10β-Hydroxyestra-1,4-diene-3,17-dione as potential antiproliferative agent: in vitro biological evaluation and in silico studies

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ABSTRACT
10β-Hydroxyestra-1,4-diene-3,17-dione (HEDD) is a natural product described as having neuroprotective activity. However, the cytotoxic properties of this quinol are barely studied. Thus, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed in six cell lines (MCF-7, T47-D, LNCaP, HepaRG, Caco-2 and NHDF). Additionally, an in vitro estrogenicity assay and a cell viability analysis together with in silico molecular docking studies were carried out in order to understand the potential mechanism of cytotoxicity. Computational predictions of its pharmacokinetic and toxicity properties were also performed. Surprisingly, HEDD displayed marked cytotoxic activity, particularly against hormone-dependent cancer cells and the flow cytometry analysis revealed that HEDD markedly reduced the viability of hepatic cancer cells. Molecular docking studies suggested a high affinity towards the estrogen receptor α and 17β-hydroxysteroid dehydrogenase type 1. Moreover, it was predicted that HEDD may have good oral bioavailability and a low maximum tolerated dose in humans.
1. Introduction

Steroids play a crucial physiological role in metabolism and neuroprotection (Simpkins et al. 2004). 10β-Hydroxyestra-1,4-diene-3,17-dione (HEDD) is a steroidal para-quinol formed in the human body which can be considered a estrone prodrug in the central nervous system (CNS) without ER affinity in the peripheral tissues (Prokai et al. 2003; Prokai-Tatrai and Prokai 2019). Interestingly, HEDD was screened against melanoma (Fem-X), cervix carcinoma (HeLa) and leukemia (K562) cells, displaying a weak antiproliferative effect (IC$_{50} >$ 100 μM) (Milić et al. 2001). However, in CCRF-CEM leukemia cells some cytotoxicity (IC$_{50} = 26.4$ μM) was found (Milić et al. 1999). Taking into account these results, we prepared HEDD and in vitro explored its cytotoxic effects against six cell lines. In addition, an estrogenicity assay and a flow cytometry study were performed. Based on the results observed, we also performed molecular docking studies, including the estrogen receptor (ERα), androgen receptor (AR), 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1), aromatase (CYP450$_{19}$) and 17α-hydroxylase/17,20-lyase (CYP17A1). Furthermore, a computational prediction of the most relevant pharmacokinetic and toxicity properties was also carried out.

2. Results and discussion

The cytotoxic effects of HEDD were only barely studied and therefore, this work was designed to improve the current knowledge of its bioactivity and potential toxicity. HEDD (compound 2) was prepared by our research group by the general synthetic procedure described in Supplementary material Scheme S1 and was structurally characterized by spectral analysis (IR, 1H- and 13C-NMR) (Supplementary material: Chemistry, Figures S1 and S2). In the present work we used potassium permanganate to prepare this product in relatively good yields and in a short time, similarly to which was previously described (Lista et al. 2006).

Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, a first screening at 30 μM showed a very high cytotoxicity of HEDD in all
cell lines [breast (MCF-7 and T47-D), prostate (LNCaP), hepatic (HepaRG), colon (Caco-2) and normal human dermal fibroblasts (NHDF)] tested (Supplementary material Figure S3). Then, a relevant anti-proliferative effect was observed for HEDD (IC$_{50}$ values ranging from 4.11 to 18.64 $\mu$M) (Table 1) when compared with the parent compound, estrone (IC$_{50}$ values between 29.53 and 61.82 $\mu$M).

These results are new and very interesting because no relevant cytotoxicity (IC$_{50}$ > 100 $\mu$M) was observed by Milić et al. (2001). Our results also evidenced that HEDD has higher cytotoxicity against hormone-dependent cancer cells. Hepatic and colon cancer cells were less affected by this compound, as well as NHDF cells. In this context, it is important to mention that estrone can be converted, particularly in the liver, into the corresponding quinol by CYP1A1, CYP2B6 and CYP2E1 isoenzymes (Ohe et al. 2000), which reinforces the importance of study its cytotoxic effect in hepatic cells. The selectivity index (SI) for HEDD (Supplementary material Table S1) was higher in T47-D cells (SI > 3). Additionally, HEDD did not exhibit a proliferative action for 0.001 and 0.01 $\mu$M concentrations in E-screening assay (Supplementary material Figure S4). Therefore, it can be considered that this compound in low concentrations has probably reduced or null estrogenic effects, similarly to which was previously demonstrated for DHED and other analogues (Prokai-Tatrai and Prokai 2019). A flow cytometry assay using propidium iodide evidenced that HEDD led to a drastic reduction (approximately 83% after 24 h of treatment at 50 $\mu$M) of HepaRG cells viability (Figure 1). Consequently, no further studies have been done to explore the effects of HEDD on the cell cycle.

ER$_{\alpha}$, AR, 17$\beta$-HSD1, aromatase and CYP17A1 were included in this study because HEDD was more active against hormone-dependent cancer cells (Payne and Hales 2004; Amelichev et al. 2011; Hong and Chen 2011). These results are displayed in Supplementary material Table S2. Interestingly, higher binding energy and potential stronger interaction for ER$_{\alpha}$ and 17$\beta$-HSD1 proteins were predicted for HEDD (Supplementary material Figures S5 and S6). Concerning ER$_{\alpha}$, HEDD shares with 17$\beta$-estradiol some hydrophobic interactions, involving particularly the residues Leu387, Met388, Leu391 and Phe404. However, the conventional hydrogen bonds with the residues Glu353 and His524 are absent (Fukuzawa et al. 2006). Instead, Van der Waals interactions with these residues and a hydrogen bond with the amino acid Arg394 were predicted. When merging the docked 3D structures of 17$\beta$-estradiol and HEDD, it is possible to verify that these molecules are partially overlapped. The evaluation of HEDD binding mode with 17$\beta$-HSD1 also showed similar atomic interactions with the observed for DHT, the co-crystallized ligand, including hydrophobic interactions with

| Compounds | MCF-7 IC$_{50}$ | T47-D IC$_{50}$ | LNCaP IC$_{50}$ | HepaRG IC$_{50}$ | Caco-2 IC$_{50}$ | NHDF IC$_{50}$ |
|-----------|-----------------|-----------------|-----------------|-----------------|-----------------|----------------|
| Estrone   | 41.93           | ND              | ND              | 29.53           | 42.69           | 61.82          |
| HEDD      | 5.79            | 7.72            | 4.11            | 10.68           | 18.64           | 12.57          |
| 5-FU      | 1.71            | 0.54            | 7.79            | 1.78            | 1.31            | 3.61           |

$^a$Cells were treated with different concentrations (0.1, 1, 10, 25, 50 and 100 $\mu$M) during 72 h. The cell proliferation effects were determined by the MTT assay. The data shown are representative of at least two independent experiments. 5-FU: 5-fluorouracil; ND: not determined.
the residues Val143, Leu149 and Pro187. However, when comparing the binding modes of both ligands, **HEDD** does not interact by a hydrogen bond with the essential amino acid His221 forming instead two hydrogen bonds with the residues Tyr218 and Ser222 (Day et al. 2008). Also, an *in silico* evaluation of drug-likeness properties, specifically the Lipinski’s rule of five, and a prediction of the ADMET properties were performed for the tested compound (Supplementary material Tables S3 and S4). The results showed a high Caco-2 permeability; high intestinal absorption; not be a P-glycoprotein substrate or inhibitor; not interact with the renal protein organic cation transporter 2 (OCT2); low probability to penetrate into the CNS; be a substrate of CYP3A4 isoform and low maximum tolerated dose in humans.

3. Experimental

(Supplementary material)

4. Conclusion

The anticancer potential of the quinol **HEDD** had been rarely explored and in this study was evaluated the *in vitro* cytotoxic properties of this compound. Interestingly, **HEDD** showed significant antiproliferative effects, mainly against hormone-dependent (MCF-7, T47-D and LNCaP) cancer cells. Furthermore, this steroidal quinol caused a drastic reduction in the hepatic HepaRG cell viability and did not promote the proliferation of estrogen-sensitive T47-D cells at low concentrations. *In silico* studies suggested strong interactions with ERα and 17β-HSD1 and a relatively low maximum tolerated dose, relevant data to be considered in future studies involving this compound.
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Disclosure statement
The authors confirm that this article content has no conflict of interest.

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