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New insights into the molecular mechanisms underlying sensitivity/resistance to the atypical retinoid ST1926 in acute myeloid leukaemia cells: The role of histone H2A.Z, cAMP-dependent protein kinase A and the proteasome

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Abstract ST1926 is an atypical retinoid and a promising anti-tumour agent with selective apoptotic activity on the leukaemic blast. The anti-tumour activity of the compound has been associated with its capacity to induce DNA double stranded breaks. Target profiling by affinity chromatography coupled to mass spectrometry led to the identification of histone H2A.Z as a protein capable of binding ST1926 specifically. The result was confirmed by studies involving Surface Plasmon Resonance (SPR). This indicates that H2A.Z is a primary target of ST1926 and links the perturbations of the histone pathway observed by microarray analysis to the DNA damage and apoptotic responses caused by the atypical retinoid. Comparison of the whole-genome gene-expression profiles of the ST1926-sensitive *NB4* and the ST1926-resistant *NB4.437r* cell lines demonstrated differential expression of numerous genes. Network analysis of the data indicated enrichment of the cellular pathways controlling cAMP (cyclic adenosine-monophosphate)-dependent signal transduction, proteasome-dependent protein degradation and nuclear histones in *NB4.437r* cells. Pharmacological inhibition of cAMP-dependent protein kinase A with H89 partially reverted resistance of *NB4.437r* cells to ST1926. Conversely, inhibition of the proteasome with MG132 or bortezomib blocked the apoptotic response afforded by ST1926 in the *NB4* cell line. This last effect was associated with a dramatic reduction in

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the DNA damage caused by the atypical retinoid. The results corroborate the idea that DNA damage is an important determinant of ST1926 apoptotic activity. More importantly, they demonstrate a proactive role of the proteasome in the DNA damaging and ensuing apoptotic response observed upon the challenge of acute myeloid leukaemia cells with ST1926.

1. Introduction

Atypical retinoids[1–3] are structurally related compounds inducing selective apoptosis in solid tumour and acute myeloid leukaemia (AML) cells.[4,5] The prototypes of this class of compounds are CD437[6–8] and ST1926,[9–11] which are the most promising members of the family. ST1926 is a very active anti-tumour agent in multiple *in vivo* models of myeloid leukaemia and solid cancer[1,2,11] (CP, unpublished results) and this has led to the synthesis of various chemically modified analogues which are undergoing pre-clinical development. The biodistribution of ST1926 in the mouse is favourable, as micromolar concentrations of the compound in the plasma and tumour xenografts are easily achievable following both intravenous and oral administration (CP, unpublished results). ST1926 is currently completing phase I clinical trials to define the pharmacokinetics and maximal tolerated doses.

The apoptotic activity of atypical retinoids does not involve the activation of nuclear retinoid receptors (RARs) with consequent effects on the transcription of retinoid target genes.[7,10,12–15] We demonstrated that ST1926 and CD437 induce DNA double-stranded breaks.[13] DNA damage is associated with cytotoxicity, as indicated by the data obtained in the AML model characterised by sensitivity and selective resistance to atypical retinoids represented by the pair of cell lines, *NB4* and *NB4.437r*.[16] The genetic damage caused by ST1926 and CD437 is not due to the covalent binding of these compounds or derived reactive metabolites to DNA.[13] Though largely unknown, the mechanisms underlying DNA damage are likely to be unique, as atypical retinoids do not show cross-resistance with other DNA-damaging agents.[1,16]

Here, we demonstrate that ST1926 binds to the histone variant H2A.Z specifically, suggesting the involvement of the protein in DNA damage and cytotoxicity. In addition, we provide evidence for a role of the cAMP pathway in induced resistance to atypical retinoids. Finally, our data indicate that the proteasome contributes to ST1926-induced DNA damage and apoptosis.

2. Materials and methods

2.1. Chemicals, antibodies, cell lines, colony and alkaline COMET assays

CD437, ST1926, ST2519 and ST2718 (Fig. 1) were synthesised by Sigma-Tau.[17] The following reagents were used throughout the study: anti-H2A.Z (Abcam Cambridge, MA, United States of America [USA]), anti-γH2AX (Upstate Biotechnology, Lake Placid, NY, USA) and anti-βactin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies; all-trans retinoic acid (ATRA), H89 and MG132 (Sigma, St. Louis, MO, USA); bortezomib (LC Laboratories, Woburn, MA, USA). The *NB4* and *NB4.437r* cell lines were cultured in RPMI 1640 containing 10% foetal calf serum.[16] All the experiments were performed on *NB4* and *NB4.437r* cells, which were maintained in culture for no more than 10 passages after thawing of the frozen stocks. *NB4.437r* cells were always passaged in medium without the selection agent CD437. Sensitivity of *NB4* and the resistance of *NB4.437r* cells to CD437 and ST1926 were routinely checked by measuring the number of viable cells after treatment with the two compounds (1 μM) for 18 h.[16] Standard protocols were used for the colony assays in methylcellulose. Cellular (*CCA*) and subcellular (*SCA*) alkaline COMET assays were performed as described.[13]

2.2. Determination of proteins binding to ST1926 and recombinant H2A.Z protein

To identify ST1926-binding proteins, a proteome-wide approach based on ligand-affinity chromatography and mass spectrometry was implemented[18,19] (Supplementary Methods). The human H2A.Z cDNA was inserted into the plasmid vector pGEX-4T-2 (GE Healthcare Life Sciences, Uppsala, Sweden) to express the corresponding protein in *Escherichia coli* upon induction with isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) (Supplementary Methods).

2.3. Whole genome gene-expression microarrays

These experiments were performed as described[20] using RNA extracted from *NB4* and *NB4.437r* cells grown in basal conditions. The data were deposited in the MIAMExpress database (Accession No. E-MEXP-3752).

2.4. Western blots, caspase-3 activation and Surface Plasmon Resonance (SPR)

Western blots and caspase-3 activation assays were performed as reported.[16] SPR experiments were conducted as detailed[21] (Supplementary Methods).