Tumor anti-initiating activity of some novel 3,4-dihydropyrimidinones

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Abstract:

Halting the tumor initiation process by targeting the inhibition of the carcinogens metabolic activators (CYP), the induction of the carcinogen detoxification enzymes (glutathione-S-transferases, GSTs), and the induction of antioxidant activity is an effective strategy. Since several dihydropyrimidine derivatives (Biginelli compounds) are therapeutically active, the present study aimed to synthesize some dihydropyrimidines with multifunctional aromatic substitutions and to investigate their effects as anti-initiating agents. Twelve compounds were synthesized and structurally elucidated. The results revealed that compound **10** was a non-cytotoxic inhibitor of cytochrome P 450 1A (Cyp1A) activity, inducer of GST activity, scavenger of OH and inhibitor of DNA fragmentation. Compounds **1** and **9** were radical scavengers of OH and inhibitors of DNA fragmentation. On the other hand, all compounds were not toxic against different tumor cells, except compounds **2**, **4**, and **5** possessed non specific cytotoxicity against both liver and colon carcinoma cells, while **7** possessed specific cytotoxicity only against colon carcinoma cells. Compound **1** was a non-cytotoxic inducer of GST activity, scavenger of OH and ROO, and inhibitor of DNA fragmentation. The present study proved that compounds **10** and **1** were active and safe tumor anti-initiating and multi-potent blocking agent.

Key words:

cancer chemoprevention, tumor anti-initiating, 3,4-dihydropyrimidinone, Biginelli compounds

Introduction

Carcinogenesis is a multistage process consisting of what appears to be three major steps: initiation, promotion and progression. In humans it takes many years for normal cells to transform into complete malignancy [18]. This journey can be slowed by the use of preventive agents that target those steps. Using this approach, it has become clear that there could be many opportunities to intervene in the development of cancer. Chemoprevention, a promising strategy to prevent cancer, is the use of either natural or synthetic

substances or their combination to block, reverse or retard the process of carcinogenesis [3]. Therefore, it is of interest to find new substances to use as chemopreventive agents. Further, the study of the biological effects of these agents at the cellular level provides the molecular basis for their function and helps to establish policies for generating more potent chemopreventive regimens with potential for chemotherapeutic efficacy.

Initiation is a key event in carcinogenesis, during which a cell is prone to clonal growth under the influence of promoters inducing a change that is persistent

and passed on to subsequent cell generations. The initiation phase is a rapid (within hours or days), irreversible event that occurs when a normal cell is exposed to a carcinogen that causes un-repairable or misrepaired DNA damage [5]. DNA damage itself is not mutagenic unless the resulting somatic mutation is recapitulated *via* mitosis to yield a clone of the mutated cell [4]. Because the initiation and progression phases are irreversible and relatively transient events, they may provide the best targets for cancer prevention [5]. Halting the initiation by targeting the inhibition of the carcinogens metabolic activators (CYP), the induction of the carcinogen detoxification enzymes (glutathione-S-transferases, GSTs), and the induction of antioxidant activity is an effective strategy.

In the last few years a variety of dihydropyrimidine derivatives (the so called Biginelli compounds [6, 20]) have received considerable attention as therapeutically active compounds. Thus, in addition to their

characteristic properties as anti-hypertensives [19, 26], these compounds show a broad range of biological effects, including anti-viral [12, 21], anti-inflammatory [31], anti-bacterial [24] and anti-tumor [4, 27] activities. The discovery of monastrol, 4-(3-hydroxyphenyl)-6-methyl-5-ethylcarboxy-2-thioxo-1,2,3,4-tetrahydropyrimidine, as a mitotic kinesin Eg5 motor protein inhibitor [7], encouraged the search for and development of more anticancer compounds from the dihydropyrimidinone series. In efforts to obtain compounds with higher equilibrium binding constants, much work has gone into the synthesis of molecules having several chromophoric groups, and several of such compounds has proven to be of higher potency than those of monomers.

In the present study, we have synthesized some dihydropyrimidinones of the Biginelli type in which the aromatic part of the molecule is polyfunctionalized, and we investigate their effects as tumor anti-initiating

Scheme 1

agents, aiming to provide potential cancer chemopreventive agents for high-risk populations. Therefore, we utilized *in vitro* chemopreventive strategies that encompass relevant markers for measuring inhibition of carcinogenesis during the initiation stage.

Materials and Methods

Chemistry

The compounds were prepared by the reaction between an aldehyde, urea (or thiourea) and ethyl acetoacetate (or acetylacetone), by the method reported by Biginelli [6, 20] with little modification. The aldehydes used include 4-hydroxy-3-methoxybenzaldehyde, 4-nitrobenzaldehyde and 4-cyanobenzaldehyde. The 3,4-dihydropyrimidin-2(1H)-ones (thiones) thus obtained were subjected to different substitution and condensation reactions through which the compounds under investigation were obtained (Scheme 1). Derivatives of quinoline and acridine were incorporated into the dihydropyrimidinone molecule by simple condensation reaction; these two chromophores are known by their ability to bind with the DNA cancer cells (Scheme 2).

Melting points were taken on a capillary melting point apparatus and are uncorrected. The IR spectra were recorded with a Philips Infracord Spectrophotometer Model PU9712 in KBr discs. ¹H-NMR spectra were measured in CDCl₃ and DMSO-d₆ on JEOL-270 spectrometer with Me₄Si as an internal standard. Mass spectra were obtained with a Shimadzu GCS-QP 1000 EX spectrometer at 70eV. Elemental analy-

sis was performed at the Microanalytical Laboratory of the National Research Centre, Dokki, Cairo, Egypt.

Preparation of the Biginelli compounds

A mixture consisting of the aldehyde derivative (10 mmol), urea or thiourea (30 mmol), ethylacetoacetate or acetylacetone (20 mmol), ethyl alcohol (50 ml) and 2 ml of acetic acid, was stirred at the reflux temperature (24–36 h, monitored by TLC). After completion of the reaction, solvent was removed under vacuum and the product was treated with water and filtered, washed with water, dried and then was crystallized from the proper solvent. Physical data of some prepared compounds are matched with those previously reported.

4-(4-Hydroxy-3-methoxyphenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (1)

Yield 88%, m.p. 231–233°C; ¹H-NMR (DMSO-d₆, δ ppm): 9.15 (s, 1H, NH), 8.92 (s, 1H, OH), 7.65 (s, 1H, NH), 6.80 (s, 1H, Ar-H), 6.74 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.61 (d, *J* = 8.4 Hz, 1H), 5.05 (d, *J* = 3.2 Hz, 1H), 3.98 (q, *J* = 7.2 Hz, 2H, OCH₂CH₃), 3.72 (s, 3H, CH₃), 2.21 (s, 3H, CH₃), 1.11 (t, *J* = 7.2 Hz, 3H, OCH₂CH₃).

4-(4-Hydroxy-3-methoxyphenyl)-6-methyl-2-thioxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (2)

Yield 75%, m.p. 267–269°C; ¹H-NMR (CDCl₃, δ ppm): 9.55 (s, 1H, OH), 8.20 (s, 1H, NH), 7.82 (s, 1H, NH), 6.80–6.74 (m, 3H, Ar-H), 5.35 (d, *J* = 3.2 Hz, C4-1H), 4.12 (q, *J* = 7.2 Hz, 2H, OCH₂), 3.78 (s, 3H, OCH₃), 2.31 (s, 3H, CH₃), 1.24 (t, *J* = 7.2 Hz, 3H, CH₃).

Scheme 2

5-Acetyl-4-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine (3)

Yield 85%, mp. 235–237°C ¹H-NMR (DMSO-d₆, δ ppm): 9.10 (s, 1H, NH), 8.84 (s, 1H, OH), 7.62 (s, 1H, NH), 6.82 (d, *J* = 3 Hz, 1H, ArC2'-H), 6.74 (d, *J* = 12 Hz, 1H, Ar-C5'-H), 6.63 (dd, *J* = 6 Hz, 1H, ArC6'-H), 5.18 (d, *J* = 5 Hz, 1H, C4-H), 3.75 (s, 3H, OCH₃), 2.25 (s, 3H, COCH₃), 2.15 (s, 3H, CH₃).

4-(4-Hydroxy-3-methoxy-5-piperidin-1-ylmethylphenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (4)

Yield 75%, m.p. 182–185°C; ¹H-NMR (CDCl₃, δ ppm): 9.65 (br, 1H, OH), 9.03 (s, 1H, NH), 7.55 (s, 1H, NH), 6.71 (s, 1H, Ar-H), 6.52 (d, *J* = 6 Hz, 1H, Ar-H), 5.05 (d, *J* = 6 Hz, 1H, C4-H), 3.98 (q, *J* = 4.5 Hz, 2H, CH₂), 3.70 (s, 3H, OCH₃), 3.54 (d, *J* = 12 Hz, 2H, -CH₂-N), 2.38 (m, 4H, 2CH₂), 2.23 (s, 3H, CH₃), 1.51 (m, 4H, 2CH₂), 1.41 (d, *J* = 12 Hz, 2H, CH₂) and 1.1 (t, *J* = 6 Hz, 3H, CH₃).

4-(4-Hydroxy-3-methoxy-5-morpholin-4-ylmethylphenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (5)

Yield 70%, m.p. 211–213°C; ¹H-NMR (CDCl₃, δ ppm): 9.55 (br s, 1H, OH), 8.00 (s, 1H, NH), 7.56 (s, 1H, NH), 6.68 (s, 1H, Ar-H), 6.50 (d, *J* = 6 Hz, 1H, Ar-H), 5.15 (d, *J* = 6 Hz, 1H, C4-H), 3.98 (q, *J* = 4.5 Hz, 2H, CH₂), 3.70 (s, 3H, OCH₃), 3.66 (d, *J* = 6 Hz, 4H, 2 CH₂-O), 2.62 (q, *J* = 5 Hz, 4H, N-CH₂), 2.23 (s, 3H, CH₃) and 1.12 (t, *J* = 4 Hz, 3H, CH₃).

4-(4-Hydroxy-3-hydroxymethyl-5-methoxyphenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (6)

Yield 73%, m.p. 111–113°C; ¹H-NMR (DMSO-d₆, δ ppm): 9.13 (br, 1H, OH), 8.60 (s, 1H, NH), 7.62 (br, 1H, NH), 6.76 (s, 2H, Ar-H), 5.10 (d, *J* = 4.5 Hz, 1H, C4-H), 4.35 (s, 2H, CH₂-O), 3.80 (q, *J* = 4 Hz, 2H, CH₂-ester), 3.72 (s, 3H, 2 OCH₃), 2.42 (s, 3H, CH₃), 2.28 (s, 3H, CH₃) and 1.05 (t, *J* = 4.5 Hz, 3H, CH₃-ester).

5-Acetyl-4-(4-cyanophenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine (7)

Yield 65%, m.p. 180–82°C; ¹H-NMR (DMSO-d₆, δ ppm): 8.60 (s, 1H, NH), 7.61 (br s, 1H, NH), 7.40 (s, 2H, Ar-H), 7.16 (s, 2H, Ar-H), 5.40 (d, *J* = 4 Hz, 1H, C4-H), 2.32 (s, 3H, CH₃), 2.22 (s, 3H, CH₃).

4-(4-Cyanophenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (8)

Yield 73%, m.p. 118°C; ¹H-NMR (DMSO-d₆, δ ppm): 8.82 (s, 1H, NH), 7.56 (d, *J* = 5 Hz, 2H, Ar-H), 7.48 (d, *J* = 6 Hz, 2H, Ar-Hs), 6.60 (br s, 1H, NH), 5.10 (d, *J* = 4 Hz, 1H, C4-H), 3.80 (q, *J* = 4.5 Hz, 2H, CH₂-ester), 2.24 (s, 3H, CH₃) and 1.0 (t, *J* = 3 Hz, 3H, CH₃-ester).

4-(4-Carboxyphenyl)-6-methyl-2-oxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (9)

Yield 55%, m.p. 146°C; ¹H-NMR (DMSO-d₆, δ ppm): 10.20 (s, 1H, OH), 8.6 (s, 1H, NH), 7.86 (d, *J* = 6 Hz, 2H, Ar-H), 7.45 (d, *J* = 6 Hz, 2H, Ar-H), 7.10 (br s, 1H, NH), 5.10 (d, *J* = 4 Hz, 1H, C4-H), 4.10 (q, *J* = 4 Hz, 2H, CH₂-ester), 2.24 (s, 3H, CH₃) and 1.1 (t, *J* = 4 Hz, 3H, CH₃-ester).

4-(4-Carboxyphenyl)-6-methyl-2-thioxo-3,4-dihydropyrimidine-5-carboxylic acid ethyl ester (10)

Yield 75%, m.p. 132°C; ¹H-NMR (DMSO-d₆, δ ppm): 10.10 (s, 1H, OH), 8.7 (s, 1H, NH), 7.66 (d, *J* = 5 Hz, 1H, 2H, Ar-H), 7.45 (d, *J* = 4 Hz, 1H, 2H, Ar-H), 6.50 (br s, 1H, NH), 5.10 (d, *J* = 4 Hz, 1H, 1H, C4-H), 4.10 (q, *J* = 4.5 Hz, 1H, 2H, CH₂-ester), 2.24 (s, 3H, CH₃) and 1.00 (t, *J* = 4.5 Hz, 1H, 3H, CH₃-ester).

5-Acetyl-4-[4-(7-chloroquinolin-4-ylamino)-phenyl]-6-methyl-3,4-dihydro-1H-pyrimidin-2-one (11)

Yield 65%, m.p. 178–180°C, ¹H-NMR (DMSO-d₆, δ ppm): 9.44 (br s, 1H, NH-quin.), 9.18 (s, 1H, NH), 8.46 (d, *J* = 5 Hz, 1H, 2H, Ar-H), 7.9 (s, 1H, Ar-H), 7.73 (s, 1H, NH), 7.59 (d, *J* = 4 Hz, 1H, Ar-H), 7.30 (m, 4H, Ar-H), 6.85 (d, *J* = 5 Hz, 1H, Ar-H), 5.18 (d, *J* = 4 Hz, 1H, C4-H), 2.35 (s, 3H, CH₃), 2.27 (s, 3H, CH₃).

5-Acetyl-4-[4-(6-chloro-2-methoxy-acridin-9-ylamino)-phenyl]-6-methyl-3,4-dihydro-1H-pyrimidin-2-one (12)

Yield 66%, m.p. 181–183°C, $^1\text{H-NMR}$ (DMSO-d_6 , δ ppm): 9.7 (br, 1H, NH-quin.), 9.1 (s, 1H, NH), 8.40 (d, $J = 5$ Hz, 2H, Ar-H), 7.92 (s, 1H, Ar-H), 7.76 (s, 1H, NH), 7.60 (d, $J = 4.5$ Hz, 2H, Ar-H), 7.30 (m, 5H, Ar-H), 5.10 (d, $J = 5$ Hz, 1H, C4-H), 3.78 (s, 3H, OCH_3), 2.27 (s, 3H, CH_3), 2.20 (s, 3H, CH_3).

Cell culture

A battery of human cell lines were used throughout this study including: hepatocarcinoma (Hep G2); colon carcinoma (HCT-116), which were purchased from the American Type Culture collections and lymphoblastic leukemia (1301), a generous gift from The Training Center of DakoCytomation, Elly, UK. Cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM). Media was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 U/ml penicillin G sodium, 100 U/ml streptomycin sulphate, and 250 ng/ml amphotericin B. Cells were maintained in humidified air containing 5% CO_2 at 37°C. The compounds were dissolved in DMSO (99.9%) and diluted 1,000 fold in the assays. In all the cellular experiments, results were compared with DMSO-treated cells. The monolayer cells were harvested using trypsin/EDTA. All experiments were repeated four times, unless mentioned, and the data were represented as the mean $\pm$ SD. All cell culture material was obtained from Cambrex, BioScience (Copenhagen, Denmark).

Anti-initiation activity**Inhibition of cytochrome P450 1A**

Cytochrome P450 1A (Cyp1A) activity was determined by the rate of dealkylation of 3-cyano-7-ethoxycoumarin (CEC) to the fluorescent 3-cyano-7-hydroxycoumarin based on [11] and modified by [14]. Homogenates from cultured Hep G2 cells induced with β -naphthoflavone were used as a source of Cyp1A. A final concentration of 1 μM of each compound was used. The rate of CEC conversion was measured kinetically at an excitation of 408/20 nm and emission of 460/40 nm by microplate fluorescence reader (FluoStarOptima, BMG). Inhibition of

Cyp1A activity was calculated in comparison with the initial fluorescence of a complete reaction mixture with cell homogenate and buffer instead of the tested compound.

Evaluation of GSTs activity

GSTs activity was measured according to [16] and based on GSTs-catalyzed reaction between GSH and 1-chloro-2,4-dinitrobenzene that acts as an electrophilic substrate for GSTs. Hep G2 cells (1×10^6) were incubated with different compounds (10 μM) for 48 h. In the kinetic analysis, the absorbance was assessed at 340 nm using a microplate reader (Biorad, USA). GSTs were normalized to the protein content as measured by bicinchoninic acid assay [28].

Oxygen radical absorbance capacity (ORAC)

The radical absorbance capacity of the synthesized compounds was tested against two physiological radicals: peroxy (ROO) and hydroxyl (OH) by a kinetic ORAC assay [9] as modified by [13]. β -Phycoerythrin (β -PE) was used as a radical-sensitive fluorescent indicator protein, 2,2'-azobis-(2-amidinopropane) dihydrochloride as ROO initiator and H_2O_2 - CuSO_4 as OH generator. The decay of β -PE fluorescence was measured using a microplate fluorescence reader at excitation 540 nm and emission 565 nm. One ORAC unit equals the net protection of α -PE produced by 1.0 μM Trolox. In this assay, different concentrations (1, 2, and 4 μM) of each compound were used, and their antioxidant capacity against the physiological radicals ROO and OH was calculated.

Determination of total cellular thiols

The total cellular thiol concentration was determined by the glutathione disulfide reductase/5,5'-dithiobis-(2-nitrobenzoic acid) enzymatic cycling procedure [15]. Hep G2 cells (1×10^6) were incubated with each compound (10 μM) for 48 h. The extent of 2-nitro-5-thiobenzoic acid formation was measured in cell lysate at 340 nm using a microplate reader. The thiol content in the cell lysate was calculated in comparison with a GSH standard curve.

DNA fragmentation

DNA fragmentation was essentially induced in Hep G2 cells by incubation with 3% H₂O₂ for 3 h in the presence and absence of the tested compounds (10 μ M), and then the fragmentation was assayed as reported previously [23]. Briefly, the cell pellets were re-suspended in 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 48°C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation for 15 min at 13,000 $\times$ g. Pellets were re-suspended in TE-buffer and samples were precipitated by 10% trichloroacetic acid at 48°C. Samples were pelleted at 4000 rpm for 10 min, mixed with 5% trichloroacetic acid, and then boiled for 15 min. DNA content was quantified using the diphenylamine reagent [8]. The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet.

Cytotoxicity assay

The cytotoxicity of the tested compounds against various tumor cell lines was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which is based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells [17]. Cells (5×10^4 cells/well) of different cell lines were incubated for 48 h with serial concentrations of each compound at 37°C in a serum free medium, before submission to MTT assay. The relative cell viability was expressed as the mean percentage of viable cells compared with DMSO-treated cells. The half maximal inhibitory concentration (IC₅₀) was calculated using the curve of dose/% of viability.

Statistical analysis

Tests data were analyzed by using one-way ANOVA followed by the Tukey *post-hoc* test; $p < 0.05$ indicated statistical significance.

Results and Discussion

Tumor anti-initiating activity

Cancer chemopreventive agents are typically natural products or their synthetic analogs that inhibit the

transformation of normal cells to premalignant cells or the progression of premalignant cells to malignant cells. These agents are believed to function by modulating processes associated with xenobiotic biotransformation, with the protection of cellular elements from oxidative damage, or with the promotion of a more differentiated phenotype in target cells [1]. The modulation of enzymes involved in metabolic activation by phase I enzymes, conjugation by phase II enzymes, and excretion of carcinogens is one of the best investigated mechanisms of chemopreventive agents and is of importance in the prevention of tumor initiation. We examined Cyp1A activity, as one of the phase I enzymes involved in the activation of procarcinogens to ultimate carcinogens. Our results indicated that, **10** can be identified as a potent inhibitor of Cyp1A activity ($p < 0.05$) with an inhibition percentage of 41.38% (Fig. 1), compared with the initial activity of β -naphthoflavone-stimulated cells. The structure of **10** as an organosulfur compound is suggested to be responsible for its inhibitory activity, however, **2** is also an organosulfur compound with similar structure and similar substitutions in the heterocyclic ring, but it is obvious that the substitutions in the side ring

Fig. 1. Anti-initiating activity through the modulation of the carcinogen metabolism: The inhibitory effect of 1 μ M of each compound on Cyp 1A activity. The symbol (#) represents compounds that could not be assayed for Cyp1A activity due to their high auto-fluorescence that interfered with the assay results at Ex. 408/20 nm and Em 460/40, * $p < 0.05$. Data expressed as the mean $\pm$ SD and control represents 100% activity

reduce its CYP inhibitory activity. However, the rest of compounds in this group possessed insignificant inhibition of Cyp1A activity to $<10\%$ ($p > 0.05$), while compounds **9** and **12** could not be assayed for Cyp1A activity due to their high auto-fluorescence, which strongly interfered with the assay results, at excitation 408/20 nm and emission 460/40 nm.

Essentially, the cellular response to oxidative stress relates to the level and form of GSH, the major cytosolic thiol, which helps in destruction of hydrogen peroxide, lipid peroxides and free radicals, and in preserving the levels of cellular active forms of β -carotene, ascorbic acid, and α -tocopherol [1]. A major factor that affects GSH homeostasis is its utilization by conjugation, primarily *via* GSTs [7]. GSTs are a superfamily of enzymes, responsible for the detoxification of a wide range of substrates including xenobiotics, and occupational and environmental carcinogens such as pesticides and polycyclic aromatic hydrocarbons; by catalyzing the nucleophilic attack of the sulfur atom of GSH on electrophilic compounds and have evolved as a cellular protection system against their toxins and carcinogens [7].

The conjugation and detoxification of active carcinogens is one of the important mechanisms used to halt the tumor initiation step. The total GSTs was investigated in cultured Hep-G2 cells as a representative for Phase II enzymes. After 48 h of incubation with 10 μM of different compounds, the results revealed that the total GSTs activity was significantly induced by **1**, **6**, **7**, **8**, and **10** ($p < 0.05$), while **11** insignificantly inhibited the enzyme activity ($p > 0.05$) (Fig. 2). These findings indicated that the substitution of ethyl ester by an acetyl group in the similar compounds **7** and **8** did not change the GST-inducing activity of the 4-cyanophenyl-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine skeleton and also that the substitution of a hydroxymethyl group in the phenyl ring in the similar compounds **1** and **6** did not affect their GST-inducing activity. The enhancement of the non-enzymatic antioxidants, total thiols, helps in counteracting the deleterious effect of ROS. The treatment with different compounds led to an insignificant increase in the total thiol content by compounds **1**, **6**, and **12** (Fig. 2).

In oxidative stress and inflammation, excessive production of reactive oxygen and nitrogen species results in DNA damage and contributes to tumor initiation and promotion, which might ultimately lead to carcinogenesis. Cell protection from external damage

Fig. 2. Anti-initiating activity through the modulation of the carcinogen metabolism: The effect of 10 μM of each compound on GSTs activity (bars) and on the non-enzymatic antioxidant GSH (circled line) in Hep-G2 cells. Data expressed as the mean $\pm$ SD and control represents 100% of the scale; * $p < 0.05$

largely depends on the availability and activity of cellular antioxidants, which maintain homeostatic control of ROS [4]. An altered balance of ROS directly affects cellular proliferation, apoptosis, and senescence [5].

Consequently, scavenging of the physiologically relevant ROS, including OH, and ROO represents an effective strategy in preventing tumor initiation and promotion. Compounds **1** and **4** were found to be the strongest ROO and OH scavengers as indicated from their ORAC units compared to the Trolox capacity and the high dose-dependent slope. Although **9** and **11** exhibited a relatively strong scavenging affinity against OH (Fig. 3A), they showed very low to absent affinity against ROO (Fig. 3B). Generally, the compounds were stronger radical scavengers than Trolox, especially at the highest tested dose, in the following order **1** > **4** > **11** > **9** > **5** > **8** = **6** > **10** against OH (Fig. 3A), and **1** > **2** > **4** > **3** > **5** > **12** > **6** > **11** against ROO (Fig. 3B). Compounds **3**, **7**, and **12** were not represented in Figure 3A and compounds **7**, **8**, **9**, and **10** were not represented in Figure 3B, due to their weak antioxidant activity as revealed from their low ORAC values (< 1). Before the ORAC assay, we tested **9** and **12** and found that they both had no auto-fluorescence at that assay excitation and emission wavelength.

In the investigation of the possible preventive activity of the compounds against the induced-DNA damage, we find that the degree of DNA fragmenta-

Fig. 3. The antioxidant capacity of different concentration of the compounds, as assayed by ORAC assay, against OH (A) and ROO radicals (B)

tion, which was assayed by diphenylamine, was dramatically inhibited by some compounds in the following order $1 > 9 > 11 > 10$ ($p < 0.05$) (Fig. 4). On the other hand, the rest of the compounds resulted in no significant inhibition of DNA fragmentation.

Cell proliferation can influence carcinogenesis by a number of mechanisms, such that cell proliferation *per se* may be carcinogenic [3]. Proliferation is considered to play an important role in several steps of the carcinogenic process, initiation, promotion, and progression [3], where it is involved in the fixation of

Fig. 4. The effect of the compounds on the induced DNA fragmentations in Hep G2 cells, as assayed by diphenylamine. The results were compared with the H_2O_2 treated-control (black bars). Data expressed as the mean $\pm$ SD; * $p < 0.05$

a miscoding lesion in the newly made DNA [2]. Anti-cancer drugs are able to kill cancer cells by multiple mechanisms: interaction into DNA, inhibition of DNA replication, cell membrane damage, or free radical generation. Cells respond to cytotoxic stress and DNA damage either by cell-cycle arrest and repair or by undergoing apoptotic cell death, depending on the cell type and the extent of the damage [13].

Exploring the cytotoxicity of the tested compounds by MTT assay indicated that all of the compounds were relatively safe and not toxic to the hematopoietic 1301 cells (T-lymphocytes) with IC_{50} values $> 20 \mu M$ (Fig. 5). While using solid tumor cell lines, 2, 4, 5, and 7 exhibited a cytotoxic effect against Hep-G2 cells as well as 2, 4, and 5 against HCT-116 cells, as indicated from their low IC_{50} values (around $20 \mu M$), while the other compounds were relatively safe for both types of cells (Fig. 5). Since chemoprevention is described as the suppression, retardation or inversion of carcinogenesis by agents with low side effects and low toxicity during their neutralization of the carcinogens, compounds 1, 3, 6, 8, 9, 10, and 11 were cell-safe chemopreventive agents.

Most of the known chemopreventive agents are subdivided into blocking agents, which inhibit the initiation step by preventing carcinogen activation and suppressing agents, which inhibit malignant cell proliferation during promotion and progression steps [30].

Fig. 5. The cytotoxic effect of the compounds on Hep G2 (black bars), HCT-116 (white bars) and 1301 (gray bars) cells, which were treated with different concentrations of the tested compounds for 48 h, as followed by MTT assay. The data are represented by IC₅₀ values (the mean $\pm$ SD, μ M)

Taken together, **10** was a non-cytotoxic promising inhibitor of Cyp activity, inducer of GST activity, scavenger of OH and inhibitor of DNA fragmentation. Compounds **1** and **9** were radical scavengers of OH and inhibitors of DNA fragmentation. On the other hand, none of the compounds were toxic to different tumor cells, except **2**, **4**, and **5**, which possessed non-specific cytotoxicity to both liver and colon carcinoma cells, while **7** possessed specific cytotoxicity only to colon carcinoma cells. Compound **1** was a promising non-cytotoxic inducer of GST activity, scavenger of OH and ROO, and inhibitor of DNA fragmentation. The present study proved that compounds **10** and **1** were active tumor anti-initiating and multi-potent blocking agents.

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