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Mechanism of antiproliferative action of a new D-secoestrone-triazole derivative in cervical cancer cells and its effect on cancer cell motility

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ABSTRACT

Cervical cancer is the fourth most frequently diagnosed tumor and the fourth leading cause of cancer death in females worldwide. Cervical cancer is predominantly related with human papilloma virus (HPV) infection, with the most oncogenic types being HPV-18 and -16. Our previous studies demonstrated that some D-secoestrone derivatives exert pronounced antiproliferative activity. The aim of the current investigation was to characterize the mechanism of action of D-secoestrone-triazole (D-SET) on three cervical cancer cell lines with different pathological backgrounds.

The growth-inhibitory effects of D-SET were determined by a standard MTT assay. We have found that D-SET exerts a pronounced growth-inhibitory effect on HPV 18-positive HeLa and HPV-negative C-33 A cells, but it has no substantial inhibitory activity on HPV 16-positive SiHa or on intact fibroblast MRC-5 cell lines. After 24 h incubation, cells showed the morphological and biochemical signs of apoptosis determined by fluorescent double staining, flow cytometry and caspase-3 activity assay. Besides the elevation of the ratio of cells in the subG1 phase, flow cytometric analysis revealed a cell cycle arrest at G2/M in both HeLa and C-33 A cell lines. To distinguish the G2/M cell population immunocytochemical flow cytometric analysis was performed on HeLa cells. The results show that D-SET significantly increases the ratio of phosphorylated histone H3, indicating cell accumulation in the M phase. Additionally, D-SET significantly increased the maximum rate of microtube formation measured by an *in vitro* tubulin polymerization assay. Besides its direct antiproliferative activity, the antimigratory property of D-SET has been investigated. Our results demonstrate that D-SET significantly inhibits the migration and invasion of HeLa cells after 24 h incubation.

These results suggests that D-SET is a potent antiproliferative agent against HPV 16+ and HPV-negative cervical cancer cell lines, with an efficacious motility-inhibiting activity against HPV 16+ cells. Accordingly D-SET can be regarded as a potential drug candidate with a promising new mechanism of action among the antiproliferative steroids, potentially allowing for the design of novel anticancer agents.

1. Introduction

Cancer is one of the major health problems worldwide. In 2012, around 8.2 million cancer deaths occurred and 14.1 million new cancer cases were detected. Cervical cancer is the fourth most frequently diagnosed cancer in women and the fourth leading cause of death worldwide, accounting for 527,600 new cases and 265,700 deaths in 2012 [1]. In agreement with these global cancer statistics, 58,300 new cervical cancer cases were diagnosed, and 13,400 cervical cancer death occurred in Europe in 2012, with 38,800 all new cases occurring in Central European countries. These data imply that cervical cancer was the fifth most common cancer in Europe, and the fourth most frequently diagnosed tumor type in Central Europe in 2012 [2]. Cervical cancer is predominantly related with human papilloma virus (HPV) infection, epidemiological studies indicating

Abbreviations: D-SET, D-secoestrone-triazole; HPV, Human Papilloma Virus; 2-ME, 2-methoxyestradiol; PAC, paclitaxel; Bid, BH3 interacting-domain death agonist; Bcl-2, B-cell CLL/lymphoma 2; FADD, Fas-Associated protein with Death Domain; DISC, death-inducing signalling complex.

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that more than 18 variants of HPV are associated with carcinogenesis, of which HPV-18 and -16 are responsible for approximately 50% and 20% of cervical cancerous cases, respectively [3,4]. In developed countries vaccination and screening methods are widely available, and the number of death cases have therefore decreased during the last few years, but at the same time cervical cancer is still the second most commonly diagnosed malignancy in less developed countries, nearly 90% of cervical cancer deaths occurring in those countries [1,5]. Vaccination is recommended under the age of 25 or before the initiation of sexual activity, because its effectiveness is highest before any exposure to HPV infection [6,7]. Women over 25 years and antivaccination groups in developed countries are therefore still compromised, and even malignant cervical lesions diagnosed in the early stage need rapid and aggressive cytotoxic treatment utilizing novel anticancer agents with a more tolerable side-effect profile.

Estrogens play important roles in the normal functions of both male and female reproductive systems, including the regulation of growth and differentiation [8]. 17β-Estradiol increases the proliferation of its target cells in the reproductive system and also in gynecological tumors by stimulating RNA synthesis [9]. Besides this established fact, since the 1990's more and more experimental results have indicated that structural modifications of 17β-estradiol may lead to estrone analogs with antiproliferative activity. A-Ring-modified 2-methoxyestradiol (2-ME) is one of the first discovered antiproliferative metabolites without steroidal activity against several cancerous cell lines [10–15] owing to its apoptosis-activating, microtubule-disrupting, antimigratory and antiangiogenic effects [16]. On the basis of these results, many new derivatives with anticancer properties have been synthetized and analyzed. The majority of published studies have focused on substitutions in multiple positions of the steroid structure or ring modifications. Several sets of newly synthetized secoestrones have been reported which include modifications to avoid estrogenic activity through opening of their D-ring [17–19]. Our previous studies demonstrated that some D-secoestrone derivatives exert pronounced antiproliferative activity thanks to their influence on microtubule formation from tubulin dimers [20].

The triazole ring can be regarded as a pharmacophore when built onto a steroid and other types of skeletons in order to obtain innovative antiproliferative agents. Several nonsteroidal triazole derivatives are biologically active, including many that possess antiproliferative and proapoptotic properties [21–24]. The most frequent positions for substitution of the sterane skeleton are C-2, C-3 and C-17. Moreover, steroids with a triazole moiety in different positions have also been revealed to exert potent growth-inhibitory effects [25–29]. Furthermore, incorporating a triazole structure into the estrone skeleton may improve stability, solubility and bioavailability of the compound [30]. A recent study has shown that triazole substitution in position C-3 dramatically enhanced the antiproliferative activity of D-secooxime derivatives [31].

These promising results encouraged us to continue and extend our investigations of D-secoestrones. In view of the previously mentioned most active secoalcohol, new analogs have been designed and proliferation-inhibitory effects have been detected. As expected, the derivatives with triazole substituents demonstrated the most pronounced anticancer effect on several malignant tumor cell lines tested [32].

Besides the antiproliferative and proapoptotic capacities, an antimetastatic effect is another important characteristic in question during the investigation of the anticancer mechanism of new compounds. The presence of metastasis is highly associated with poor patient prognosis, and the most frequent cause of cancer death [33]. Metastasis formation is a complex, multistage process, including changes in cell adhesion, migration and invasion [33,34]. The antimetastatic activity of the anticancer compounds is a major highlight of tumor-specificity as antimetastatic drugs have no cytotoxic effect on intact cells (i.e. on cells without motility changes). Previous studies have revealed that 17β-estradiol may induce cell invasion via the PI3K signaling pathway in cervical [35], endometrial [36] and ovarian cancer [37] cell lines. In addition, it has been reported that the above-mentioned A-ring-modified estrogen metabolite, 2-ME, has an antimetastatic effect on several cancer cell lines [38,39]. Stander et al. have reported that another synthetic sulfamoylated estrogen analog also has an antimetastatic potential, associated with the acidotic microenvironmental conditions in tumors [40]. To date, there has been no detailed investigation of the effects of secoestrogens on cell motility.

The aim of the present study was to characterize the mechanism of action of D-secoestrone-triazole (D-SET), the most active member of a recently designed and synthetized D-secoestrone library on three cervical cancer cell lines with different pathological backgrounds. Besides investigating its antiproliferative activity, we also examined the changes in nuclear morphology, the alterations in cell cycle progression, and the proapoptotic properties of the test compound. We also investigated its action on cell migration and invasion, to reveal D-SET's influence on cancer cells motility.

2. Materials and methods

2.1. Chemicals

D-SET was synthetized as described elsewhere [32]. Its chemical structure is presented in Fig. 1. All other chemicals and kits, if otherwise not specified, were purchased from Sigma Aldrich Ltd. (Budapest, Hungary).

2.2. Cell cultures

The human cervical cancer cell line HeLa (HPV 18+ adenocarcinoma) was purchased from ECACC (European Collection of Cell Cultures, Salisbury, UK), while SiHa (HPV 16+ squamous cell carcinoma) and C-33 A (HPV negative carcinoma) were purchased from ATCC (American Tissue Culture Collection, Manassas, Virginia, USA) [41]. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% non-essential aminoacids, and 1% penicillin-streptomycin. All media and supplements were obtained from Lonza Group Ltd. (Basel, Switzerland). The cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2.

2.3. Antiproliferative MTT assay

The growth-inhibitory activity of D-SET was determined by standard MTT dye uptake methods on three cervical cancer cell lines with differences in HPV status: HeLa, C-33 A and SiHa cells.

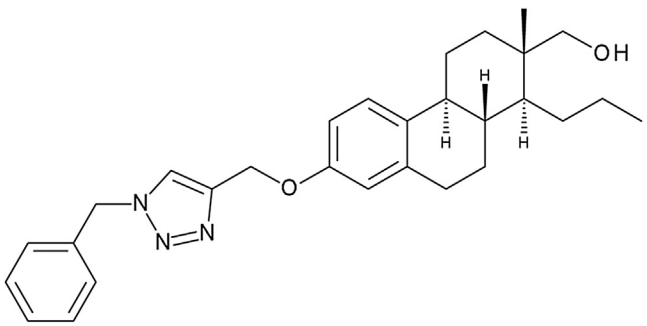

Fig. 1. Chemical structure of D-secoestrone-triazole (D-SET).