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4-(1-Ethyl-4-anisyl-imidazol-5-yl)-*N*-hydroxycinnamide – A new pleiotropic HDAC inhibitor targeting cancer cell signalling and cytoskeletal organisation

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ABSTRACT

Histone deacetylases (HDAC) which play a crucial role in cancer cell proliferation are promising drug targets. However, HDAC inhibitors (HDACi) modelled on natural hydroxamic acids such as trichostatin A frequently lead to resistance or even an increased agressiveness of tumours. As a workaround we developed 4-(1-ethyl-4-anisyl-imidazol-5-yl)-*N*-hydroxycinnamide (etacrox), a hydroxamic acid that combines HDAC inhibition with synergistic effects of the 4,5-diarylimidazole residue. Etacrox proved highly cytotoxic against a panel of metastatic and resistant cancer cell lines while showing greater specificity for cancer over non-malignant cells when compared to the approved HDACi vorinostat. Like the latter, etacrox and the closely related imidazoles bimacroxam and animacroxam acted as pan-HDACi yet showed some specificity for HDAC6. Akt signalling and interference with nuclear beta-catenin localisation were elicited by etacrox at lower concentrations when compared to vorinostat. Moreover, etacrox disrupted the microtubule and focal adhesion dynamics of cancer cells and inhibited the proteolytic activity of prometastatic and proangiogenic matrix metalloproteinases. As a consequence, etacrox acted strongly antimigratory and antiinvasive against various cancer cell lines in three-dimensional transwell invasion assays and also antiangiogenic *in vivo* with respect to blood vessel formation in the chorioallantoic membrane assay. These pleiotropic effects and its water-solubility and tolerance by mice render etacrox a promising new HDACi candidate.

1. Introduction

The development of small-molecule inhibitors of human histone deacetylases (HDAC) is a promising approach to the treatment of cancer [1–6]. The enzyme family of HDAC contributes to post-translational protein modifications by catalysing the deacetylation of lysine residues in their target proteins [7,8]. These include not only DNA-associated histones but also a great number of non-histone proteins such as transcription factors and regulators, signal transduction mediators, as well as chaperone and structural proteins [9–11]. HDAC belonging to class I (HDAC1, 2, 3 and 8), class IIa (HDAC4, 5, 7, 9), and class IIb (HDAC6 and 10) [2,12] share a zinc(II) cation in the centre of their catalytic cavity, yet differ in their cellular function, (tissue) localisation and protein substrates [2,10]. For instance, HDAC1 and 2 are nuclear enzymes that catalyse the deacetylation of histones in nucleosome complexes mediating silencing of target genes [13–15]. In contrast, HDAC6 is a cytoplasmic microtubule-associated deacetylase of alpha-tubulin and a moderator of microtubule dynamics and vesicle transport along microtubules [16–20]. Other non-histone substrates are the epidermal growth factor receptor (EGFR), [20,21] the transcription factor beta-catenin, [22] STAT1/3 (signal transducers and activators of transcription), [23] and angiogenesis-relevant proteins such as the vascular endothelial growth factor receptor (VEGFR), hypoxia inducible factor-1alpha (HIF-1alpha), and cortactin, a promotor of F-actin rearrangement [24–27].

Inhibitors of zinc-dependant HDAC mimic the natural substrate acetyllysine and act by irreversibly chelating the zinc(II) centre with high-affinity ligands such as hydroxamic acids, benzamides, or carboxylates [2,5]. They are more or less specific for individual HDAC subclasses [28–30]. Fig. 1 shows prominent examples. Vorinostat (a.k.a. SAHA, suberoyl anilide hydroxamic acid) is an FDA-

Abbreviations: HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; Etacrox, 4-(1-ethyl-4-anisyl-imidazol-5-yl)-N-hydroxycinnamide × HCl

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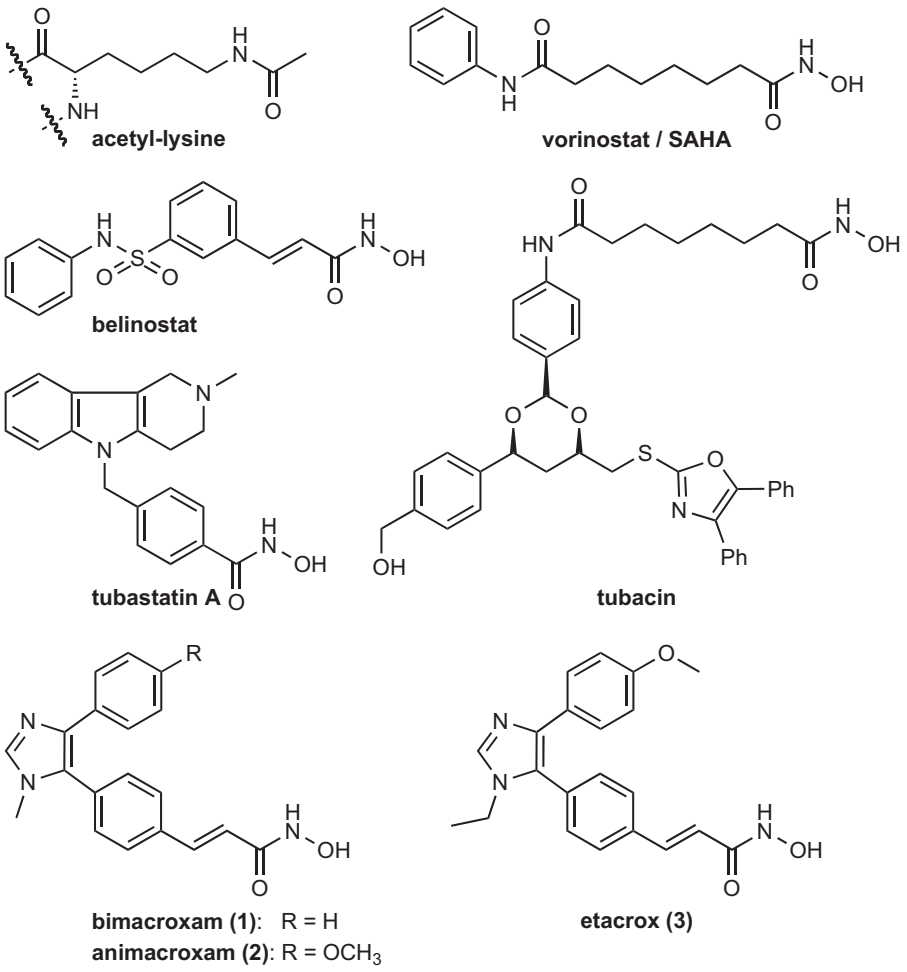

Fig. 1. Chemical structures of HDAC substrate acetyllysine and HDAC inhibitors. The known non-isoform-specific HDACi vorinostat/SAHA (suberoyl anilide hydroxamic acid) and belinostat, natural HDAC6-specific inhibitors tubacin and tubastatin A, and the new HDACi conjugates bimacroxam (**1**), animacroxam (**2**), and etacrox (**3**).

approved pan-HDACi clinically employed against cutaneous T-cell lymphoma [10,31]. Belinostat (PXD101) shows specificity for HDAC classes I and II and has been approved in the USA for the treatment of peripheral T-cell lymphoma [32]. Tubacin is a HDAC6 specific inhibitor currently in advanced clinical trials [17]. Regardless of their specificity most of the clinically used HDACi tend to induce resistance in tumour cells [33,34]. As a potential workaround we recently developed a new HDACi motif that links the common 2nd-generation *para*-cinnamylhydroxamate pharmacophore to 4,5-diphenylimidazoles, derived from the natural vascular-disrupting *cis*-stilbene combretastatin A-4 [35]. Such conjugates showed pronounced pan-HDAC inhibition exceeding that of vorinostat, but they lacked the tubulin affinity typical of the original imidazoles and of combretastatin A-4 [35–37]. Moreover, the 4,5-diaryl imidazole moiety confered an improved water-solubility and chemical stability. In the current publication, we investigate in detail the effects of three new HDACi (**1**–**3**) of this imidazole-cinnamylhydroxamic acid type on cancer cell signalling pathways, in particular those affected by vorinostat, on protein turnover, and on cytoskeletal integrity and dynamics. With the current discussion [38,39] of HDACi as potential inducers of an epithelial-to-mesenchymal transition (EMT) in mind, we also took a closer look at their antimetastatic potential by means of three-dimensional *in vitro* models.

2. Materials and methods

2.1. Materials

Vorinostat and tubastatin A were purchased from LC Laboratories and used without further purification. Stock solutions were prepared in DMSO at a final concentration of 10 mM. The primary antibodies for the detection of acetylated proteins (anti-acetyl-alpha-tubulin (Lys40) rabbit monoclonal antibody (mAb), anti-acetyl-histone H2B (Lys5) rabbit mAb; anti-acetylated lysine rabbit polyclonal antibody (pAb)) as well as the anti-Akt (pan) rabbit mAb, anti-phospho-Akt (Ser473) rabbit mAb were from Cell Signalling Technology. The anti-caspase-9 mouse mAb was bought from Calbiochem, the anti-alpha-tubulin mouse mAb from invitrogen. Focal adhesion staining and detection of beta-catenin was done with anti-paxillin mAb or beta-catenin mAb, both from BD Transduction Laboratories. All secondary antibody–horseradish peroxidase (HRP) conjugates (anti-mouse IgG, HRP-linked antibody; anti-rabbit IgG, HRP-linked antibody) were from Cell Signalling Technology. Secondary antibodies for immunofluorescence microscopy were from Pierce/Thermo Scientific (goat anti-mouse IgG (H+L), cross adsorbed secondary antibody-DyLight 550 conjugate) or invitrogen/life technologies (goat anti-mouse IgG (H+L) sec. antibody–AlexaFluor488 conjugate, goat anti-rabbit IgG secondary antibody–AlexaFluor488 conjugate). The Phalloidin–AlexaFluor594 conjugate for staining filamentous actin was from invitrogen/life.