

RESEARCHES ON THE ANTIOXIDANT ROLE OF TWO NEW SYNTHESIZED PROSTAMIDES ON HUMAN HEPATOCELLULAR CARCINOMA CELL LINE (HepG2)

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Abstract

The prostamides are a part of a large and continuously expanding series of neutral lipids. The prostamides role in the organism is not yet completely understood. Nevertheless, significant progresses have been made since the discovery of the synthetic product, bimatoprost. This synthetic prostamide is considered nowadays the most efficient drug used for the treatment of glaucoma. Recent *in vitro* studies demonstrated that some classes of prostaglandins and prostamides are implicated in the protection against induced oxidative stress and also have a cytoprotective role. Most researches that demonstrated the biological importance of prostamides were focused especially on their action at the ocular level but less is known about their biological effect on other tissues.

The goal of the present study was to determine the effect of the treatment with two novel prostamides in human hepatocellular liver carcinoma cell line (HepG2) on cellular viability, lactate dehydrogenase and some intracellular antioxidant systems activity. The study results indicated that the new synthesized prostamides have a possible antioxidant effect due to an indirect mechanism by activation of the endogenous antioxidative defense systems in HepG2 cell line.

Rezumat

Prostamidele (prostaglandin-etanolamidele) sunt o categorie de mediatori locali recent descoperită din categoria lipidelor neutre. Până în prezent, rolul acestora în organism nu a fost complet elucidat. Progrese semnificative în această direcție s-au făcut odată cu introducerea în terapeutică a medicamentului bimatoprost (Lumigan®). Acesta este o prostamidă de sinteză utilizată cu succes în tratamentul glaucomului. Numeroase studii *in vitro* au demonstrat că unele clase de prostaglandine și prostamide au rol citoprotector și sunt implicate în protecția împotriva stresului oxidativ. Majoritatea studiilor fac referire la acțiunea la nivel ocular și foarte puține aspecte se cunosc despre efectele la nivelul altor țesuturi. Scopul lucrării a fost de a determina

efectele a doua prostamide de sinteză asupra unei linii celulare de carcinom hepatic uman (HepG2). S-a studiat viabilitatea celulară, activitatea lactat dehidrogenazei, precum și unele sisteme antioxidante de la nivel celular (glutathion peroxidaza, superoxid dismutaza și glutathionul total). Rezultatele studiului au indicat un posibil efect antioxidant, datorat unui mecanism indirect de activare a apărării antioxidante endogene la nivelul liniei celulare HepG2.

Keywords: prostamide, antioxidant effect, glutathione peroxidase, superoxide dismutase, HepG2 cell line

Introduction

Prostamides (or prostaglandin-ethanolamides) are cellular acting local-messenger molecules within the neutral lipids mediators. In spite of their structure similarity with prostaglandins, prostamides have a different mechanism of action, not completely known [12,8]. This novel discovered class of eicosanoids may have unique pharmacological properties rather than serving as latent sources of prostaglandins at sites remote from their tissue of origin. [8] Significant progresses in elucidating the biological importance have been made since the discovery of bimatoprost (Lumigan®), a prostaglandin-ethanolamide with a similar structure of the prostaglandin-F class. This synthetic prostamide is considered nowadays the most efficient drug used for the treatment of glaucoma [4,10,12].

Based on the scientific facts that one of the most incriminated causes of glaucoma is oxidative stress, many *in vitro* studies demonstrated that some classes of prostaglandins and prostamides have a cytoprotective role and an antioxidant effect. Most research studies that demonstrated the biological importance of prostamides were focused especially on their action at the ocular level but less is known about their biological effect in other tissues [6,7,12].

The anticancer treatments are considered to mediate cell death by activating key elements of the apoptosis program and the cellular stress response. The human hepatocellular liver carcinoma cell line HepG2 has been grown successfully in large scale cultivation systems, being a suitable model to study different biological processes including the antioxidant action.

The present research assesses the effect of the newly synthesized prostamides after the treatment of the human hepatocyte-derived cell line (HepG2) on cell viability and the enzymatic activity of lactate dehydrogenase. Some antioxidant systems with an important intracellular role: superoxide dismutase, glutathione peroxidase and total glutathione were also studied.

Materials and Methods

The new prostamides (D-Cloprostenol-1-N-ethylamide and D-Cloprostenol-1-N-phenylglycinolamide) were obtained by stereocontrolled synthesis within the Department of Biosynthesis of Natural Products from the National Institute for Chemical and Pharmaceutical Research and Development, Bucharest, Romania. These compounds are derivatives of D-Cloprostenol, a prostaglandine-F2 α analogue already used in veterinary medicine (Figure 1). The purity of synthesized prostamides was checked by high-performance liquid chromatography, and the results indicated 99.9% analytical purity.

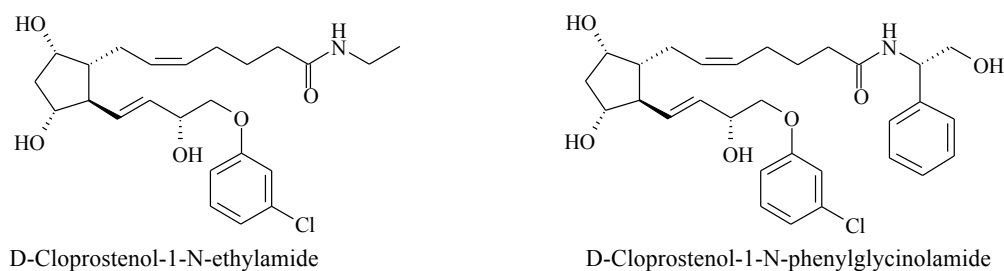


Figure 1

The chemical structure of the new synthesized prostamides

Cell culture and treatments

HepG2 cells, a human hepatocyte-derived cell line, were purchased from ATCC (Figure 2). HepG2 is a perpetual cell line which was derived from the liver tissue of a 15 years old Caucasian American male with a high differentiated hepato-cellular carcinoma. These cells are epithelial in morphology, have a model chromosome number of 55 and are not tumorigenic in nude mice. The cells were cultured at an initial density of 105 cells/cm² into 24-well tissue culture plates in Dulbecco's Modified Eagle's Medium supplemented with 10% heat inactivated fetal bovine serum, and maintained in a humidified atmosphere with 5% CO₂ at 37°C.

They were treated for 24 h with different concentrations of both prostamides. The stock solution concentration of the new synthesized prostamides was related to the therapeutic dose of bimatoprost used for the treatment of glaucoma.

D-cloprostenol-1-N-phenylglycinolamide is a very lipophilic compound and was dissolved using a tensioactive agent (Tween 80). The prostamide was prepared as 0.03% stock solution and used on cell culture in 2 concentrations: 5.5 μ M (F10- 100x dilution of stock solution) and 55 μ M (F100- 10x dilution of stock solution).

D-cloprostenol-1-N-ethylamide, prepared as 0.03% stock solution was added on cell culture at final concentrations: $7.7\mu\text{M}$ (E10-100x dilution of stock solution) and $77\mu\text{M}$ (E100-10x dilution of stock solution). Data obtained were compared to control- untreated cells and cells treated with Tween 80.

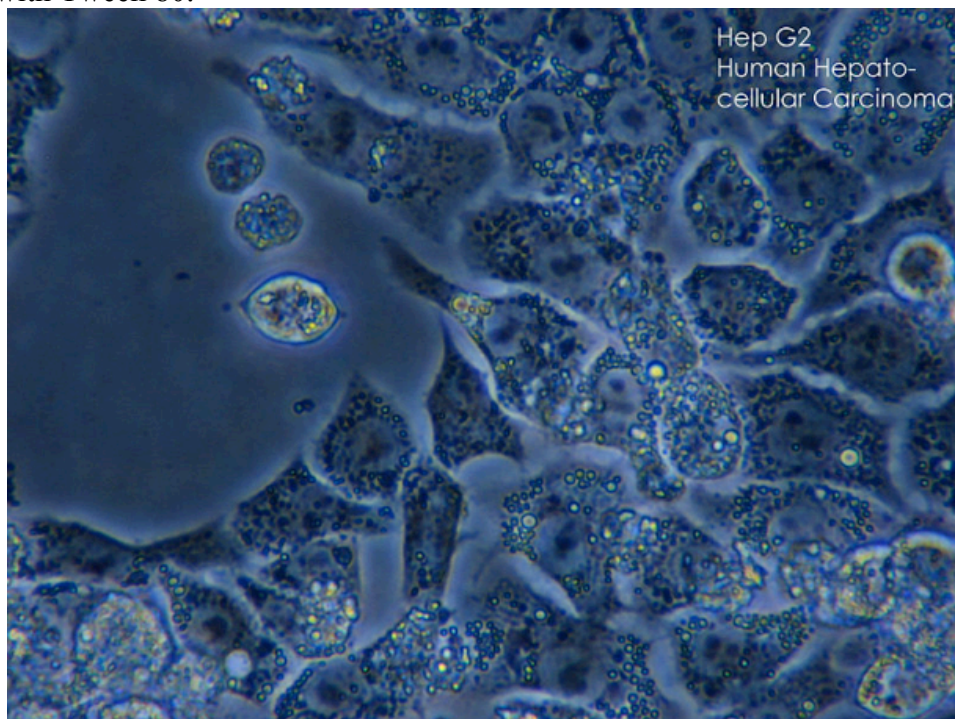


Figure 2

Morphological aspect of HepG2 cell line in culture

Cell Viability Assessment

Cell viability was assessed on HepG2 culture after 24h exposure at different treatments with prostamides. Cells were incubated with 1mg/mL MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) solution at 37°C for 2h. The yellow dye [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) was reduced to insoluble purple formazan granules by metabolically active cells. The precipitated formazan was dissolved in dimethyl sulfoxide, and after 10 min of slow shaking, the absorbance was recorded at 550 nm. Absorbance values, that are lower than those displayed by control cells, indicated a reduction in the cell activity and viability. Conversely, higher absorbance values indicated an increase in cell viability [2,1,11].

Cytotoxicity Assay

HepG2 cell line was routinely grown, treated, and assessed for leakage of lactate dehydrogenase (LDH) out of the cytoplasm of the damaged cells. Briefly, after 24 h of various treatments with prostamides, the culture media were recovered and used to measure the loss of cell viability and LDH release using an *in vitro* toxicology assay kit (TOX-7, Sigma-Aldrich Co.) according to the manufacturer's instructions.

Superoxide dismutase Assay

The assay of superoxide dismutase (SOD) activity was carried out by the method described by Paoletti and Mocali [11] and consisted of purely chemical reaction sequence, which generated superoxide from molecular oxygen in the presence of EDTA, manganese (II) chloride, and mercaptoethanol. NAD(P)H oxidation (pursued by the diminution of absorbance at 340 nm) was linked to the availability of superoxide anions in the medium. Values of ΔA_{340} recorded for samples with SOD activity progressively decreased depending on the amount of enzyme in the assay mixture. The percent change in enzyme activity was calculated using the assay data from treated and control cells. HepG2 cells were treated for 24 h with prostamides, washed with phosphate-buffered saline (PBS), resuspended in PBS, and lysed by sonication. Appropriate volume of aliquots from the total cell extract were used for assay of superoxid dismutase (SOD) activity. The assay volume (213 μ L) contained 160 μ L 100 mM triethanolamine–diethanolamine–HCl buffer (pH 7.4), 8 μ L of 7.5mM NADPH, 5 μ L of 100mM EDTA–50mM MnCl₂ solution (pH 7.0), 20 μ L of the cell extract and was kept at room temperature for 5 min to stabilize. Then, 20 μ L of 10mM mercaptoethanol was added and mixed well. Reagent blanks, which contained all assay components except cell extract glucose oxidase were used to calibrate the spectrophotometer. The absorbance decrease at 340nm was monitored for 20 min over a 5-min interval at 25°C in a Beckman DU 7500 spectrophotometer. All determinations were expressed as specific SOD activity in units per milligram (U/mg) protein. The assay of protein concentration was carried out by the Bradford method [3].

Total glutathione was measured using a TECAN microplate reader according to the procedure described in the Glutathione Assay Kit (Sigma). The assay is based on the enzymatic recycling method, using glutathione reductase and 5',5'-dithiobis-2-nitrobenzoic acid (DTNB). Briefly, after the treatment, the cells were washed twice with phosphate-buffered saline (PBS) and the cells were recovered by centrifugation at 200×g, for 10 minutes at 4°C. The resulting cell pellet was lysed by adding 80 μ L HCl 10

mM and freeze-thawing. The lysate was deproteinized by addition of 20 μ L 5-sulfosalicylic acid 5 %. Precipitated proteins were removed by centrifugation at 8000 $\times$ g, for 10 minutes and the supernatant was submitted immediately to the analysis according to the manufacturer's protocol. All the samples and total glutathione standard were treated the same way and the absorbance at 412nm was registered for 5min at 30sec intervals. The intracellular levels of total glutathione were calculated based on cellular protein concentration.

Glutathione peroxidase

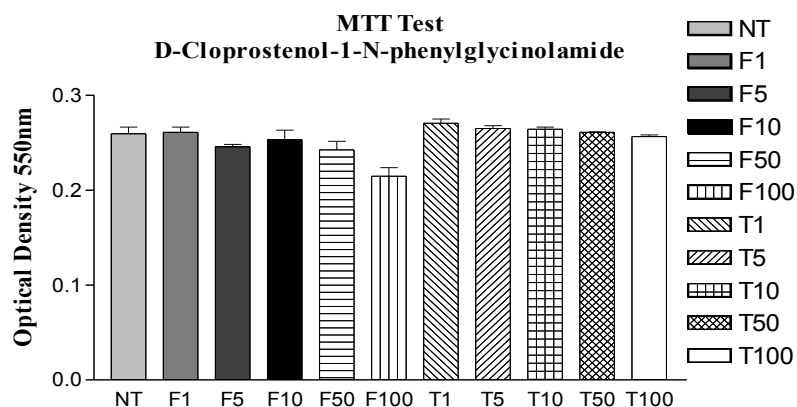
Glutathione peroxidase (GPx) activity of the cell supernatants was measured by assessing the oxidation of reduced glutathione (GSH) in the presence of t-butyl hydroperoxide, glutathione reductase and NADPH [5]. The rate of glutathione disulphide (GSSG) formation was measured by means of glutathione reductase activity, and the oxidation of NADPH was analysed at 340 nm. Both sample and reference tubes contained 0.05 M phosphate buffer, pH 7.2, 4.3mM EDTA, 0.28mM NADPH, 0.5U of glutathione reductase, 4mM glutathione, 0.18mM t-butyl hydroperoxide and the appropriate amount of lysates. One mol of NADPH is oxidized for each mol of t-butyl hydroperoxide reduced. One unit of GPx was defined as 1 μ M substrate converted per minute. Protein concentration was determined by the Bradford method [3].

Statistical Analysis

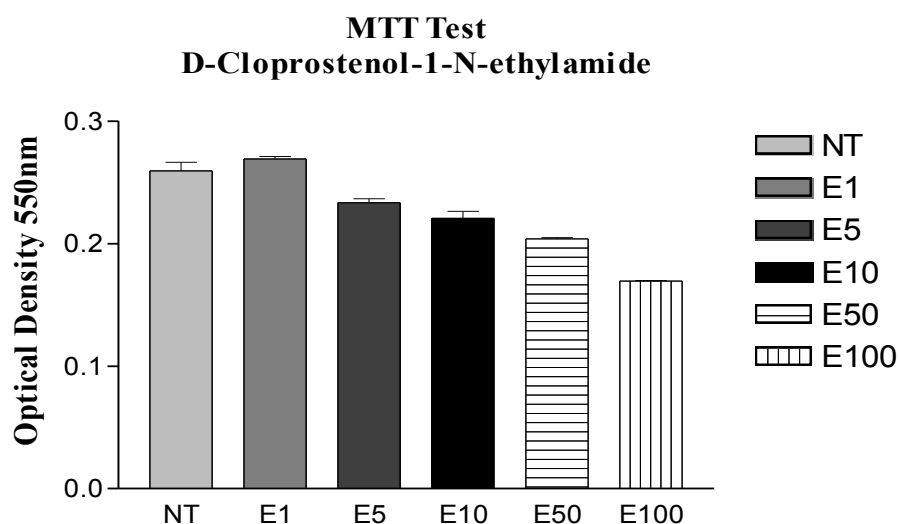
For the statistical evaluation of the obtained data, one-way ANOVA with Bonferroni's multiple comparison tests were performed. All values were expressed as means $\pm$ SEM. Statistical analysis was performed using the Prism software (GraphPad, San Diego, CA).

Results and Discussion

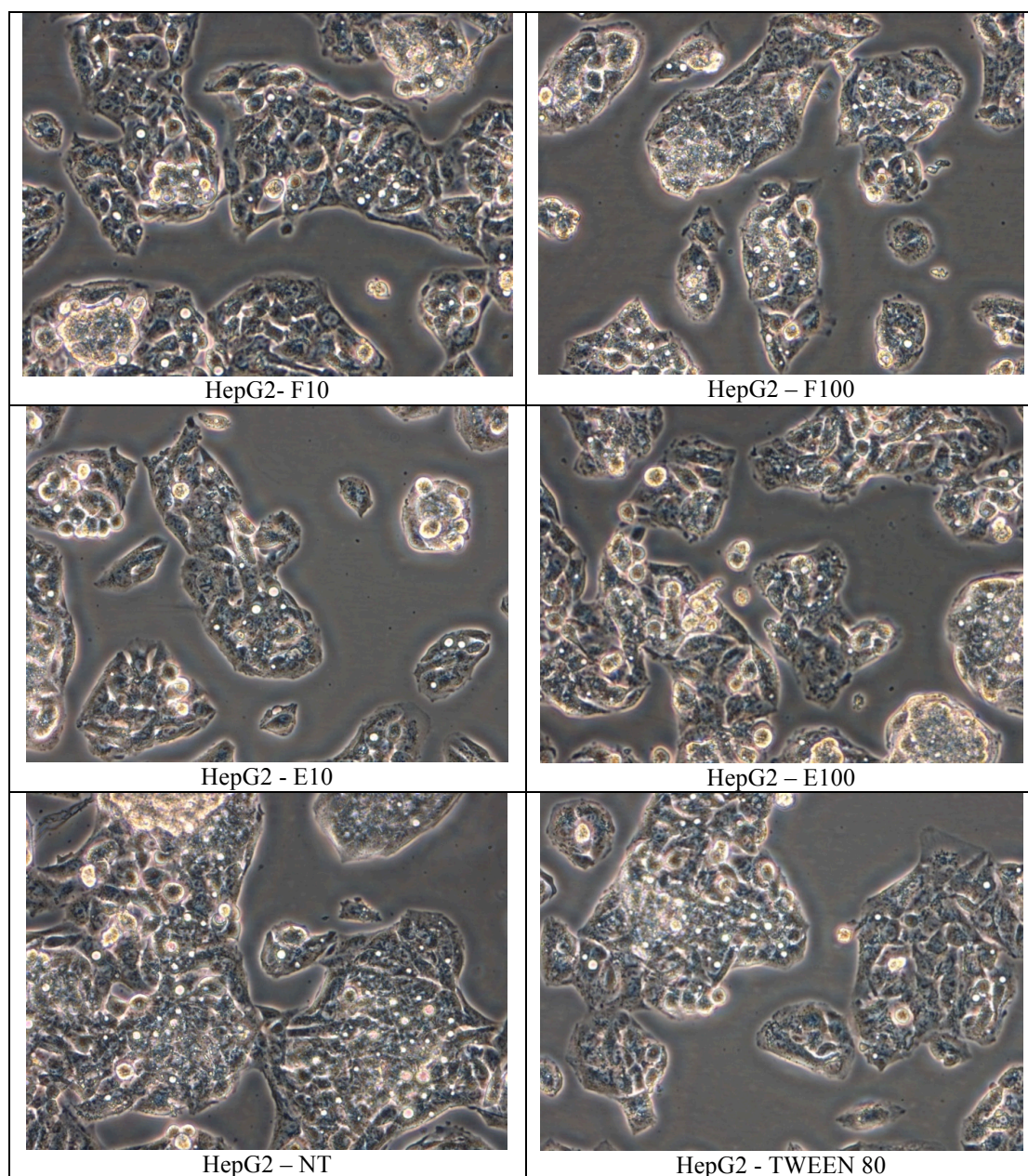
In order to examine the cell viability, we performed the MTT test. In Figures 3 and 4, we showed the effects of two concentrations of prostamide on HepG2 cell line. The results are confirmed by phase-contrast light microscopy (Figure 5). Light microscopy analysis indicated that the cell viability decreased with the increase of the prostamide concentration. The most obvious cytotoxic effects ($p < 0.001$) were observed at the maximum concentration of D-Cloprostenol-1-N-ethylamide (E100) used in the study (Figure 5). No significant differences were noticed between control and treated cells with both Tween 80 ($p > 0.05$) and low concentration of D-Cloprostenol-1-N-phenylglycinolamide (F10 $p > 0.05$).

**Figure 3**

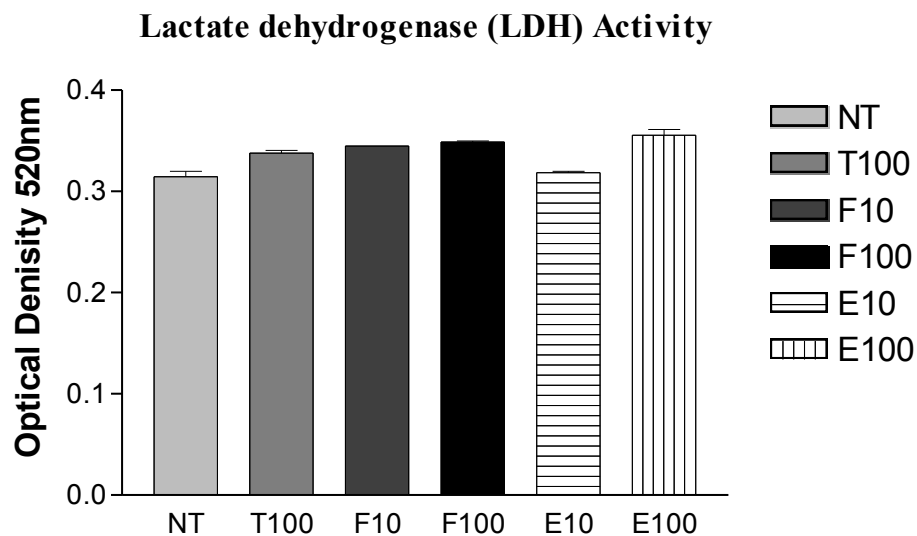
The prostamides effect on cell viability (MTT test). HepG2 were treated with different concentrations of prostamides for 24h. Data are expressed as mean $\pm$ SEM of two different experiments performed in triplicate. NT=not treated, F10 = 100x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution ($p>0.05$), F100= 10x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution ($p<0.01$), T100= dilution 10x of Tween 80 solution ($p>0.05$)

**Figure 4**

The prostamides effect on cell viability (MTT test). HepG2 were treated with different concentrations of prostamides for 24h. Data are expressed as mean $\pm$ SEM of two different experiments performed in triplicate. NT=not treated, E10= 100x dilution of D-Cloprostenol-1-N- ethylamide stock solution ($p<0.001$), E100= 10x D-Cloprostenol-1-N-ethylamide dilution of stock solution ($p<0.001$)

**Figure 5**

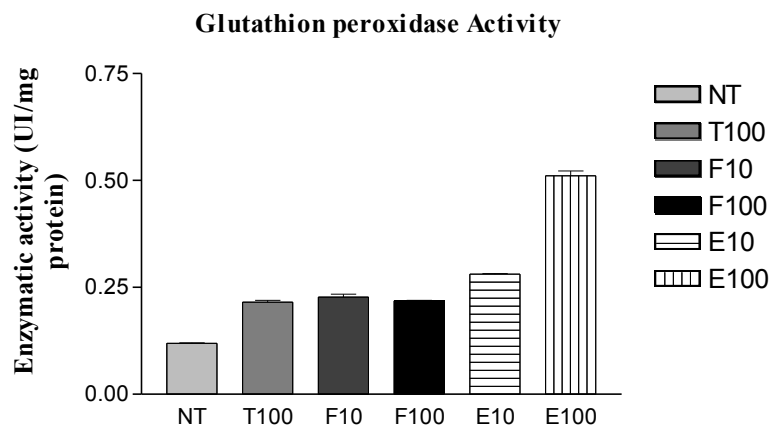
Morphological appearance in phase-contrast light microscopy of HepG2 cells control and treated for 24h with different concentrations of prostamide – D-Cloprostenol-1-N-phenylglycinolamide F10 (dilution 100x) and F100 (dilution 10X); D-Cloprostenol-1-N-ethylamide E10 (dilution 100x) and E100 (dilution 10x), NT= not treated

**Figure 6**

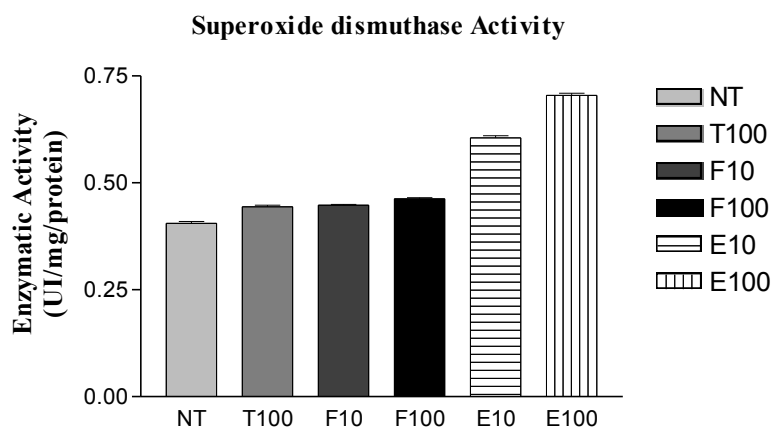
Lactate dehydrogenase activity in HepG2 cells treated with different concentrations of prostamides for 24h. Data are expressed as mean $\pm$ SEM of two separate experiments performed in triplicate. NT=not treated, F10 = 100x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution ($p < 0.05$), F100= 10x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution ($p < 0.01$), T100= dilution 10x of Tween 80 solution ($p > 0.05$), E10= 100x dilution of D-Cloprostenol-1-N- ethylamide stock solution ($p > 0.05$), E100= 10x D-Cloprostenol-1-N- ethylamide dilution of stock solution ($p < 0.01$)

The decrease of cell viability was associated with the cell membrane damage followed by a release of lactate dehydrogenase (LDH) in the cell culture and an increase of the enzymatic activity (Figure 6). The enzymatic activity was slightly increased in all treatments. The most significant increase of LDH activity was observed in the case of high concentration of D-Cloprostenol-1-N-ethylamide (E100 $p < 0.01$).

The enzymatic activities of both GPx and SOD (Figures 7, 8) were slightly increased in the case of D-Cloprostenol-1-N-phenylglycinolamide but no significant differences were observed comparing to the cells treated with solvent. The enzymatic activity was more increased in the case of D-Cloprostenol-1-N-ethylamide and a significant increase was noticed in the case of the cells treated with a high dose of prostamide ($p < 0.01$).

**Figure 7**

Glutathion peroxidase activity in HepG2 cells treated with different concentrations of prostamides for 24h. Data are expressed as mean $\pm$ SEM of two separate experiments performed in triplicate. NT=not treated, F10 = 100x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution, F100= 10x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution, T100= dilution 10x of Tween 80 solution, E10= 100x dilution of D-Cloprostenol-1-N- ethylamide stock solution, E100= 10x D-Cloprostenol-1-N- ethylamide dilution of stock solution

**Figure 8**

Superoxide dismutase in HepG2 cells treated with different concentrations of prostamides for 24h. Data are expressed as mean $\pm$ SEM of two separate experiments performed in triplicate. NT=not treated, F10 = 100x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution($p<0.01$), F100= 10x dilution of D-Cloprostenol-1-N-phenylglycinolamide stock solution($P<0.001$), T100= dilution 10x of Tween 80 solution($p<0.01$), E10= 100x dilution of D-Cloprostenol-1-N- ethylamide stock solution ($p<0.001$), E100= 10x D-Cloprostenol-1-N- ethylamide dilution of stock solution ($p<0.001$)

No significant differences were observed between the control and the HepG2 cells treated with both concentrations of D-Cloprostamol-1-N-phenylglycinolamide and solvent. An increase of the total glutathione level was observed in the case of D-Cloprostamol-1-N-ethylamide in a high concentration ($p < 0.001$).

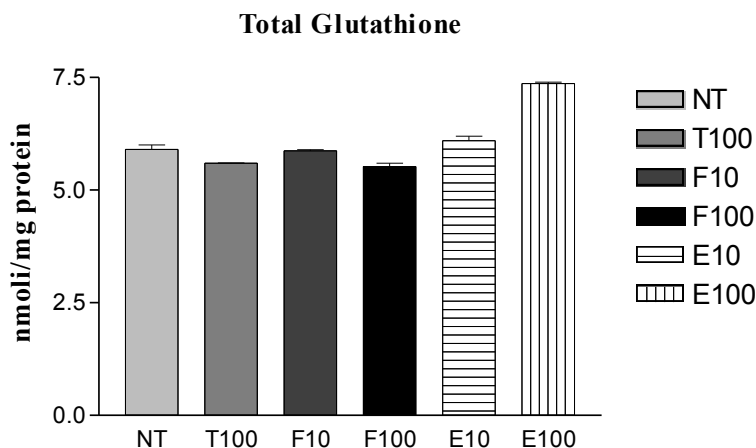


Figure 9

The intracellular level of total glutathione after 24h treatment of the HepG2 cell culture with the new synthesized prostamides. Data are expressed as mean $\pm$ SEM of two separate experiments performed in triplicate. NT=not treated, F10 = 100x dilution of D-Cloprostamol-1-N-phenylglycinolamide stock solution, F100= 10x dilution of D-Cloprostamol-1-N-phenylglycinolamide stock solution, T100= dilution 10x of Tween 80 solution, E10= 100x dilution of D-Cloprostamol-1-N-ethylamide stock solution, E100= 10x D-Cloprostamol-1-N-ethylamide dilution of stock solution

Oxidative products and free radicals, which are highly reactive products of the normal metabolism and immune defence system, can cause extensive damage to nucleic acids, proteins and lipids if an organism lacks sufficient antioxidant protection. To control and neutralise free radicals, animals maintain systems of defences based on different antioxidants by expressing the most important endogenous antioxidants (enzymes such as catalase, superoxide dismutase and glutathione peroxidase) [6].

It is widely known that the cell response to an oxidative injury of a xenobiotic compound depends on the activity of antioxidant enzymes. Among them, superoxide dismutase (SOD) and glutathione peroxidase (GPx) play a central role. SOD is a critical enzyme responsible for the elimination of superoxide radicals and is considered to be a key antioxidant in aerobic cells.

Glutathione (GSH) is the most abundant non-protein thiol in mammalian cells and has many critical functions, including defense against oxidative stress as a scavenger of reactive oxygen species (ROS) and electrophiles. Thus, GSH plays a role in maintaining cellular redox homeostasis, scavenging lipid peroxides, and detoxifying reactive intermediates of xenobiotics [6,7].

Our results indicated no cytotoxic effects of D-cloprostenol-1-N-phenylglycinolamide on HepG2 cells, only a slight decrease (~15%) of cell viability in the case of F10. The cytotoxic effect was proportional with the release of lactate dehydrogenase (LDH) in medium (Figure 6). The activity of glutathion peroxydase was 1.8 fold increased but the same effect was observed in the case of the cells treated with solvent. Further studies will be necessary to demonstrate if the antioxidant effect resulted from this experiment was caused exclusively by D-Cloprostenol-1-N-phenylglycinolamide and not by the solvent.

A cytotoxic effect and an increased enzymatic activity were observed in the case of D-Cloprostenol-1-N-ethylamide. The effects were proportional to the prostamide dose used to treat the HepG2 cell culture. In the case of E10 (dilution 100x), the cellular viability was slightly decreased (only 15%) and the enzymatic activities of glutathione peroxidase and superoxide dismutase were increased 2.4 folds and respectively 1.5 folds (Figures 7, 8).

In higher concentrations (E100), D-cloprostenol-1-N-ethylamide had a cytotoxic effect (65% cellular viability). A significant increase of the intracellular enzymatic activity was noticed in the case of GPx (4.3 folds) and SOD (1.75 folds). The intracellular level of total glutathione was also increased 1.25 folds (Figure 9).

Conclusions

The study results indicated that the new synthesized prostamides have a possible antioxidant effect due to an indirect mechanism of the endogenous antioxidative defense systems activation. The prostamide D-Cloprostenol-1-N-phenylglycinolamide could not be correctly assessed due to solvent interference. The solvent Tween 80, a tensioactive agent is very commonly used for dissolving prostaglandins in drug formulation but had its own effect that could interfere with the active substance.

All results are in agreement with data from literature obtained for bimatoprost. Future researches are necessary to demonstrate the antioxidant

effect of the prostamides considering that oxidative stress is implicated in the pathology of many diseases, including hepatic neoplasms.

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