1st International Conference
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ICCBIKG 2021

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BOOK OF PROCEEDINGS

October 26-27th, 2021,
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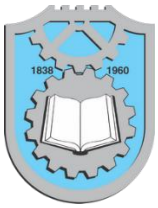

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DISCLOSING THE TRUE NATURE OF HESPERETIN'S ANTIGENOTOXICITY *IN VIVO* WITHIN THE *DROSOPHILA MELANOGASTER* SOMATIC CELLS THROUGH THE EXTENSIVE GENOTOXICAL AND STRUCTURE-BASED STUDIES

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Abstract:

Previously unreported genotoxic and antigenotoxic potentials of hesperetin (**Hes**) were revealed by treating the *Drosophila melanogaster* (*dm*) whose DNA has been altered by means of O^6-ethylguanine (*dm*GO^6-Et) and O^4-ethylthymine (*dm*TO^4-Et) lesions appearance, caused by ethyl methanesulfonate (**EMS**), a proven alkylating agent and mutagen. Therefore, **Hes** potencies were determined by means of the comet assay on somatic cells level, where compound exerted no genotoxic effects but acted genotoxically as a Topoisomerase IIα (*dm*TopIIα) catalytic inhibitor by invading the Binding and Cleavage Domain and stabilizing the noncovalent *dm*TopIIα-plasmid DNA (*dm*PDNA) complex, as verified by the kinetoplast DNA (*dm*K-DNA) decatenation assays. **Hes**'s structure-based alignment caused compound's A and C rings to occupy the area normally invaded by **EMS**, thus making a spatial barrier for the *dm*GO^6-Et or *dm*TO^4-Et lesions formation: the A ring C7-OH group formed hydrogen bonds (HBs) with either *dm*GO^6 (d_{HB} = 2.576 Å) or guanine's N^7 nitrogen (*dm*GN^7, d_{HB} = 2.737 Å), whereas the A ring C5-OH group formed an HB with *dm*TO^4 (d_{HB} = 3.548 Å). Furthermore, **Hes** likewise acted as a mixed-type competitive inhibitor of *dm*ATPase, as verified by the catalytic, FRET, and structure-based studies where it affected the *dm*ATPase dimerization and the hydrolysis of ATP, denying the metabolic energy for the catenation of ethylated G-*dm*DNA segment, the formation of *dm*TO^4-Et-G-*dm*DNA phosphotyrosine intermediate (*dm*TO^4-Et-G-*dm*DNA-PTyr785I), and the passage of ethylated T-*dm*DNA segment through the temporarily broken *dm*TO^4-Et-G-*dm*DNA-PTyr785I, processes seen as comets. Conclusively, **Hes** may be used in anticancer therapy controlling the effects of alkylating agents.

Keywords: Hesperetin, *Drosophila melanogaster*, molecular modelling, comet assay

1. Introduction

Hesperetin (**Hes**) is a flavanone with adverse biological effects [1-5], but with little known genotoxic or antigenotoxic effects [4]. To the best of the authors' knowledge, there are no

reports on **Hes**'s genotoxic potential on *Drosophila melanogaster* (*dm*) as a model organism. Herein, **Hes**'s overall influence on the *dm*DNA was appraised by virtue of alkaline comet assay in the presence of ethyl methanesulfonate (**EMS**), the well-known endogenous alkylating agent. Therefore, the premise of this report was to outline the **Hes**'s antigenotoxic potential on somatic cells level, as well as to give an insight into the related pharmacology *in vivo* using the *in vitro* and the *in silico* methods.

2. Results and discussion

2.1 Hes's influence on somatic cells. The individual treatment of fruit fly's somatic cells with **Hes** caused no significant difference in the total score of *dm*DNA damage related to the negative control, while **EMS**'s induced significant increase in the percentage of damaged cells, as well as by the formation of comet classes 1 and 4 (Table 1). Being applied simultaneously with **EMS**, **Hes** exerted the antigenotoxic potency by reducing the *dm*DNA damage by more than 70% and significantly lowering the frequencies of comet classes 2 to 4.

Table 1. Genotoxic and antigenotoxic activities of **Hes** against **EMS**, determined using the comet assay

Treatments	Comet classes					Total score[a]	%R[b]
	0	1	2	3	4		
NC[c]	82.70±0.26	17.30±0.43	0.00±0.00	0.00±0.00	0.00±0.00	17.3±0.4†	/
EMS[d]	13.00±0.2	23.20±0.41	17.60±0.34	17.30±0.25	28.90±0.31	225.9±1.04*	/
Hes[e]	82.30±0.34	12.60±0.71	5.10±0.90	0.00±0.00	0.00±0.00	22.8±0.82†	/
EMS+**Hes**[f]	60.20±0.61	22.9±0.57	11.00±1.32	4.80±0.81	1.10±0.24	63.7±0.34*†	77.8

[a]The values are mean ± S.D. from three independent experiments; [b]%R; percentage of reduction; [c]Negative control; [d]Ethyl methanesulfonate, 1mM; [e]**Hes**, 1 mM; [f]**EMS**+**Hes** (1mM+1mM). $^{*}p$ <0.05 when compared with the negative control group; $^{\dagger}p$<0.05 when compared with the positive control group.

2.2 Hes's pharmacology on the dmTopIIα binding and cleavage domain level. To validate **Hes**'s pharmacology on *dm*TopIIα-*dm*DNAcomplex, kinetoplast DNA (*dm*K-DNA) decatenation assays were performed. While **EMS** (Figure 1A, lane 3) behaved in the genotoxic fashion related to *dm*K-DNA, **Hes** inhibited the *dm*TopIIα mediated decatenation, in a dose-dependent manner with an IC_{50} equal to 680μM. Therefore, **Hes** likely acted as the stabilizer of the noncovalent *dm*TopIIα-*dm*K-DNA complex [8].

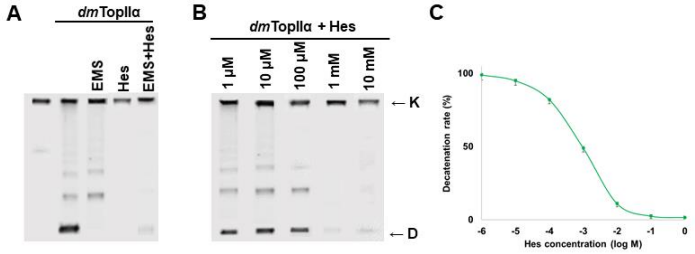

Fig.1. TheK-DNA decatenation assay (A). The repeated decatenation assay in presence of **Hes** (1μM to 10mM) (B). An inhibition curve (C). Abbreviations: K - catenated K-DNA; D - decatenated K-DNA

2.3 Hes's binding mode within the dmTopIIα binding and cleavage domain. To perform the electrophilic alkylation and comet formation, **EMS** has adopted the bio-pose above either *dm*G*O*^{6} or *dm*T*O*^{4}, placing the ethyl group in a position to be easily transferred to guanine (Figure 2). As antigenotoxic agent, **Hes** has made a spatial barrier for the alkylating agent, inasmuch as its A ring C7-OH group formed hydrogen bonds with either *dm*G*O*^{6} (d_{HB} = 2.576 Å), guanine's *N*^{7} nitrogen (*dm*G*N*^{7}, d_{HB} = 2.737 Å), or **Hes**'s A ring C5-OH group and *dm*T*O*^{4} (d_{HB} = 3.548 Å).

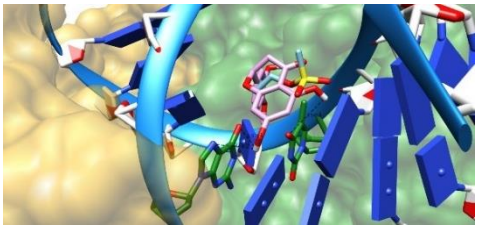

Fig. 2. The structure-based alignment of **Hes** (light purple) and **EMS** (light blue) into the *dm*PDNA

2.4 Hes's pharmacology within dmTopIIα ATPase domain. The ATPase assay, for estimation of **Hes**'s impact on ATP hydrolysis through, pointed out compound as a mixed-type ATPase inhibitor with a more competitive character. Afterwards, experiment performed on dual-labeled *dm*ATPase showed that **Hes** induced a significant decrease in FRET, forcing the not dimerized *dm*ATPase conformation (Figure 3), and that **Hes** does not affect *dm*ATPase catalytic activity if the holoenzyme is pre-dimerized with AMPPNP.

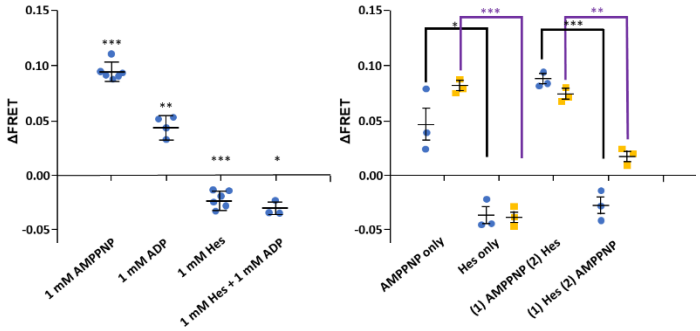

Fig. 3. FRET-based monitoring of ATPase domain closure in the presence of **Hes** (left), and quantification of AMPPNP and **Hes** order of addition assays (right).

2.5 The binding of Hes within the dmATPase. As a *dm*ATPase substrate, ATP adopted complex bioactive conformation, while **Hes** was located at the very top of the *dm*ATPase domain (Figure 4). **Hes**'s A ring formed electrostatic interactions with the *dm*Ser309 and *dm*Thr217, whereas C and B rings were stabilized by means of hydrophobic interactions with *dm*Phe218 and *dm*Phe313. Further, **Hes**'s B-ring's C3'-OH and C4'-methoxy portions forced the induced dipole interactions with the *dm*Ile329, *dm*Val306, *dm*Phe307, and *dm*Leu328, respectively (Figure 4).

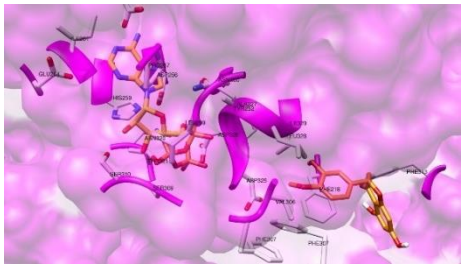

Fig. 4. Molecular docking of ATP and Hes within *dm*ATPase domain

3. Materials and methods

3.1 Chemicals. All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

3.2 Comet assay. Genotoxic and antigenotoxic potential of **Hes**, in comparison with **EMS**, were detected using the alkaline comet assay according to Singh et al. [6].

3.3 Statistical analyses. The obtained results were statistically analyzed using SPSS statistical software package (version 13.0) as described by Mladenovic et al. [7].

3.4 Decatenation assay. To validate **Hes** as a *dm*TopIIα catalytic inhibitor, *Drosophila melanogaster*'s kinetoplast DNA (*dm*K-DNA) decatenation assays were conducted, following the experimental setup as described by Lee et al. [8].

3.5 Molecular modeling. The experimental setups for homology modeling of *dm*TopIIα, the *dm*TopIIα-*dm*PDNA complex construction and molecular docking were described elsewhere [7].

4. Conclusions

The enclosed study showed that **Hes** has not exerted genotoxic potential in somatic cells, and when applied simultaneously with **EMS** significantly reduced the DNA damage induced by genotoxic agent. Decatenation assays and structure-based studies revealed that **Hes** acts as *dm*TopIIα catalytic inhibitor by stabilizing the noncovalent *dm*TopIIα-PDNA complex. Furthermore, verified by FRET-based assays and structure-based studies, **Hes** acted as a mixed-type competitive inhibitor of *dm*ATPase, where it affected the *dm*ATPase dimerization and the hydrolysis of ATP, denying the metabolic energy for the decatenation and catenation of alkylated *dm*DNA segments, unnatural G=T and T=G bonding and mutagenic, toxic, and carcinogenic effects after the replication. Obtained results indicate that **Hes** may be used in anticancer therapy to prevent the effects of alkylating agents.

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