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Original Article

A novel histone deacetylase inhibitor TMU-35435 enhances etoposide cytotoxicity through the proteasomal degradation of DNA-PKcs in triple-negative breast cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) treatment offers only limited benefits, and it is very relevant given the significant number of deaths that it causes. DNA repair pathways can enable tumor cells to survive DNA damage that is induced by chemotherapeutic or radiation treatments. Histone deacetylase inhibitors (HDACi) inhibited DNA repair proteins. However, the detailed mechanisms for this inhibition remain unclear. In the present study, we investigated whether a newly developed HDACi, TMU-35435, could enhance etoposide cytotoxicity by inhibiting DNA repair proteins in triple-negative breast cancer. We found synergistic cytotoxicity following treatment of 4T1 cells with etoposide and TMU-35435. Furthermore, TMU-35435 enhances etoposide-induced DNA damage by inhibiting the DNA repair pathway (non-homologous end joining, NHEJ). TMU-35435 suppresses the NHEJ pathway through the ubiquitination of DNA-dependent protein kinase catalytic subunit (DNA-PKcs). In addition, TMU-35435 ubiquitinated DNA-PKcs by inducing the interaction between RNF144A (an E3 ligase) and DNA-PKcs. The combined treatment induced apoptosis and autophagic cell death in 4T1 cells. In an orthotopic breast cancer model, combined treatment with TMU-35435 and etoposide showed anti-tumor growth through the increase of DNA damage and cell death. Taken together, our data suggest that TMU-35435 enhances etoposide cytotoxicity by regulating ubiquitin–proteasome system and inhibiting the DNA repair pathway in TNBC.

Introduction

Breast cancer affects over one million patients every year. A subgroup of patients has what is called “triple-negative breast cancer (TNBC)” which is defined by the absence of receptors for estrogen, progesterone and human epithelial receptor 2 (HER2) [1]. TNBC exhibits characteristics distinct from other breast cancers in that it is particularly aggressive and frequently recurs, becoming metastatic. TNBC is associated with very poor prognosis and limited

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treatment options are available [2]. Therefore, this situation signifies an urgent need for novel therapeutic approaches. Recently, several clinical strategies were developed, including modified chemotherapy approaches targeting the DNA damage response, angiogenesis inhibitors, immune checkpoint inhibitors, or anti-androgens in phase 1–3 clinical studies of TNBC [3]. Although DNA damage reagents may cause DNA double-strand breaks (DSBs) and kill cancer cells, they induce DNA repair pathways. Cells have developed mechanisms to repair such DSBs through two major pathways: non-homologous end joining (NHEJ) and homologous recombination (HR) [4]. NHEJ is the prevalent DSB repair pathway and can process the direct ligation of broken DNA ends throughout the whole cell cycle. This process can be regulated by assembly and disassembly of DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and Ku70/Ku80 heterodimer on DSB sites [5]. DNA-PKcs may be to phosphorylate protein kinase B (also known as Akt) at Ser-473 and lead to NF-κB activation to cause proinflammatory response [6,7]. DNA repair pathways can enable tumor cells to survive DNA damage that is induced by chemotherapeutic treatments. Therefore, inhibitors of specific DNA repair pathways might prove efficacious when they are used in combination with DNA-damaging chemotherapeutic drugs. This approach also has the potential to be selective for tumor cells and have fewer side effects [8]. Acetylation plays an important role in modulating the expression of genes that act on cell cycle control and contribute to the development and progression of tumors [9]. Histone acetylation is performed by enzymes called histone acetyltransferases (HATs), which add acetyl radicals to the lysine residues of histone proteins. In contrast, histone deacetylases (HDACs) act by removing acetyl radicals and by recruiting corepressor complexes. More recently, data accumulated by our group and others have revealed that HDAC inhibitors (HDACi) inhibited DNA repair proteins [10,11]. However, the mechanism by which HDACi inhibit DNA repair proteins is unclear.

Accumulating evidence suggests that autophagy can be activated by DNA damage [12]. Autophagy is a catabolic process in which long-lived proteins and organelles are sequestered for lysosomal degradation by means of double-membrane-bound cytosolic vesicles, termed autophagosomes [13,14]. Previous research has shown that defects or deregulation of this process were observed in various human diseases, such as neurodegenerative, cardiovascular and immune disorders, infections, diabetes, and cancer [15]. Recent studies seeking pharmacological approaches to enhance or inhibit autophagy have yielded promising results in animal models of human diseases. Not surprisingly, autophagy has a dual role in cancer therapy [16,17]. Malignant cells can harness autophagy to survive in adverse microenvironmental conditions and to resist therapeutic challenges [18]. However, one theory suggests that the proper amount of autophagy promotes cell survival and that a high level of autophagy results in autophagic cell death [19]. Many anti-cancer agents induce autophagy to restrain proliferation in various cancers. Therefore, inducting autophagic cell death may serve as a novel therapeutic tool to kill cancer cells [20].

The ubiquitin–proteasome system (UPS) is a large set of machinery consisting of numerous components that selectively degrade damaged or abnormal proteins through ubiquitination. Ubiquitination occurs through a three-step process involving ubiquitin (Ub)-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes [21]. E3 in the UPS are considered to be primarily responsible for the specific recognition of a large number of target proteins, acting as the substrate recognition component of the UPS pathway. This specificity is crucial when considering the prospect of designing drugs for the entire ubiquitination pathway because such drugs can be designed to target specific substrates of the E3 ligase without affecting other substrates [22,23]. Here, we used a newly developed HDACi, TMU-35435 (N-Hydroxy-6-(5-methyl-4-acridinecarbamoyl) hexanamide), which was been optimized for HDAC inhibition using structure-based analyses [24]. Etoposide is one of the well-known chemotherapeutic drugs that inhibits topoisomerase II activity, thereby leading to DSBs and cytotoxicity [25,26]. We demonstrate the anti-cancer effects of etoposide combined with this novel HDACi (TMU-35435) in TNBC cells *in vitro* and in an orthotopic mouse model. TMU-35435-inhibited DNA repair pathways enhanced DNA damage when used in combination with etoposide. Moreover, TMU-35435 inhibited DNA repair proteins via UPS. Finally, we examined the possible role of autophagy in combined treatment-induced cell death.

Materials and methods

Preparation of TMU-35435

The complete chemical name of TMU-35435 is N-Hydroxy-6-(5-methyl-4-acridinecarbamoyl) hexanamide. Requests for this compound should be sent to wjhuang@tmu.edu.tw.

Cell culture

The murine breast cancer cell line 4T1 (ATCC CRL-2539) and human breast cancer cell line MDA-MB-231 (ATCC HTB-26) were obtained from the American Type Culture Collection (ATCC). The luciferase-expressing murine breast cancer cell line 4T1-Luc was obtained from Dr. M.L. Kuo (Institute of Toxicology, National Taiwan University, Taipei, Taiwan) [27]. The cells were cultured in Dulbecco's modified essential medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with an antibiotic mix containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Gibco BRL, Grand Island, NY, USA) and 10% fetal bovine serum (HyClone, South Logan, UT, USA). The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Exponentially growing cells were detached using 0.05% trypsin–EDTA (Gibco BRL, Grand Island, NY) in DMEM.

Cell viability assay

The MTT assay [(1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan)] (Sigma–Aldrich Co. LLC, MO, USA) was used to determine cell viability. 4T1 cells were seeded into 96-well plates, grown overnight, and treated with drugs. Next, cells were incubated with an MTT solution (100 μl/well) for 1 h in a humidified atmosphere containing 5% CO_2 at 37 °C. After incubation, the MTT solution was removed and DMSO solution (100 μl/well) was added for 30 min. Cell viability was measured using a microplate spectrophotometer system (Molecular Devices Inc., CA, USA) at 570 nm.

Drug interaction analysis

The effect of drug combination was evaluated by the combination index (CI) method using the CalcuSyn software (Biosoft), which is based on the median effect model of Chou and Talalay [28]. The cells were exposed to etoposide at concentrations ranging from 0.125 to 1 μM and to TMU-35435 at concentrations ranging from 0.4 to 2.0 μM. Then, a cell viability assay was performed as described above. Experimental data were entered into the CalcuSyn interface and used to calculate the CI values. $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additive effect, and antagonism, respectively.

Comet assay

To detect DNA damage in individual cells, we adopted a comet assay. Briefly, cells were collected after the treatments and resuspended in 0.2 ml of PBS containing 0.5% low-melting-point agarose. Eighty-five microliters of the mixture was applied to the slides, which were then submerged in cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100 (pH 10)). Electrophoresis was performed at 300 mA and 25 V for 20 min. After electrophoresis, the slides were neutralized with 0.4 M cold Tris–HCl buffer (pH 7.5) and then stained using ethidium bromide. Comets were visualized using a fluorescence microscope (Olympus, Japan). DNA damage was assessed in 100 cells, where the tail moment (tail length multiplied by the fraction of DNA in the tail) was quantified using Image-Pro Plus (Media Cybernetics Inc.).

Determination of early apoptosis

Apoptosis was assessed by quantifying the translocation of phosphatidylserine to the cell surface, detected with an Annexin V apoptosis detection kit (Calbiochem, CA, USA) as described previously [29].