the DHF pool and thus counteracts the effect of GA. Alternatively, irreversible methotrexate binding to DHFR may affect the interaction between GA and the enzyme.

Prolonged combined treatment with methotrexate and GA resulted in a clear synergistic effect: 70% of cells died when treated with concentrations of the two drugs that did not affect cell survival when applied independently. The GA

concentrations used in these experiments did not inhibit DHFR or did so only very weakly, so this synergistic effect of methotrexate and GA is not explained by simple additive inhibition of DHFR activity by the two drugs. Thus, the combination of DHFR inhibition by methotrexate and GA-mediated decrease of DHF affinity for the enzyme acts synergistically such that cell lethality is enhanced.

The effects of combined treatments with methotrexate and GA were also tested on various cancer cell lines from different species (human, rat and mouse) and of diverse

Histogenetic origins. The synergistic effect of methotrexate and GA on cell mortality was observed for all cell types, to different extents. The three hepatoma cell lines (rat, mouse and human) were particularly sensitive to the synergistic effect of the two drugs. It may be informative to investigate whether or not this is a consequence of the metabolic traits of liver cells. *In vivo*, intravenously injected methotrexate and GA are metabolized in the liver (23, 24) and are excreted in the bile. GA is a potential anticancer drug for hepatocarcinoma, but methotrexate is not suitable due to poor efficacy and liver toxicity (25). A factor was identified which may alter the effect of GA. The experiments with C2, a non-tumorigenic variant of Fao, and its methotrexate-resistant derivatives, indicate that the reduction of the cell growth rate associated with strong overexpression of DHFR enhances GA resistance. Therefore, slow-growing tumor cells may not be good targets for GA. The substantial synergistic effect of the two drugs opens up new possibilities, particularly for using much lower and thus less toxic doses than those currently administered clinically. The methotrexate concentrations we used in the double treatments of the six cell lines (except for HepG2 which is resistant to much higher concentrations of the drug), and which were effective, were between 5 and 20 nM. These concentrations are much lower than the plasma concentrations in patients treated with methotrexate: the peak serum concentration of methotrexate in children treated for malignant diseases is between 20 and 100 µM depending on the infusion (26). Plasma concentrations >1 µM necessitate folic acid supplementation to limit liver and renal toxicity. The GA concentrations we show here to be effective covered a substantial range (5 nM to 0.5 µM) depending on the cell line, but were nevertheless again substantially lower than the plasma concentrations recorded in patients treated with GA (about 3 µM; 27).

GA and 5-fluorouracil (5-FU) have been described to act synergistically on human gastric carcinoma cell survival: GA inhibits TYMS, also a target of 5-FU (12). Here, we report that the effects of a combination of methotrexate and GA on DHFR activity accounts for the synergistic effects of the two drugs. Therefore, the effects of GA at two metabolic steps of the folate pathway may serve as a basis for the development of effective anticancer combinations.

We describe a novel biological effect of GA: it affects the affinity of DHFR for DHF and moderately inhibits the activity of the enzyme. Our subsequent demonstration of the efficacy of combined GA and methotrexate treatment of cancer cells leads us to propose this combination of drugs as a potentially potent approach to cancer chemotherapy, including that for liver carcinoma.

## Acknowledgements

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