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Cytotoxicity assay and intracellular localization of 2-carbamido-1,3-indandione in Balb/c 3T3 cells

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Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th anniversary

Cytotoxicity assay of 2-carbamido-1,3-indandione (CAID) has been performed by means of the validated Balb/c 3T3 Neutral Red Uptake Test. In addition, the intracellular localization of CAID in murine embryonic fibroblasts, studied by fluorescence microscopy, is reported. The results indicate complete absence of toxicity of CAID to immortalized normal mammalian embryonic cells and high affinity for binding to nucleic acids. Fluorescence microscopic examination demonstrate the affinity of the 2-carboxamide-indane-1,3-dione to DNA- and RNA-containing cellular structures. To elucidate the affinity of CAID to nucleic acids, the B3LYP/6-31G(d,p) calculations were performed. According to the calculations CAID is preferably associated to nucleotides phosphate group.

Key words: 2-carbamido-1,3-indandione; anti-proliferative activity; malignant cells

INTRODUCTION

2-Substituted 1,3-indandiones have been a subject of investigations due to their pharmacological properties [1] and a wide range of biological activities such as anti-inflammatory [2] and antitumor [3,4] activity. Dimmock et al. [4] show that 2-benzylidene-1,3-indandiones are cytotoxic to several malignant cell lines and also display preferential toxicity to various neoplasms rather than to the normal cells.

2-Carbamido-1,3-indandione (CAID) belongs to the 1,3-indandione group. CAID shows relatively high photostability and because of its strong absorption in UVA and UVB spectral region it is a promising sunscreen candidate. Our previous work [5] also indicates that the compound has a potential and is suitable for use as a fluorescent molecular probe for investigation of different biomolecules.

Thus, the aim of this study, as an essential part of an ongoing investigation on the antitumor activity of 2-carbamido-1,3-indandione is to present data about its cytotoxicity to normal cells such as Balb/c 3T3 cell line. In addition, the intracellular localization of CAID in murine embryonic fibroblasts, studied by fluorescence microscopy, is reported.

MATERIALS AND METHODS

Synthesis of 2-carbamido-1,3-indandione

2-Carbamido-1,3-indandione was synthesized applying a known procedure [6]. Condensation of diethyl phthalate with acetonitrile in the presence of sodium methoxide afforded 1,3-dioxo-2-indancarbonitril in 91% yield. Subsequent hydrolysis with concentrated sulfuric acid resulted in the desired amide in 64 % yield after recrystallized from methanol.

Steady-state spectral measurements

The photophysical properties, absorbance and fluorescence, of CAID were investigated in ethanol and dimethylsulfoxide, solvents suitable for biological applications. UV-visible spectra were recorded with a Perkin Elmer Lambda 25 UV/Vis Spectrometer. Fluorescence spectra were recorded with a Perkin Elmer LS-55 Luminescence Spectrometer.

Cell lines and culture conditions

Balb/c 3T3 cell line was used for assessment of the cytotoxicity of CAID. Cells were cultured in Dulbecco's Modified Eagle's Medium with low glucose (1.0 g/L) containing L-glutamine and

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sodium pyruvate (DMEM, Sigma-Aldrich). The culture medium was supplemented with 10% fetal bovine serum - FBS (Gibco; BioWhittaker) and antibiotics (penicillin 100 UI/ml and streptomycin, 100 μg/ml, AppliChem). The cultures were maintained in plastic flasks with a growth surface area 25 cm^2 and 75 cm^2 (Orange Scientific, Belgium). Cultivation was performed in an incubator at 37ºC, 5% CO_2 and 95% relative humidity. Solution containing 0.05% trypsin (Gibco) and 0,025% ethylenediamine tetraacetic acid (EDTA, FlowLab, Australia) was used for cell dissociation. Disposable plastic consumables (tissue culture flasks, containers, filtration systems, tissue culture plates, etc.) were purchased from Orange Scientific, Belgium.

Cytotoxicity testing (Balb/c 3T3 Neutral Red Uptake Test)

Cytotoxic effect of the 2-carboxamide-indane-1,3-dione was studied by Balb/c /c 3T3 Neutral Red Uptake Test (3T3 NRU test) as described previously [7,8]. Briefly, cells were suspended in growth medium with 10% FBS and seeded in 96-well plates ($1x10^4$ cells/well). After 24-hour incubation period, the cell cultures were treated with indandione in growth medium containing 5% FBS. Cells were treated with eight different concentrations of the tested compound (dilution factor - $7\sqrt{10}$ = 1.389; 6 wells for each concentration) for 24 hours. Untreated (control) cultures were grown for the same conditions in the growth medium with 5% FBS. The morphology of the cell cultures were monitored by inverted microscope. After washing with PBS control and treated cell cultures were incubated for 3 hours in a culture medium containing 0.25 μg/mL neutral red. The absorbed intracellular vital dye was extracted by adding a solution containing 50% ethanol, 49% water and 1% acetic acid. The cytotoxic effect of the test substance was presented as a concentration-dependent decrease of the amount of absorbed vital dye – neutral red. The optical density of the control and treated cultures was measured using an ELISA spectrophotometer (TECAN, SunriseTM, Grödig / Salzburg, Austria) at a wavelength of 540 nm. The relative survival of the treated cells compared to the control was calculated for each concentration using the following formula:

Cell viability (%) = Optical density $(OD)_{540}$ (experimental)/OD_{540} (control) x 100.

Fluorescent microscopy

BALB/c 3T3 cells were suspended in growth medium on sterile diagnostic slides with teflon rings and incubated in a moist chamber at 37.5°C and 5% CO_2 for 24 hours. Growth medium was removed and cells were treated with four different concentrations of 2-carboxamide-indane-1,3-dione - 100 μM, 50 μM, 10 μM and 1 μM. After 24 h treatment, cells were washed with PBS, dried at room temperature, fixed in ice cold acetone for 20 min. and covered with glass coverslips. The preparations were observed by a fluorescence microscope Leica DM 5000 B.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical significance of differences between the viability of the cells treated with different concentrations and the control cells was evaluated by one-way analysis of variance (ANOVA), followed by Bonferroni's test using the GraphPAD PRISM software, version 5 (GraphPad Software Inc., San Diego, USA). Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered statistically significant.

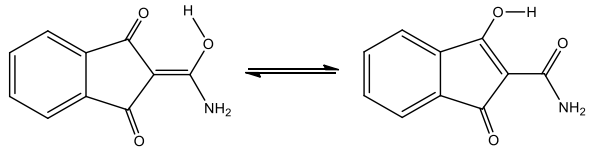
Quantum chemical calculations

The geometries of the two 2-carbamido-1,3-indandione tautomers were optimized at the MP2/6-31+G(d,p) level. Solvent effect (ethanol and dimethylsulfoxide, DMSO) was accounted by using the self-consisted reaction field method with the conductor polarizable continuum model (CPCM) formalism. The stationary-point geometries were fully optimized in the reaction field of the implicit solvent. The minima and transition state were confirmed to have zero and one imaginary frequency, respectively. The values of Gibbs free energies (ΔG) and activation barrier ($\Delta G^{\#}$) were calculated at temperature 298.15 K. The percent content of tautomers is: content % = $100.p_i$ where $p_i = e^{-\Delta G_i / RT} / \sum_i e^{-\Delta G_i / RT}$. The classical rate constants of the forward (k_f) and the reverse (k_r) tautomerization reactions were obtained using the Eyring equation. Because of the size of the structures studied, the geometries of complexes of 2-carbamido-1,3-indandione with uridine mono-phosphate or guanosine monophosphate were optimized at B3LYP/6-31G(d,p) level without any constrains.

According to Alcolea Palafox [9-11], the B3LYP functional and 6-31G(d,p) basis set represents a compromise between accuracy and computational cost and thus B3LYP/6-31G(d,p) has been used in the majority of the calculations. The program package GAMESS [12] was used to perform the quantum chemical calculations.

RESULTS AND DISCUSSION

Two enol tautomeric forms of 2-carbamido-1,3-indandione coexist in different solutions [4, 12]: 2-(hydroxyl-aminomethylidene)-indan-1,3-dione (A) and 2-carboamide-1-hydroxy-3-oxo-indan (B) (Fig. 1). According to our CPCM/MP2/6-31+G(d) calculations in ethanol and DMSO solutions the ratio of the tautomers is A : B = 60 : 40 (Table 1). Tautomer **A** converts into tautomer **B** and vice versa by means of very fast intramolecular proton transfer reaction. The rate constants are of order 10^8 (Table 1).



A *B*

Fig. 1. Equilibrium between tautomeric forms 2-(hydroxyaminomethylidene)-indan-1,3-dione (**A**) and 2-carboamide-1-hydroxy-3-oxo-indan (**B**).

Table 1. CPCM/MP2/6-31+G(d) calculated energy difference ΔG_{293} for the tautomers of **CAID** and energy barrier $\Delta G^{\#}$ (kcal mol^{-1}) of intramolecular proton transfer in ethanol or DMSO solution, % contents of the tautomers and rate constants of the forward (k_f) and reverse (k_r) reactions (s^{-1}).

Solvent	ΔG_{293}	% content	$\Delta G^{\#}$	k
ethanol	0.26	60.8 % A	1.23 A→B	$k_f = 6.08 \times 10^8$
		39.2 % B	0.97 B→A	$k_r = 9.93 \times 10^8$
DMSO	0.21	58.8 % A	1.95 A→B	$k_f = 1.56 \times 10^8$
		41.2 % B	1.74 B→A	$k_r = 2.32 \times 10^8$

The electronic absorption spectra of CAID were recorded in ethanol (EtOH) and dimethylsulfoxide (DMSO). The absorption spectra (Fig. 2) showed characteristic absorption band in the UV-A region with slightly dependence on solvent. The absorption maxima for CAID solved in EtOH are at 374 nm and in DMSO - at 376 nm.

Fluorescence emission spectra for CAID were recorded at excitation 330 nm in both solvents at concentration 10 μM (Fig. 2). The fluorescence emission maxima are red shifted as compared to the absorption maxima with a shift of 140 nm for both solvents**.** The fluorescence spectra are stable in time with good fluorescence quantum yield, which is an advantage in further studies for biological applications of CAID.

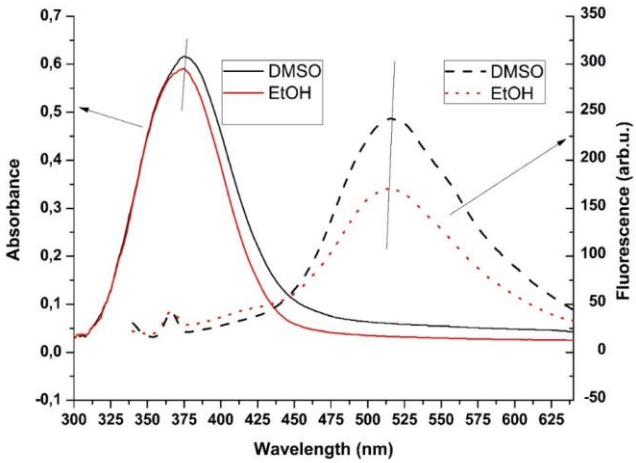


Fig. 2. Absorption (left) and emission (right) spectra of CAID in ethanol (EtOH) and dimethylsulfoxide (DMSO)

Balb/c 3T3 Neutral Red uptake test

Cytotoxic effect of 2-carboxamide-indane-1,3-dione was studied on non-tumorigenic cell line of mouse embryo fibroblasts Balb/c 3T3 by applying Neutral Red Uptake test. The cells were exposed to eight different concentrations of the tested compound for 24 hours. Light microscopic examination showed that CAID did not induce significant changes in morphology and growth properties of the cells. Results of the Neutral Red Uptake test confirmed the data from the microscopic examination and indicated that 2-carboxamide-indane-1,3-dione did not induce statistically significant reduction of the cell viability as compared to the control (Fig. 3).

The results of the study show that the 2-carboxamide-indane-1,3-dione is not toxic to the cells of the Balb/c 3T3 cell line. Fluorescence properties of the 2-carboxamide-indane-1,3-dione were used for study of CAID ability to penetrate through cellular membranes and to establish its intracellular localization (Fig. 4).

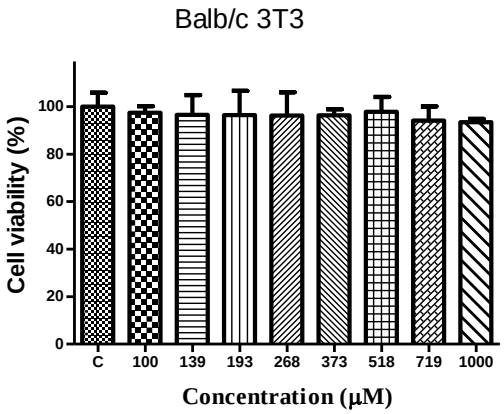


Fig. 3. Cytotoxicity of 2-carboxamide-indane-1,3-dione on Balb/c 3T3 cells line after 24 hours of treatment.

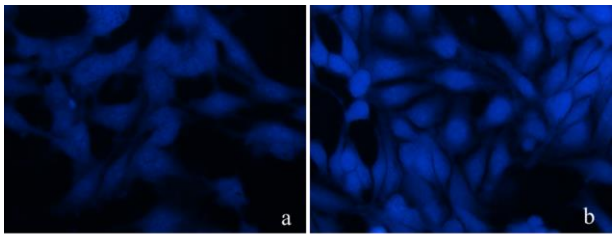


Fig. 4. Fluorescence microscopy of Balb/c 3T3 cells after 24-hour treatment with 1 μM CAID: a) control; b) cells treated 2-carboxamide-indane-1,3-dione.

Fluorescence microscopic examination indicated that the tested substance penetrates into Balb/c 3T3 cells and strongest fluorescent signal was established in the cell nucleus and nucleoli, demonstrating the affinity of the 2-carboxamide-indane-1,3-dione to DNA- and RNA-containing cellular structures as nuclei and nucleoli.

To elucidate the affinity of CAID to nucleic acids, the DFT calculations were performed. We have modeled complexes between CAID and two nucleotides (with purine and pyrimidine nitrogen-containing nucleobases) – uridine monophosphate (UMP) and guanosine monophosphate (GMP). There are two possible site of association of CAID to nucleotide by hydrogen bonding - to nucleobases (UMP-CAID and GMP-CAID) or to phosphate group (CAID-GMP and CAID-GMP). The complexes formed are shown in Fig. 5. According to B3LYP/6-31G(d,p) calculations the more stable complex is CAID-UMP (by 3.09 kcal mol^{-1}) where 2-carboxamide-indane-1,3-dione is associated to UMP by phosphate group. In the case of GMP-complexes the two complexes are close by energy but the GMP-CAID are the more stable one by 0.12 kcal mol^{-1}.

To account for the possibility of the formation of intermolecular hydrogen bonding between CAID and nucleotides, the interaction energies (E_{int}'s) for the complex are considered: luorescence microscopic examination indicated that the tested substance penetrates into Balb/c 3T3 cells and strongest fluorescent signal was established in the cell nucleus and nucleoli, demonstrating the affinity of the 2-carboxamide-indane-1,3-dione to DNA- and RNA-containing cellular structures as nuclei and nucleoli.

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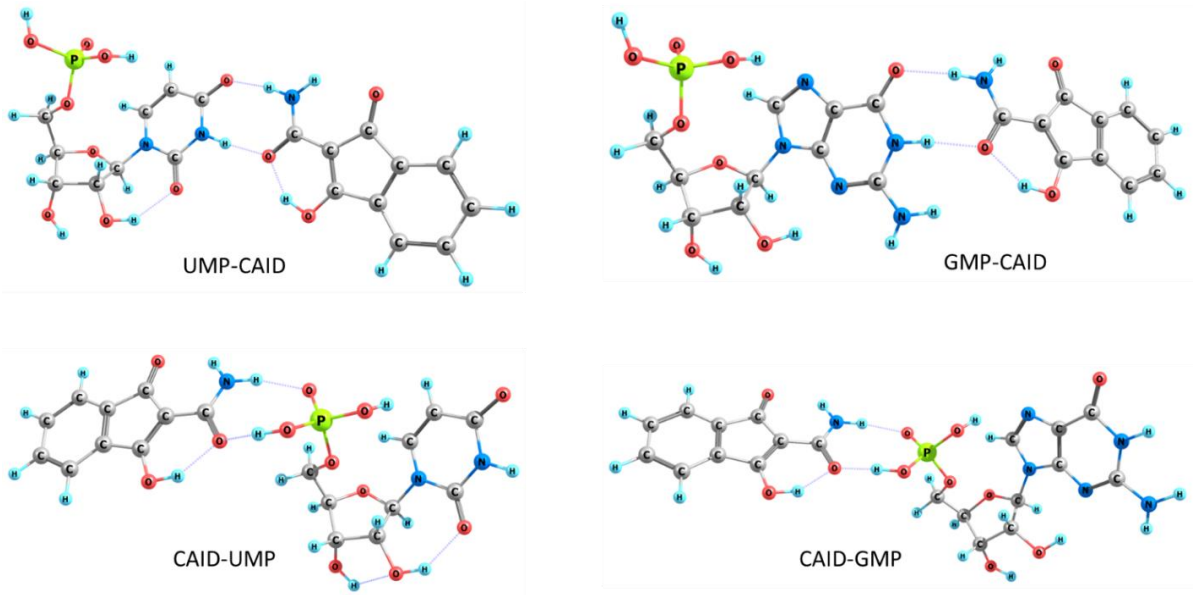

Fig. 5. Complexes of tautomer **B** (2-carboamide-1-hydroxy-3-oxo-indan) (CAID) with: guanosine monophosphate (GMP) and uridine monophosphate (UMP).

To elucidate the affinity of CAID to nucleic acids, the DFT calculations were performed. We have modeled complexes between CAID and two nucleotides (with purine and pyrimidine nitrogen-containing nucleobases) – uridine monophosphate (UMP) and guanosine monophosphate (GMP). There are two possible site of association of CAID to nucleotide by hydrogen bonding - to nucleobases (UMP-CAID and GMP-CAID) or to phosphate group (CAID-GMP and CAID-GMP). The complexes formed are shown in Fig. 5. According to B3LYP/6-31G(d,p) calculations the more stable complex is CAID-UMP (by 3.09 kcal mol^{-1}) where 2-carboxamide-indane-1,3-dione is associated to UMP by phosphate group. In the case of GMP-complexes the two complexes are close by energy but the GMP-CAID are the more stable one by 0.12 kcal mol^{-1}.

To account for the possibility of the formation of intermolecular hydrogen bonding between CAID and nucleotides, the interaction energies (E_{int}'s) for the complex are considered:

$$\Delta E_{int} = E_{CAID} + E_{GMP(UMP)} - E_{complex,} \quad (1)$$

where the complexes are GMP-CAID, CAID-GMP, CAID-UMP and UMP-CAID (Figure 5).

According to eq. (1) the obtained values for E_{int} (in kcal mol^{-1}) are: 18.10 (GMP-CAID), 17.97 (CAID-GMP), 14.69 (UMP-CAID) and 17.77 (CAID-UMP). The results show that the association of CAID to the phosphate group in the two types of nucleotides is more probable. However, the favorite nucleotide is GMP

CONCLUSION

The results from the present study, namely complete absence of toxicity towards immortalized normal mammalian cells and high affinity for binding to nucleic acids makes 2-carboxamide-indane-1,3-dione a promising candidate for investigations on its antiproliferative activity to malignant cells. Fluorescence microscopic examination demonstrate the affinity of the 2-carboxamide-indane-1,3-dione to DNA- and RNA-containing cellular structures. According to our DFT calculations tautomer B of CAID is preferably associated to nucleotides phosphate group.

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ИЗСЛЕДВАНЕ НА ЦИТОТОКСИЧНОСТТА И ВЪТРЕКЛЕТЪЧНАТА ЛОКАЛИЗАЦИЯ НА 2-КАРБАМИДО-1,3-ИНДАНДИОН В BALB/C 3T3 КЛЕТКИ

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(Резюме)

Изследването за цитотоксичност на 2-карбамидо-1,3-индандион (CAID) към Balb/c клетки беше извършено чрез валидизирания Balb/c 3T3 Neutral Red Uptake Test. Представени са и данни от флуоресцентното микроскопско изследване за вътреклетъчната локализация на CAID в ембрионални фибробласти от мишка. Резултатите показват пълно отсъствие на токсичен ефект на CAID към иммортализирани ембрионални клетки от бозайник и подчертан афинитет за свързване с нуклеиновите киселини. Изследването с флуоресцентна микроскопия показва, че 2-карбоксамид-индан-1,3-дион се свързва приоритетно към клетъчни органели, съдържащи ДНК и РНК. За да се изясни свързването на CAID към нуклеиновите киселини, бяха проведени теоретични изследвания на ниво B3LYP/6-31G(d,p), които показват, че CAID се свързва приоритетно с фосфатната група от нуклеотида..