Bioorganic & Medicinal Chemistry Letters 28 (2018) 420–424

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovering alkylamide derivatives of bexarotene as new therapeutic agents against triple-negative breast cancer

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ARTICLE INFO

Article history:
Received 25 August 2017
Revised 25 November 2017
Accepted 13 December 2017
Available online 16 December 2017

Keywords:
Triple-negative breast cancer
Apoptosis
Cancer stem cell
Epithelial-mesenchymal transition
Anti-cancer therapeutics

ABSTRACT

Triple-negative breast cancer (TNBC) has been reported to be correlated with high expression of proliferation markers as well as constitutive activation of metastasis-relevant signaling pathways. For many years, breast cancer researchers have been investigating specific and effective methods to treat or to control the development of TNBC, but promising therapeutic options remain elusive. In this study, we have demonstrated that alkylamide derivatives of bexarotene DK-1–150 and DK-1–166 induce apoptotic cell death in TNBC cell lines without causing cytotoxicity in the normal mammary epithelial cell line. Furthermore, the bexarotene derivatives also showed significant effects in inhibiting TNBC cell proliferation and migration, modulating cancer stem cell markers expressions, as well as limiting the epithelial-mesenchymal transition (EMT) activities of TNBC cell lines in terms of downregulating EMT marker and blocking nuclear translocation of β-catenin. Therefore, we propose the alkylamide derivatives of bexarotene as potential candidates for novel anticancer therapeutics against TNBC.

Triple-negative breast cancer (TNBC), characterized by mammary tumors that lack detectable expressions of estrogen receptor (ER), progesterone receptor (PR) and human epithermal growth factor receptor 2 (HER2),[1] contributes to 15–20% of all breast cancer cases diagnosed.[2,3] This breast cancer subtype is associated with high expression of proliferation markers such as Ki-67,[4] and activation of the β-catenin pathway.[5] TNBC is further classified into 6 subtypes based on the gene expression profiles, namely basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) subtype. Among these TNBC subgroups, mesenchymal (M) and mesenchymal stem-like (MSL) subtypes are enriched in genes associated with the epithelial-mesenchymal transition (EMT) and growth factor signaling pathways.[6] In contrast to other subtypes of breast cancer, TNBC is more aggressive and invasive, more resistant to chemotherapies, and also possesses elevated EMT activity and a higher percentage of cancer stem cell (CSC) population. Such properties of TNBC tumors give rise to resistance to conventional anti-cancer therapies, metastasis and tumor relapse.

Although a variety of single agents and combination regimens are available for breast cancer prevention and/or treatment, none of them are recommended specifically for TNBC.[7–9] Without effective treatments, most TNBC patients with advanced diseases experienced relapse shortly after neoadjuvant chemotherapy, frequently with visceral metastases and a short life expectancy.[10] Particularly, advanced TNBC has a higher rate of early recurrence and distant metastasis to the brain and lungs, as compared to other breast cancer subtypes.[11–13] Thus, identifying effective, tailored and less toxic therapeutic options for TNBC is a pressing need. In this study, we are demonstrating the alkylamide derivatives of bexarotene as a new class of therapeutic agents targeting TNBC, imposing anti-cancer activities through induction of apoptotic cell death, suppression of cell proliferation and migration, as well as limiting EMT and CSC properties of TNBC cells.

The bexarotene alkylamide derivatives (Fig. 1A), DK-1–150 and DK-1–166 were synthesized by reacting acyl chloride of bexarotene with 1-(2-aminoethyl)pyrrolidine or *N*,*N*-diethylethylenediamine, respectively. Cellular toxicity of the alkylamide derivatives of bexarotene was assessed in two of the invasive TNBC cell lines, MDA-MB-231 (MSL subtype) and BT549 (M subtype), via the evaluation of post-treatment cell viabilities (Fig. 1B). In addition to the TNBC cell lines, we also used normal mammary epithelial cell line MCF10A and non-TNBC breast cancer cell MCF-7, in order to examine if the cytotoxic effects induced by bexarotene derivatives are selectively high in TNBC cells. MTT cell viability assay results showed that 5 μM of bexarotene failed to cause significant reduction in cell viabilities in MCF10A, and in the three breast cancer cell lines. However, 5 μM of DK-1–150 or DK-1–166 was

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https://doi.org/10.1016/j.bmcl.2017.12.033
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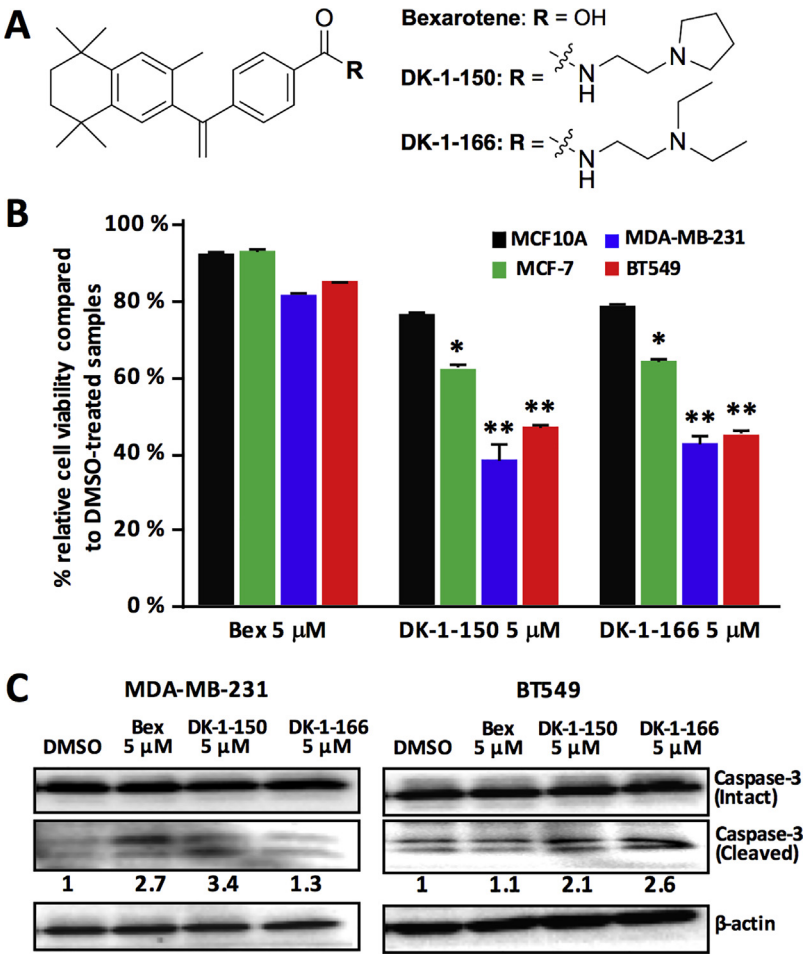

Fig. 1. Bexarotene derivatives display cytotoxicity against TNBC cell lines. A) Chemical structures of bexarotene and its alkylamide derivatives, DK-1–150 and DK-1–166. B) Cytotoxicity evaluation by MTT assay comparing post-treatment cell viabilities among MCF10A and breast cancer cell lines. ($^{*}p < .05$, $^{**}p < .005$). C) Activation of caspase-3 of TNBC cell lines by the bexarotene derivatives. β-Actin was used as endogenous loading control. Densitometry measurement for quantification was performed, and the results were normalized to DMSO-treated samples. Numbers stated underneath indicate expression fold-change values from ImageJ analysis. See Supplementary Fig. 2 for the activation of caspases-8 and -9.

able to induce about 40% reduction in MCF-7 cell viability, and about 60% reduction in cell viability of MDA-MB-231 and BT549 cells. The EC_{50} of bexarotene on TNBC cells is estimated to be higher than 56 μM while the EC_{50} of DK-1–150 and DK-1–166 on TNBC cells are less than 10 μM (Fig. 1B; Supplementary Fig. 1). Importantly, both derivatives showed minimal cytotoxicity on MCF10A. These results suggest the two bexarotene derivatives are more cytotoxic than bexarotene to breast cancer cells, but may not be harmful to the normal breast epithelial cells.

To examine if the cytotoxicity of the derivatives is due to induction of apoptosis, the activation of caspases in TNBC cells was evaluated. Bexarotene and the two derivatives significantly induced activation of caspase-8, caspase-9, and caspase-3 in TNBC cell lines by at least 2 folds (Fig. 1C; Supplementary Fig. 2), suggesting that the derivatives are able to initiate both intrinsic and extrinsic apoptotic cascades, as well as induce both early and late apoptosis in TNBC cells. In contrast, no significant activation of caspases was observed in MCF10A and MCF-7 cell lines, upon treatments with bexarotene or either of the derivatives (Supplementary Fig. 2). Interestingly, BT549 cell line responds better to DK-1–150 or DK-1–166 than to bexarotene of the same dosage (Fig. 1C; Supplementary Fig. 2). Furthermore, both MDA-MB-231 and BT549 cells treated with 5 μM DK-1–150 showed increased levels of cleaved PARP (Fig. 4B), suggesting induction of DNA damage by the derivative through apoptosis mechanism.

To assess the effect of the bexarotene derivatives on breast cancer cell proliferation, we performed adherent colony-forming assay and found that both DK-1–150 and DK-1–166 led to 80% reduction of colony formation in MCF-7 cell line at 5 μM, while bexarotene did not affect the colony-forming capacity of MCF-7 (Fig. 2A). To further evaluate if the derivatives can inhibit anchorage-independent proliferation, we conducted the soft-agar colony-forming assay. Similar to the adherent colony-forming assay, soft-agar colony-forming assay results reflected that both bexarotene derivatives at 2 μM inhibited the formation of colonies by at least 90%, while 10 μM bexarotene failed to inhibit colony formation in MDA-MB-231 cells (Fig. 2B; Supplementary Fig. 3). TNBC is well known for its high metastatic potential. To assess the *in vitro* anti-metastatic activity of bexarotene derivatives on TNBC cell lines, we performed wound-healing assay on MDA-MB-231 cells. While DMSO- or bexarotene (5 μM)-treated MDA-MB-231 cells were able to migrate, and eventually close the "wound gap" after 24 h incubation, cells treated with 2 μM DK-1–150 or 2 μM DK-1–166 had significantly lowered capability of cell migration (Fig. 2C; Supplementary Fig. 4). Collectively, these data prove that the bexarotene derivatives are able to suppress TNBC cell proliferation and migration.

It has been proposed that CSCs, while existing as a minor population, exert resistance to the contemporary anti-cancer therapies, and hence become a potential root of tumor recurrence or