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Efficacy of hybrid vitamin D receptor agonist/histone deacetylase inhibitors in vitamin D-resistant triple-negative 4T1 breast cancer

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ABSTRACT

Hormonal 1,25-dihydroxyvitamin D (1,25D) and its analogues have shown efficacy in some preclinical models of cancer. However, many models are resistant to the antiproliferative effects of 1,25D or its analogues *in vitro* or *in vivo,* and such compounds have failed in the clinic as monotherapies because of tumor resistance. Given the observed synergism between 1,25D analogues and histone deacetylase inhibitors (HDACi) in 1,25D-resistant cells, we previously developed a series of hybrid secosteroidal and easily assembled non-secosteroidal analogues that combined agonism for the vitamin D receptor and HDACi in a single backbone. These compounds displayed enhanced efficacy against 1,25D-resistant malignant cells *in vitro*. Structure/function studies led to synthesis of several non-secosteroidal variants in which HDACi potency was optimized without substantially sacrificing VDR agonism. Here, we present the first studies of efficacy *in vivo* of two of these compounds, DK-366 and DK-406, in the aggressive mouse 4T1 model of triple-negative breast cancer, a form of the disease for which treatment options are limited. 4T1 cells are resistant *in vitro* to the cytostatic and cytotoxic effects of 1,25D and the potent HDACi SAHA individually up to concentrations of 1 μM and 50 μM, respectively, whereas combinations of the two are efficacious. *In vitro*, DK-366 or −406 induced dose-dependent arrest of cell proliferation and cytotoxicity at 10–20 μM. *In vivo,* the maximum tolerated dose (MTD) of DK-366 and DK-406 were 2.5 and 5.0 mg/kg, respectively. Although the compounds induced hypercalcemia at elevated doses, consistent with VDR agonism *in vivo*, they both reduced tumor burden at doses below their MTD's. Moreover, in a separate experiment, DK-406 at 5 mg/kg reduced 4T1 lung metastases by at least 50%. Under the same conditions, 1,25D (0.25 μg/kg) and SAHA (25 mg/kg) combined had no effect on tumor burden or on lung metastases. These experiments show that hybrid compounds are bioavailable and efficacious against a particularly aggressive model of metastatic breast cancer, providing strong support for the therapeutic potential of the hybrid concept.

1. Introduction

Vitamin D_3 (cholecalciferol) was initially identified for its role in preventing nutritional rickets in children [1], and is a key regulator of calcium homeostasis and bone biology. It undergoes successive hydroxylations at the 25 and 1α positions to produce the bioactive metabolite 1α,25-dihydroxyvitamin D_3 (1,25D (**1**), Fig. 1), which is an agonist of the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand-regulated transcription factors [2,3]. The VDR is expressed in numerous tissues and genomics studies have identified VDR binding sites in the regulatory regions of genes responsible for cell cycle regulation and differentiation as well as production of antimicrobial peptides [2,4,5]. Consistent with these findings, 1,25D and its analogues have shown potential for treatment of proliferative disorders, such as cancer and psoriasis, as well as enhancing innate immunity [6].

While 1,25D and its analogues are efficacious antiproliferative agents in some cancer models, they have failed in the clinic because of tumor resistance. However, several studies have shown that the combination of 1,25D and histone deacetylase inhibitors (HDACi) is synergistic in cancer models, notably including those which are resistant to 1,25D alone [7–11]. Others have shown that VDR signaling and the HDACi butyrate can cooperate to induce colon cancer cell

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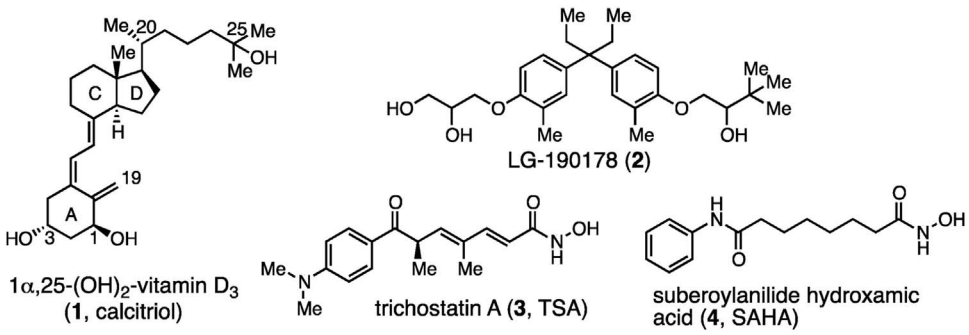

Fig. 1. Structures of VDR agonists and HDAC inhibitors. Structures of 1,25-dihydroxyvitamin D_3, VDR agonist LG-190178 and HDAC inhibitors trichostatin A and SAHA.

differentiation [12]. HDACs, along with histone acetyl transferases (HATs), control the acetylation state of histones as well as transcription factors and cofactors, and can thus regulate gene transcription. In addition, HDACs and HATs regulate acetylation of other non-histone proteins such as tubulin and HSP90 [13]. HDAC inhibitors (HDACi) are known to block cell cycle progression and induce apoptosis or differentiation depending on the cell type and thus have been developed as anticancer agents [14–16,17]. The prototypical HDACi is trichostatin A (TSA, **3**), a natural product originally isolated for its antifungal activity but later found to have potent antiproliferative activity in vitro. Subsequently, several HDACi have been approved for clinical use including suberoylanilide hydroxamic acid (SAHA, vorinostat, **4**) and romidepsin for cutaneous T-cell lymphoma, belinostat and romidepsin for peripheral T-cell lymphoma, and panobinostat for the treatment of multiple myeloma. Other HDACi's such as entinostat (MS-275) are in clinical trials [15,18].

We have previously shown that the combination of 1,25D and TSA was highly cytostatic and cytotoxic in 1,25D-resistant SCC4 cells, inducing cell death by autophagy [19]. Moreover, the combination induced significant morphological changes not observed with monotherapy, including polynucleated cells and intercellular tubulin bridges consistent with mitotic catastrophe. Based on the synergy between 1,25D and TSA, we developed bifunctional hybrid molecules which act simultaneously as VDR agonists and HDACi [19–23]. Hybrid or polyfunctional drugs are single molecules that are designed to bind to multiple biochemical targets [24,25]. Combination therapy achieved with a single drug may have significant advantages over multiple agents, as it can simplify analysis of pharmacokinetic profiles and, above all, optimization of dose/toxicity relationships. In addition, hybrid molecules have the potential to localize activity against one target based on affinity for a second target [26–29]. Moreover, a single hybrid drug may improve compliance relative to more complex treatment regimens inherent in combination therapy.

The first of these hybrids, triciferol (**5**, Fig. 2), combined the secosteroid backbone of 1,25D with a side-chain consisting of the dienylhydroxamic acid found in TSA [19]. Triciferol possessed enhanced cytostatic and cytotoxic activity compared to both 1,25D and the combination of 1,25D and TSA in several cell models. Although triciferol and its analogues were efficacious, their synthesis was lengthy, requiring 25–30 steps from vitamin D_2. Thus we subsequently developed hybrids based on the framework of non-secosteroidal analog LG-190178 (**2**, Fig. 1) [22,23]. Non-secosteroidal hybrids were designed by replacing the diol in **2** that mimics the A-ring of 1,25D with a hydroxamic acid. The first of these hybrids, JF-B01 (**6**, Fig. 2) possessed nanomolar agonist activity for the VDR but only mid-micromolar activity towards HDAC. Virtual docking suggested that HDACi potency was attenuated due to the aromatic ring proximal to the hydroxamic acid being forced into the narrow HDAC access channel. In a follow-up study, HDACi potency of the non-secosteroidal hybrids was improved by removing the problematic *ortho*-methyl group or by lengthening the side-chain such that the aromatic would bind at the HDAC surface [23]. Two hybrids of interest resulted from this study, DK-366 and DK-406. The former is a simple des-methyl analog of JF-B01. It maintains good agonist activity towards the VDR and possesses low μM HDACi potency. The latter, DK-406, possessed slightly attenuated VDR agonist activity compared to DK-366 while possessing further improved HDACi potency. In this paper, we describe the *in vitro* and *in vivo* properties of these hybrids towards a 1,25D-resistant triple negative breast cancer cell line.

2. Materials and methods

2.1. Cell culture

Mouse 4T1 cells were split at 60–70% confluence. For treatments, cells were split and 24 h later medium was changed to DMEM + 10% charcoal-stripped FBS. 24 h after that media was changed and cells were incubated in DMEM-F12 + 10% charcoal-stripped FBS and 1,25D (Sigma), SAHA or hybrid compounds, as indicated in the figures.

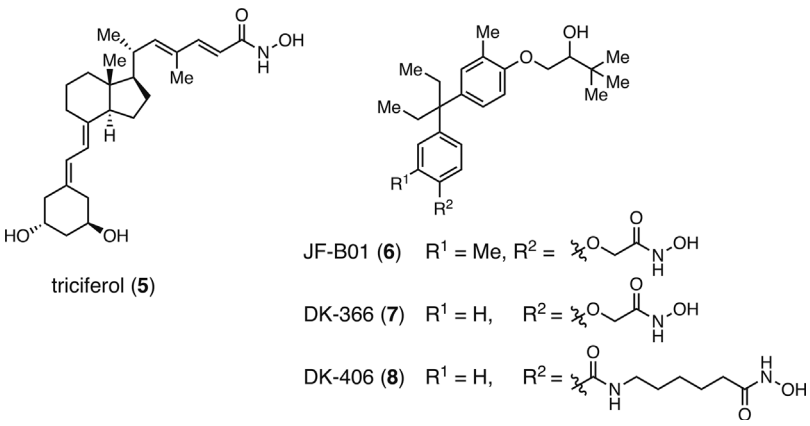

Fig. 2. Structures of VDR agonist/HDACi hybrids. Triciferol combines structural features of 1,25D and TSA while JF-B01, DK-366 and DK-406 combine features of LG-190178 and SAHA.