Drug Targeting to Monocytes and Macrophages Using Esterase-Sensitive Chemical Motifs


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ABSTRACT

The therapeutic and toxic effects of drugs are often generated through effects on distinct cell types in the body. Selective delivery of drugs to specific cells or cell lineages would, therefore, have major advantages, in particular, the potential to significantly improve the therapeutic window of an agent. Cells of the monocyte-macrophage lineage represent an important target for many therapeutic agents because of their central involvement in a wide range of diseases including inflammation, cancer, atherosclerosis, and diabetes. We have developed a versatile chemistry platform that is designed to enhance the potency and delivery of small-molecule drugs to intracellular molecular targets. One facet of the technology involves the selective delivery of drugs to cells of the monocyte-macrophage lineage, using the intracellular carboxylesterase, human carboxylesterase-1 (hCE-1), which is expressed predominantly in these cells. Here, we demonstrate selective delivery of many types of intracellularly targeted small molecules to monocytes and macrophages by attaching a small esterase-sensitive chemical motif (ESM) that is selectively hydrolyzed within these cells to a charged, pharmacologically active drug. ESM versions of histone deacetylase (HDAC) inhibitors, for example, are extremely potent anticytokine and antiarthritic agents with a wider therapeutic window than conventional HDAC inhibitors. In human blood, effects on monocytes (hCE-1-positive) are seen at concentrations 1000-fold lower than those that affect other cell types (hCE-1-negative). Chemical conjugates of this type, by limiting effects on other cells, could find widespread applicability in the treatment of human diseases where mono-ocyte-macrophages play a key role in disease pathology.

Introduction

One of the recurring issues with modern, intracellularly acting drugs is that their target enzymes are often part of signaling pathways that are active in most cells in the body. Although pharmacological benefits are often evident with such agents in disease models, their effects on cells unin-volved in the disease process often lead to levels of toxicity in humans that limit their utility to the most severely affected patients. It has long been a “holy grail” of pharmacology to target agents specifically to the cell type responsible for disease development. A range of approaches has previously been investigated in this regard including immunoliposomal systems based on monoclonal antibodies, sugar-dependent systems that take advantage of cell-specific lectins, and receptor-dependent systems that allow the targeting of cells bearing specific integrins by RGD-containing peptides (Pasqualini et al., 1997; Dubowchik and Walker, 1999; Paulos et...
al., 2004; Kontermann, 2006; Aouadi et al., 2009). Although some success has been achieved by these means, the approaches have been limited to parenterally acting drugs, often chemotherapeutic agents that are administered acutely for life-threatening diseases such as cancer. There remains a need for a more robust and general method of selective drug delivery that can be applied to multiple drug types administrable by mouth on a long-term basis. If such an approach were found, it would have the potential to improve the therapeutic window of drugs by limiting their effects to those cells specifically involved in the disease pathology. This could lead to the resurrection of certain drug classes whose pharmacological effects on other cell types have led to toxicity that has precluded drug development.

Human cells express three intracellular carboxylesterases that convert neutral, membrane-permeant esters into charged acid products (Imai, 2006). One of these, human carboxylesterase-1 (hCE-1), has a restricted expression profile in humans (Satoh et al., 1999; Uphoff and Drexler, 2000; Su et al., 2004; Li et al., 2005), with cells of the monocyte/macrophage lineage being the principal source of the enzyme outside the hepatocyte. We postulated that tissue exposure to a drug-ester conjugate that is a specific substrate for hCE-1 might lead to intracellular ester hydrolysis and production of a pharmacologically active acid only within those cell types. Because of its charged nature, the acid would have limited ability to leave the cell and, over time, would be expected to accumulate to high intracellular levels. This would yield benefits in terms of drug potency and longevity of action but, importantly, would also direct the pharmacological effects to cells expressing hCE-1, such as monocytes and macrophages.

Tangential support for the proposed approach comes from our studies with the novel anticancer agent tosedastat (2S-[2R-(S-hydroxy-hydroxycarbamoyl-methyl)-4-methylpentanoyl-aminol-2-phenylethanoic acid cyclopentyl ester (CHR-2797)), which contains an amino acid ester motif that undergoes intracellular hydrolysis to its acid (Krige et al., 2008). Administration of tosedastat to human cells in vitro and in vivo leads to the intracellular build-up of the corresponding acid and improved potency and longevity of action. In the case of tosedastat, however, the amino acid ester moiety is hydrolyzed by all three carboxylesterases and, in addition, is required for high-affinity binding to its pharmacological target. To deliver a wide range of intracellularly acting drugs to hCE-1-expressing cells, an hCE-1-selective motif has to be attached to a drug at a position that does not significantly disrupt the target enzyme-inhibitor interaction. In this article, we describe a robust method of selectively delivering a range of intracellularly acting agents to monocytes and macrophages using an hCE-1-selective amino acid ester motif.

**Materials and Methods**

**Chemistry.** 1H NMR spectra were determined with Bruker (Newark, DE) AV spectrometers at 300 or 400 MHz. Chemical shifts are reported in ppm relative to residual chloroform (7.26 ppm) or dimethyl sulfoxide (2.49 ppm) as internal reference with coupling constants (J) reported in Hz. The peak shapes are reported as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad. Analytical HPLC/MS was performed on an Agilent HP1100 LC system (Agilent Technologies, Santa Clara, CA) using reverse-phase Luna C18 columns (3 μm, 50 × 4.6 mm), gradient 5 to 95% B (A = water/0.1% formic acid; B = acetonitrile/0.1% formic acid) over 2.25 min, flow = 2.25 ml/min. UV spectra were recorded at 220 and 254 nm using a G1315B DAD detector (Agilent Technologies). Mass spectra were obtained over the range m/z 150 to 800 on a LC/MSD SL G1956B detector (Agilent Technologies). Data were integrated and reported using ChemStation and ChemStation Data Browser software (Agilent Technologies). The purity of all the compounds was found to be above 95%. Reverse-phase HPLC purifications were performed on Gilson (Villier Le Bel, France) preparative systems using reverse-phase Axis prep Luna C18 columns (Phenomenex, Torrance, CA) (10 μm, 100 × 21.2 mm), gradient 0 to 100% B (A = water/0.05% trifluoroacetic acid, B = acetonitrile/0.05% trifluoroacetic acid) over 10 min, flow = 25 ml/min, monitored by UV detection at 254 nm. Thin-layer chromatography analysis was performed with Kieselgel 60 F254 (Merck, Darmstadt, Germany) plates and visualized using UV light. Details of the synthesis of parental and esterase-sensitive motif (ESM)-related molecules are in Supplemental Data.

**Animals.** The hCE-1 knock-in transgenic mouse was generated by Genoway (Lyon, France) by targeted insertion of the expression cassette into the expression permissive hprt locus on the X chromosome by homologous recombination. Expression of the hCE-1 transgene was driven by the human CD68 promoter (provided by Dr. David Greaves, Oxford University, Oxford, United Kingdom), which has previously been shown to direct transgene expression in macrophages of transgenic mice (Gough et al., 2001). Details are provided in Supplemental Data.

**Assessment of the Accumulation of Ester-Derived Acid in Intact Human Tumor Cells.** Cells (4 × 10^5/ml) were incubated at 37°C in culture medium containing 6 μM compound. Incubations were terminated by centrifugation (300g; 5 min; 4°C). Supernatants were added to four volumes of HPLC-grade acetonitrile. After decanting the supernatant, the residual cell pellet (1 × 10^5 cells) was extracted into 1 ml of acetonitrile. Samples were analyzed for the ester and acid metabolite at room temperature by LC/MS/MS (Sciex API3000; Sciex, Warrington, UK). Chromatography was based on an AceCN (Hichrom, Theale, UK) (75 × 21 mm) column with a 5 to 95% (v/v) acetonitrile, 0.1% (v/v) formic acid mobile phase.

**Assessment of the Effect of Drugs on LPS-Induced TNFα Production in Human Blood.** Human heparinized blood was incubated with an equal volume of RPMI 1640 medium and then aliquoted into 96-well microtiter plates (100 μl/well). Inhibitors (5–10,000 nM; diluted in RPMI 1640 medium) were added and, after incubation for 2 h at 37°C, TNFα production was stimulated by the addition of LPS (100 ng/ml) for 6 h at 37°C. Plates were then centrifuged (3 min, 800g) and TNFα present in the supernatant measured using a Quantiglo chemiluminescent enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN).

**Assessment of the Effect of Drugs on Cytokine Gene Expression in Human Blood.** Human heparinized blood was diluted with an equal volume of RPMI 1640 medium and aliquoted (0.5 ml) into 12-well dishes. Vehicle/compounds were added (triplicate samples) and incubated for 2 h at 37°C before the addition of LPS (100 ng/ml) for 6 h. Red cells were then lysed and mRNA was stabilized by the addition of PAXgene Blood RNA Reagent (PreAnalytixX, Hombrechtikon, Switzerland). RNA was isolated and purified using half reaction volumes of a PAXgene Blood RNA Kit (PreAnalytixX) DNA was eluted and used for cDNA synthesis using a High Capacity cDNA RVT Kit (Applied Biosystems, Foster City, CA). After dilution in water, cDNA was stored arrayed in 96-well deep well plates (Axygen, Union City, CA), and 10 μl of cDNA was used as a template for quantitative PCR using SYBR Green PCR Master Mix (Applied Biosystems) in a 25-μl reaction using primers (200 nM). Quantitative PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems). Expression levels were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase by the ΔΔCt methodology, and the results were expressed relative to negative controls. The primers used are described in Supplemental Data.
Assessment of the Effect of Drugs on Cell Proliferation. The method used has been described previously (Krige et al., 2008).

Assessment of the Effect of Drugs on LPS-Induced TNFα Production in Human THP-1 Cells. THP-1 cells (4 × 10⁵/well) in 96-well microtiter plates were incubated with inhibitors (5–10,000 nM) for 2 h at 37°C. TNFα production was stimulated by the addition of LPS (1 μg/ml) for 6 h at 37°C. Plates were then centrifuged (3 min, 800g) and TNFα present in the supernatant was measured using a Quantikine chemiluminescent enzyme-linked immunosorbent assay kit (R&D Systems).

Esters as Substrates for Recombinant hCE-1 and hCE-2. Recombinant hCE-1 and hCE-2 (>95% pure by SDS-polyacrylamide gel electrophoresis) were obtained from Dr. P. Sanghani (Indiana University School of Medicine, Indianapolis, IN) and stored at 4°C in 50 mM potassium phosphate buffer, pH 7.4/40 mM potassium chloride. The activities of the hCE-1 and hCE-2 preparations were 5.66 and 97.2 U/mg protein, respectively (1U = μmoles 4-methyl umbelliferyl acetate converted per minute per milligram at 37°C). Hydrolysis assays were conducted in triplicate at 37°C by addition of compound (2.5 μM) to recombinant hCE-1 or hCE-2 (0.0025 U) in potassium phosphate buffer, pH 7.4/40 mM potassium chloride. Samples were taken at various time points and the reaction was stopped by the addition of three volumes of acetonitrile. Samples were then analyzed for parent ester and acid metabolite at room temperature by LC/MS/MS.

Esters as Substrates for Carboxylesterases in Cell Lysates. Cell lysates (10⁶ cells in 35 ml) were prepared by nitrogen cavitation (700 psi; 50 min; 4°C) in 10 mM Tris-HCl, pH 7 buffer containing potassium phosphate buffer, pH 7.5/125 mM NaCl. After incubation for various times at 37°C, the reaction was stopped by the addition of acetonitrile. Samples were then analyzed for parent ester and acid metabolite at room temperature by LC/MS/MS.

Table 1: Hydrolysis of amino acid ester derivatives by cell lysates derived from U-937 cells (hCE-1-ve) and HCT-116 cells (hCE-1-ve)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Rate of Ester Hydrolysis by Cell Lysates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>U-937 Cells (hCE-1-ve)</td>
</tr>
<tr>
<td>CHR-2935</td>
<td><img src="image" alt="CHR-2935" /></td>
<td>2187 ± 75</td>
</tr>
<tr>
<td>CHR-2946</td>
<td><img src="image" alt="CHR-2946" /></td>
<td>1012 ± 12</td>
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Assessment of the Effect of Drugs on Anisomycin-Stimulated MAPKAPK-2 Phosphorylation in Intact U-937 and HuT-78 Cells. U-937 and HuT-78 cells, grown in RPMI 1640 medium, with 10% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin were treated with vehicle/compound for 4 h before adding anisomycin (10 μM, 30 min) or vehicle. Cells were harvested on ice, washed twice, and lysed in SDS lysis buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, and 50 mM dithiothreitol supplemented with protease and phosphatase inhibitors according to the manufacturer's instructions; Novex/Invitrogen, Carlsbad, CA). After sonication and centrifugation to remove cell debris, samples were run on 10% BT MOPS gels (Novex/Invitrogen). Phosphorylation of MAPKAPK-2 was measured from the resulting nitrocellulose membranes following the manufacturer's instructions for the specific primary antibody (Cell Signaling Technology, Danvers, MA). Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. Visualization of the signal was carried out using enhanced chemiluminescence reagent and film (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Enzyme Assays. The ability of the various parental or ESM-based drugs to inhibit enzyme [histone deacetylase (HDAC), HSP-90, IκB kinase 2, and p38 MAP kinase α] activity was measured as described in Supplemental Data.

Mouse Anticollagen Antibody Arthritis Model. An anticollagen antibody-induced arthritis model was conducted in littermate wild-type (w/t) or hCE-1 transgenic (t/g) mice. Details of the methodology and scoring system are described in Supplemental Data.

Results

Identifying an hCE-1-Selective Chemical Motif. Initial studies focused on the identification of an amino acid ester motif that was selectively hydrolyzed by hCE-1. Synthesis of a range of compounds in which a phenylalanine cyclopentyl ester was attached through a variety of linkers to a phenyl substituent led to the discovery that an amine linkage, rather than the amide linkage found in tosedostat,
led to ester hydrolysis in lysates of hCE-1-expressing, but not non-hCE-1-expressing, cells (Table 1).

**ESM-Based HDAC Inhibitors.** Next, a range of analogs of the HDAC inhibitor vorinostat (SAHA) (Richon et al., 1998) were prepared in which the phenyl ring was substituted with different amino acid esters through amine linkages. As exemplified by CHR-3111 and CHR-2875 (Table 2), ring substitution, remote from the critical hydroxamic acid binding site (Richon et al., 1998; Weinmann and Ottow, 2004), had little impact on HDAC inhibitory activity. For hCE-1<sup>−/−</sup> cell lines (KG-1, HuT-78, and MOLT-4), antiproliferative potency of the HDAC inhibitors was similar to SAHA (Table 2), but in hCE-1<sup>+/+</sup> myelomonocytic tumor cell lines (THP-1, MV4–11 and U-937 cells) (see Supplemental Table S1), however, there was a 10- to 30-fold increase in antiproliferative potency (Table 2). In an isolated enzyme assay, CHR-2875 and CHR-3111 were hydrolyzed by hCE-1 but only to a very limited extent by the ubiquitously expressed hCE-2 (Table 3). Moreover, lysates of U-937 cells, but not of non-hCE-1-expressing cells such as HuT-78 cells, also selectively hydrolyzed CHR-2875 and CHR-3111 and, when intact cells were exposed to the agents, measureable acid derived from these esters could be detected only in intact U-937 cells (Table 3). The rate of hydrolysis of CHR-3111 by different leukemic cell lysates correlated well with the extent of expression of hCE-1 in these cell lines (Supplemental Table S1). When the cyclopentyl ester found in CHR-3111 and CHR-2875 was changed to the less hydrolysable <i>t</i>-butyl ester (CHR-2908 and CHR-6511) (Table 2) neither hCE-1 nor lysates from cells expressing it were able to produce significant amounts of the acid product (Table 3). As expected, the selective antiproliferative effect on hCE-1-expressing cell lines was lost in cells treated with the <i>t</i>-butyl ester analogs (Table 2), demonstrating that significant ester hydrolysis was man-aged.

**Table 2**

The effect of parental and ESM-modified histone deacetylase inhibitors on cell proliferation in leukemic cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>HDAC Enzyme Assay (IC&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Inhibition of Cell Proliferation (IC&lt;sub&gt;50&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>U-937</td>
</tr>
<tr>
<td>CHR-2875</td>
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<td>53</td>
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<tr>
<td>CHR-6511</td>
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<td></td>
<td>73</td>
</tr>
<tr>
<td>SAHA</td>
<td>H</td>
<td></td>
<td>78</td>
</tr>
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<sup>a</sup> Values shown are means of at least three estimations of HDAC potency using HeLa cell nuclear extract as the source of HDAC activity.

<sup>b</sup> Values shown are means derived from experiments conducted over 72 h using [<sup>3</sup>H]thymidine incorporation in the final 3 h as the proliferation readout. Concentration-response curves were created in sextuplicate using eight concentrations of drug and repeated at least twice.
ESM-Based p38 MAP Kinase Inhibitors. When added to intact cells, the ESM-based p38 MAP kinase inhibitor CHR-5623 (Table 4), for example, led to accumulation of its corresponding acid, $N$-(2-{4-[6-amino-5-(2,4-difluorobenzoyl)-2-oxopyridin-1(2H)-yl]-3,5-difluorophenyl}ethyl)-L-leucine (CHR-5668), in U-937 cells (hCE-1 positive) but not in HuT-78 cells (hCE-1 negative) (Table 5). Regarding this selective accumulation, the addition of CHR-5623 to these two cell types led to very potent inhibition of anisomycin-stimulated MAPKAPK-2 phosphorylation (a downstream target of p38 MAP kinase) (Winzen et al., 1999) only in U-937 cells (Table 5). The parent p38 MAP kinase inhibitor, CHR-3464 (Table 4), failed to elicit such cell-selective effects (Table 5).

ESM-Based HSP-90 Inhibitors. Similar cell selectivity data were seen when a targeted HSP-90 inhibitor, CHR-7296 (Supplemental Table S2), was compared with a conventional inhibitor, SNX-2112 (Supplemental Table S2) (Okawa et al., 2009). Both compounds had similar potencies in an LPS-stimulated TNFα production assay carried out in the monocye/macrophage human tumor cell line, THP-1 (the IC50 values for SNX-2112 and CHR-7296 were both approximately 50 nM) or in human blood (the IC50 values for SNX-2112 and CHR-7296 were both approximately 50 nM).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Amino acid ester variants of p38 MAP kinase inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHR-3464</td>
<td><img src="image" alt="Structure" /></td>
<td>2 3 240 10 10</td>
</tr>
<tr>
<td>CHR-5623</td>
<td><img src="image" alt="Structure" /></td>
<td>210 (ester); 1 (acid) 1 58 777</td>
</tr>
<tr>
<td>SNX-2112</td>
<td><img src="image" alt="Structure" /></td>
<td>1.05 0.65 73</td>
</tr>
</tbody>
</table>

Values shown are the means derived from at least four individual experiments. Values shown are the means ± S.D. from one representative experiment, which was repeated three times.

* Values shown are the IC50, 10 min, in the presence or absence of at least six concentrations of p38 MAP kinase inhibitors.

** Values shown are the IC50 values estimated by eye from Western blots of MAPKAPK-2 phosphorylation stimulated in the two cell types by anisomycin (10 μM, 30 min), after pretreatment for 4 h in the presence or absence of at least six concentrations of p38 MAP kinase inhibitors.

Monocyte/Macrophage Targeting of Intracellular Drugs

Effects in Human Blood. In human blood, only monocytes have been shown to express hCE-1 (Su et al., 2004; Liu et al., 2006; see Supplemental Fig. S1). To investigate whether selective delivery to monocytes could be achieved by ESM targeting, samples of human blood were treated with both parental and macrophage-targeted versions of HDAC inhibitors and their impact on protein acetylation was measured directly in FACS-identified blood cells. The data in Fig. 2 demonstrate that the targeted HDAC inhibitor, CHR-2875, but not its poorly hydrolysable analog, CHR-2908, was more potent than SAHA in its ability to enhance protein acetylation in the monocyte cell population in blood and, unlike SAHA, showed selectivity for the monocyte population versus lymphocytes and granulocytes. The selective effects seen with CHR-2875 were seen to a greater extent with an unrelated HDAC inhibitor, CHR-4487 (Supplemental Table S3), which contains a strongly hydrolysable cyclohexylglycine amino acid ester motif (Supplemental Table S3). When incubated with human blood, CHR-4487 was 1000-fold selective for monocytes over either lymphocytes or granulocytes (Fig. 2). The t-butyl ester analog of CHR-4487, CHR-5355, which, like CHR-2908, is poorly hydrolyzed by lysates of hCE-1-positive cells (Supplemental Table S3), did not show any cell-selective effects (Fig. 2). HDAC inhibitors such as SAHA are known to exhibit anticytokine effects in human blood or blood cell studies (Leoni et al., 2002, 2005). The functional impact of monocyte-selective delivery can be clearly seen when the anticytokine effects of HDAC inhibitors were investigated in LPS-stimulated human blood. Agents showing monocyte-selective effects were significantly more potent as inhibitors of TNFα production than parental or unhydrolysable inhibitors: despite being 4-fold weaker than SAHA as an HDAC inhibitor, the IC50 for CHR-4487 as an inhibitor of LPS-induced TNFα production was ~20-fold lower (16 nM versus 290 nM) (Supplemental Table S3).

To study the effects of p38 MAP kinase inhibitors in human blood, it is necessary to activate the pathway and monitor the cell-selective impact of inhibitors on the phosphorylation of MAPKAPK-2, an immediate downstream substrate of p38 MAP kinase (Winzen et al., 1999). LPS activates MAPKAPK-2 phosphorylation in FACS-identified monocytes and granulocytes, but not in lymphocytes. The macrophage-
targeted p38 MAP kinase inhibitor, CHR-5623, was approximately 100-fold selective for monocytes versus granulocytes when inhibition of MAPKAPK-2 phosphorylation was measured after LPS addition (Fig. 3). Its parent, CHR-3464, showed no such selectivity and, despite its 100-fold better activity than CHR-5623 as a p38 MAP kinase inhibitor (Table 5), was 10-fold weaker than the latter as an inhibitor of LPS-stimulated monocyte MAPKAPK-2 phosphorylation (Fig. 3).

Thus, despite the fact that monocytes comprise only 2 to 8% of the leukocytes present in blood, targeted HDAC and p38 MAP kinase inhibitors, but not conventional inhibitors, were able to exert profoundly cell-selective effects on this cell population in human blood.

**Monocyte-Targeted and Parental Inhibitors Show Distinct Effects in Cytokine Gene Expression Studies.** The range of pharmacological effects of monocyte-targeted agents are likely to be distinct from those of nontargeted agents because the latter can act on cells outside of the monocyte-macrophage lineage. This was investigated by examining the effects of both types of agent on gene expression changes induced by either LPS or a combination of phorbol myristate acetate (PMA) and ionomycin in human blood. The former stimulates multiple signaling pathways predomi-
but completely unaffected by the monocyte-targeted agent, CHR-7296, even at concentrations 30-fold higher (Fig. 4b).

Creation of an hCE-1 “Knock-In” Mouse. Expression data (Lattin et al., 2008) indicate that neither mouse blood cells nor, for example, the murine monocyte-macrophage cell line RAW 264.7 selectively express an hCE-1 homolog (also see data from wild-type mice in Supplemental Fig. S2 indicating that CHR-4487 is not monocyte-selective). To circumvent this problem and assess the in vivo efficacy of these macrophage-targeted compounds, we created a transgenic mouse strain in which hCE-1 was expressed predominantly in a monocyte-macrophage lineage-selective way (driven by a human CD68 promoter; Gough et al., 2001) (see Supplemental Data). Wild-type littermate animals emerging from this breeding program served as controls and allowed a definitive assessment of how the targeting of HDAC inhibitors affected the disease process. Histone acetylation was assessed in blood from wild-type and transgenic mice treated in vitro with either SAHA or CHR-4487. SAHA treatment increased histone acetylation at approximately equivalent concentrations in all blood cell types from both strains of mice. In wild-type mouse blood, CHR-4487, similarly, showed no selective effects on histone acetylation in the FACS-identified blood cell populations (Supplemental Fig. S2). In contrast, CHR-4487 increased acetylation in the monocyte population of hCE-1 “knock-in” mice to a greater extent than the other blood cell types studied (Supplemental Fig. S2). No difference in lymphocyte protein acetylation was seen between the two mouse strains when blood was incubated with CHR-4487 (Supplemental Fig. S2). These data indicate that the selective effects of macrophage-targeted HDAC inhibitors seen in human whole blood can be recapitulated, to a large degree, in blood from these transgenic mice.

Administration of CHR-4487 (10 mg/kg, i.p.) to wild-type or transgenic mice led to the short-lived appearance of the ester in plasma, followed by a more sustained circulating level of its acid product, CHR-4495 (Supplemental Fig. S3). The impact of the selective accumulation of CHR-4495 in the circulating monocytes of the transgenic animals was assessed indirectly by measuring the level of protein acetylation in the different blood cell populations. A rapid and sustained increase in this parameter in FACS-identified monocytes, but not lymphocytes, was readily seen (Supplemental Fig. S3). A smaller, but significant, effect on granulocyte protein acetylation was also noted (Supplemental Fig. S3). As expected from previous data with tosedostat, the pharmacological effect of CHR-4487 on circulating monocytes substantially outlived circulating ester exposure because of retention of the product acid within these cells (Löwenberg et al., 2010).

In Vivo Arthritis Studies Using Monocyte/Macrophage-Targeted and Parental HDAC Inhibitors. When both wild-type and transgenic mice were inoculated with a cocktail of anticollagen type II antibodies (Nandakumar et al., 2003), followed 3 days later by an LPS injection, all animals developed clinical signs of arthritis by day 6 (Fig. 5). Treatment of mice with daily doses of SAHA inhibited arthritis development in both strains of mice only at the highest dose of SAHA studied (100 mg/kg/day). Once-daily intraperitoneal dosing with CHR-4487 (3 or 10 mg/kg/day i.p.) significantly inhibited arthritis development in transgenic animals (Fig. 5). Doses of 3 and 10 mg/
Fig. 4. The effects of conventional and macrophage-targeted HDAC inhibitors and HSP-90 inhibitors on cytokine gene expression in human blood. 
a, human blood was incubated with a conventional (SAHA, blue symbols) or macrophage-targeted (CHR-4487, red symbols) HDAC inhibitor for 2 h at 37°C. After treatment with either LPS (100 ng/ml) (left) or a combination of PMA (20 nM) and ionomycin (1 μM) (right) for 6 h, RNA was collected, stabilized, and extracted using the PAXgene Blood RNA system. After preparation of cDNA samples, real-time PCR was used to quantify mRNA expression in the presence or absence of drugs. b, human blood was incubated with a conventional (SNX-2112, blue symbols) or macrophage-targeted (CHR-7296, red symbols) HSP-90 inhibitor for 2 h at 37°C. After treatment with either LPS (100 ng/ml) (left) or a combination of PMA (20 nM) and ionomycin (1 μM) (right) for 6 h, RNA was collected, stabilized, and extracted using the PAXgene Blood RNA system. After preparation of cDNA samples, real-time PCR was used to quantify cytokine/chemokine mRNA expression in the presence or absence of drugs. Values shown are means ± S.D. for a single experiment, which was replicated two to five times.
kg/day almost completely inhibited both clinical and histological signs of arthritis (Fig. 5 and Supplemental Fig. S4). It is noteworthy that other studies demonstrated that transgenic mice tolerated at least 30 mg/kg/day i.p. CHR-4487 well, gaining ~5% of the starting weight over 7 days of treatment (Supplemental Table S4). In contrast, at the effective dose of 100 mg/kg/day, SAHA-treated animals lost ~5% body weight within 7 days (Supplemental Table S4). In this model, therefore, a therapeutic window of at least 30-fold was seen with CHR-4487. Thus, targeting of HDAC inhibitors to hCE-1-positive cells has both retained the antiarthritic effect seen with SAHA and has widened the therapeutic window of this class of agent.

Discussion

A range of approaches has previously been investigated to achieve cell targeting, including the use of immunoliposomal, sugar- and receptor-dependent systems (Pasqualini et al., 1997; Dubowchik and Walker, 1999; Paulos et al., 2004; Kontermann, 2006; Aouadi et al., 2009). In the current approach, receptor-based cell targeting is eschewed and the selective expression of an intracellular enzyme (hCE-1) is harnessed to convert a prodrug into a pharmacologically active acid species only in hCE-1-expressing cells. The irreversible nature of this hydrolysis, and the physicochemical properties of the poorly membrane-penetrant acid produced, leads to a high intracellular concentration of the active acid, which delivers the cell-selective effect. The striking cell-selective effects seen with monocyte-targeted agents such as CHR-4487 in human blood reflect the exquisite hydrolytic selectivity of hCE-1 for certain amino acid ester constructs and occur despite the fact that monocytes constitute a very small proportion of the carboxylesterase-containing cell pool present in blood. Moreover, the selectivity also occurs despite the fact that CHR-4487 is as pharmacologically active in its ester proform as it is in its acid product form. As a neutral species, the ester will penetrate all cells, and it might have been expected to generate effects on neutrophils and lymphocytes commensurate with its innate activity against the HDAC enzyme. The monocyte-specific effects seen in blood with agents such as CHR-4487 demonstrate that this is not the case and may be explained by the extremely efficient intramonicytic processing of the low concentrations of free (nonplasma protein bound) ester present in human blood that limit ester availability to other cell types. These data are reinforced by the studies conducted in blood in which cytokine gene expression patterns are examined after treatment with conventional and selective inhibitors of different intracellular targets. The results seen are entirely consistent with the notion that cell-selective drugs might be expected to exhibit a different, and potentially advantageous, therapeutic profile compared with nontargeted homologues.

Ideally, any cell-selective delivery system should be flexible, compatible with as many drug classes as possible and suitable for either oral or parenteral routes of administration. Data derived from ESM versions of many drug classes indicate that it is possible to selectively hydrolyze and, hence, deliver a range of drug classes to intracellular targets within the monocyte-macrophage lineage using hCE-1-hydrolysable chemical motifs. Such ESM-based drugs retain the enzyme potency of the parent molecule in their intracellular acid form and, as a result, demonstrate cell-selective potency advantages relative to their parents. The potency advantages seen with these targeted agents are often best seen in human blood and, using the technology, HDAC inhibitors, for example, become very potent anticytokine agents. Parental HDAC inhibitors are known to have such effects (Leoni et al., 2002, 2005), but their usefulness in diseases outside of cancer is likely to be limited by their inherent low therapeutic ratio, confirmed again in this work. The in vivo studies conducted with the hCE-1 knock-in mice demonstrate that the anticytokine effects of macrophage-targeted HDAC inhibitors translate into a powerful antiarthritic effect at well tolerated doses. Moreover, the complete absence of effect of CHR-4487 in littermate hCE-1-negative mice confirms that the limited
expression of hCE-1 in monocytes and monocyte-derived cells and, to a lesser extent, granulocytes, is both essential and sufficient for therapeutic efficacy. These data underline the central role of these cells in the pathology of arthritis. More generally, they suggest that pharmacological targeting of these cells using hCE-1-hydrolysable prodrugs can retain disease efficacy and may improve the therapeutic window of a range of anti-inflammatory agents in the many diseases where these cells contribute to the pathological process. One example of this type of agent, (S)-4-[7-hydroxy carbamoyl-3-[(pentanoylamino)-benzylamino]-phenylacetic acid cyclopentyl ester (CHR-2845), has already entered human clinical trials as a once-a-day orally administrable agent (www.clinicaltrials.gov; identifier: NCT00820508).

A more general conclusion to be derived from the work is that strikingly cell-selective effects can be achieved by harnessing an intracellular enzyme that is able to generate a charged product. There may be other enzymes with useful expression profiles different from that of hCE-1 that can be exploited in a similar way.

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We thank Dr. Steven Wood (Q3 Analytical Ltd., Porton Down, United Kingdom) for assistance with the bioanalytical assays monitoring ester and acid concentrations in human cells and mouse blood studies; the staff at GenOway (Lyon, France) and Charles River (Margate, United Kingdom) for their work in the creation and breeding of the hCE-1-transgenic mice; and Drs. Wynne Aherne and Martin Rowlands (Institute of Cancer Research, Sutton, United Kingdom) for assistance with the bioanalytical assays monitoring ester and acid concentrations in human cells and mouse blood studies; and the staffs at GenOway (Lyon, France) and Charles River (Margate, United Kingdom) for their work in the creation and breeding of the hCE-1-transgenic mice; and Drs. Wynne Aherne and Martin Rowlands (Institute of Cancer Research, Sutton, United Kingdom) for help with early work in monitoring the anti-HDAC activity of parental and ESM agents based on the SAHA series.

Authorship Contributions

Participated in research design: Needham, Davidson, Bone, Brotheron, Charlton, Davies, Day, Patel, Moffat, and Drummond.

Conducted experiments: Needham, Bawden, Belfield, Brotheron, Bryant, Clark, Donald, Legris, McDermott, Owen, Pintat, Testar, and Wells.

Contributed new reagents or analytic tools: Bone, Krige, and McGovern.

Performed data analysis: Needham, Davidson, Bone, Moffat, and Drummond.

Wrote or contributed to the writing of the manuscript: Davidson, Moffat, and Drummond.

References


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Drug targeting to monocytes and macrophages using esterase-sensitive chemical motifs

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Chroma Therapeutics Ltd., 93 Milton Park, Abingdon, OXON OX14 4RY, UK
Table S1: The relationship between expression of hCE-1 by different human leukaemic cell lines and hydrolysis of CHR-3111 by lysates prepared from these cells

<table>
<thead>
<tr>
<th></th>
<th>THP-1</th>
<th>MV4-11</th>
<th>U-937</th>
<th>HuT-78</th>
<th>KG-1</th>
<th>MOLT-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCE-1 (mRNA, x10^6 versus GAPDH)</td>
<td>20000</td>
<td>17000</td>
<td>7000</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CHR-3111 hydrolysis by cell lysates (pg/ml/min)</td>
<td>4800±90</td>
<td>1740±12</td>
<td>3410±12</td>
<td>25±10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values shown are ± SD. The experiment was repeated at least twice. For hCE-1 cell expression analysis, mRNA was extracted and purified from 10^6 cells using RNeasy kit 74106 (Qiagen). cDNA was then prepared as described i
Table S2: Potency of conventional (SNX-2112) and ESM-based (CHR-7296) HSP-90 inhibitors as inhibitors of LPS-stimulated TNFα production in THP-1 cells and human blood

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of HSP-90* (IC$_{50}$, nM)</th>
<th>Inhibition of LPS-stimulated TNFα production in THP-1 cells* (IC$_{50}$, nM)</th>
<th>Inhibition of LPS-stimulated TNFα production in human blood* (IC$_{50}$, nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNX-2112</td>
<td>17</td>
<td>53</td>
<td>125</td>
</tr>
<tr>
<td><img src="image" alt="SNX-2112" /></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHR-7296</td>
<td>42 (ester) 7 (acid)</td>
<td>45</td>
<td>128</td>
</tr>
<tr>
<td><img src="image" alt="CHR-7296" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values shown are means of 3-4 experiments, each conducted with at least 7 concentrations of the drug.
Table S3: Potency of parental and both hydrolysable and non-hydrolysable macrophage-targeted HDAC inhibitors as inhibitors of LPS-stimulated TNFα production in human blood.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition of HDAC (IC₅₀, nM) (ester/acid)*</th>
<th>Inhibition of LPS-stimulated TNFα production (IC₅₀, nM)*</th>
<th>Ester hydrolysis (pg/ml/min) by cell lysates**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorinostat</td>
<td>80</td>
<td>290</td>
<td>na</td>
</tr>
<tr>
<td>CHR-4487</td>
<td>320/180</td>
<td>16</td>
<td>1167±13</td>
</tr>
<tr>
<td>CHR-5355</td>
<td>70/180</td>
<td>1980</td>
<td>13±13</td>
</tr>
</tbody>
</table>

*Values shown are means of 3-4 experiments, each conducted with at least 7 concentrations of the drug

**Values shown are means ± S.D.
Table S4: The effect of vorinostat and CHR-4487 on the body weights of hCE-1 ‘knock-in’ transgenic mice after 7 days of once daily treatment by the i.p. route.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Day 7 body weight* (as % of day 0 value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (for vorinostat)</td>
<td>-</td>
<td>106.5±1.9</td>
</tr>
<tr>
<td>Vorinostat</td>
<td>100 mg/kg/day</td>
<td>94.9±2.7</td>
</tr>
<tr>
<td>Vehicle (for CHR-4487)</td>
<td>-</td>
<td>101.1±0.5</td>
</tr>
<tr>
<td>CHR-4487</td>
<td>30 mg/kg/day</td>
<td>105.1±0.7</td>
</tr>
</tbody>
</table>

*Values shown are means ± S.D. (4-5 animals/group). For vorinostat, the vehicle treatment was: PBS/0.05% (v/v) Tween 20 (pH 7). For CHR-4487, the vehicle treatment was: 5% (v/v) DMSO : 5% (v/v) 2-hydroxypropyl β cyclodextrin: 90% water (pH ~5).
Figure S1: Detection of hCE-1 in FACS-identified human blood cells using an hCE-1-specific antibody.

The mean fluorescent intensity (MFI) resulting from PE-conjugated rabbit serum, either pre- (pre-bleed) or post- (hCE-1) immunisation with an hCE-1-specific peptide is shown for a range of serum dilutions. In Western Blotting studies, the hCE-1 serum does not detect either hCE-2 or hCE-3 (data not shown). Detailed methods are outlined below.
Figure S2: The effect of A) a conventional HDAC inhibitor (SAHA) and B) a hydrolysable (CHR-4487) ESM-based HDAC inhibitor on protein acetylation in FACS-identified murine blood cells following incubation of either wild-type (WT) or hCE-1-transgenic (TG) mouse blood with the compounds for 6 hours.

A)

*Results from a representative experiment, repeated four times with similar results, are shown. Values are means ± s.d. Detailed methods are described below.
Figure S3: A comparison of the pharmacokinetic properties (a) of CHR-4487 and its product acid (CHR-4495) in wild-type (WT) and hCE-1-transgenic (TG) mice with its pharmacodynamic effects (b) on FACS-identified blood cell protein acetylation in hCE-1-transgenic mice.

a) Whole blood

b) Values shown are (a) means ± S.E.M. (b); means ± S.D. (b). Detailed methods are described below.
Figure S4: Representative histology from the anti-collagen antibody-induced arthritis model conducted in wild-type and hCE-1-transgenic mice treated with vehicle, SAHA (100 mg/kg/day) or CHR-4487 (10 mg/kg/day).

Representative section from hCE1-transgenic mice treated with vehicle. This mouse had a score of 2 for cell infiltrate, 2 for synovial hyperplasia and 0 for erosion (x40 magnification)

Representative section from hCE1-transgenic mice treated with 10 mg/kg CHR-4487. This mouse had a score of 0 for cell infiltrate, 0 for synovial hyperplasia and 0 for erosion.
Representative section from hCE1-transgenic mice treated with 100 mg/kg SAHA. This mouse had a score of 0 for cell infiltrate, 0 for synovial hyperplasia and 0 for erosion.

Representative section from wild type mice treated with vehicle. This mouse had a score of 1 for cell infiltrate, 2 for synovial hyperplasia and 0 for erosion.
Representative section from wild type mice treated with 10 mg/kg CHR-4487. This mouse had a score of 2 for cell infiltrate, 2 for synovial hyperplasia and 0 for erosion.

Representative section from wild type mice treated with 100 mg/kg SAHA. This mouse had a score of 0 for cell infiltrate, 0 for synovial hyperplasia and 0 for erosion.
Methods

Chemicals

The following abbreviations are used:

AcOH = acetic acid
ACN = acetonitrile
Boc = tert-butoxycarbonyl
CDI – 1,1’-carbonyldiimidazole
CO₂ = carbon dioxide
DCE = dichloroethane
DCM = dichloromethane
Dess-Martin periodinane = 1,1,1-Triacetoxy-1,1-dihydro-1,2-benziodoxol-3(1H)-one
DIPEA = diisopropylethylamine
DMAP = 4-dimethylaminopyridine
DME = dimethyl ether
DMF = dimethylformamide
DMP = Dess-Martin periodinane
DMSO = dimethyl sulfoxide
EDC = N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride
Et₂O = diethyl ether
EtOAc = ethyl acetate
EtOH = ethanol
Et₃N or TEA = triethylamine
ELS = Evaporative Light Scattering
g = gram(s)
hr = hour(s)
HATU = 2-(1H-7-Azabenzotriazol-1-yl)--1,1,3,3-tetramethyl uronium hexafluorophosphate
HCl = hydrochloric acid
HOBt = 1-hydroxybenzotriazole
KOTMS = potassium trimethylsilanolate
K₂CO₃ = potassium carbonate
LC/MS = high performance liquid chromatography/mass spectrometry
LiAlH₄ = lithium aluminium hydride
LiOH = lithium hydroxide
MeOH = methanol
MgSO₄ = magnesium sulfate
mg = milligram(s)
min = minute(s)
mol = moles
mmol = millimole(s)
mL = millilitre
MnO₂ = manganese dioxide
N₂ = nitrogen
Na₂CO₃ = sodium carbonate
NaHCO₃ = sodium hydrogen carbonate
Na₂SO₄ = sodium sulphate
NaH = sodium hydride
NaOH = sodium hydroxide
NH₃ = ammonia
NH₄Cl = ammonium chloride
NMM = 4-methylmorpholine
NMR = nuclear magnetic resonance
Pd/C = palladium on carbon
RT = room temperature
sat. = saturated aqueous solution
STAB = Sodium triacetoxyborohydride
TBAF = Tetrabutylammonium fluoride
TFA = trifluoroacetic acid
THF = tetrahydrofuran
TLC = thin layer chromatography

Scheme 1: Preparation CHR-2875
Reagents and conditions: a) K₂CO₃, CH₃CN, RT, 90%. b) 5% Pt on carbon, H₂ (balloon pressure), EtOAc. c) Suberic acid monomethyl ester, EDC, EtOAc, RT, 83% over two steps. d) NH₂OH.HCl, MeOH, LiOH (aq.), 5 °C, 83%.

Cyclopentyl (2S)-[(3-nitrobenzyl)amino](phenyl)ethanoate 2: m-Nitrobenzyl bromide (5.2 g, 24 mmol) and cyclopentyl (2S)-amino(phenyl)ethanoate tosylate (9.5 g, 24 mmol) were stirred with potassium carbonate (10.0 g, 72 mmol) under N₂ in acetonitrile (90 mL) for 22 h at RT. The solvent was removed under reduced pressure and the residue partitioned between EtOAc (100 mL) and water (100 mL). The aqueous layer was further extracted with EtOAc (3 x 100 mL) and the combined organic layers washed with water (2 x 100 mL), dried (MgSO₄) and concentrated under reduced pressure to give cyclopentyl (2S)-[(3-nitrobenzyl)amino](phenyl)ethanoate 2 (7.7 g, 90%) which was used without purification.

Cyclopentyl (2S)-[(3-aminobenzyl)amino](phenyl)ethanoate 3: Cyclopentyl (2S)-[(3-nitrobenzyl)amino](phenyl)ethanoate (7.7 g, 2.2 mmol) was dissolved in EtOAc (80 mL). The reaction flask was purged with N₂ and platinum on carbon [5 wt. %, wet] (800 mg) was added. The mixture was stirred under hydrogen (balloon pressure) at 40 °C for 4.5 h. The catalyst was removed by filtration through Celite, washing with additional EtOAc (2 x 40 mL) and the product used in the next stage without isolation or purification.

Methyl 8-[[3-{{(1S)-2-(cyclopentyloxy)-2-oxo-1-phenylethyl}amino} methyl]-phenyl]amino]-8-oxooctanoate 4: To the EtOAc solution of cyclopentyl (2S)-[(3-aminobenzyl)amino] (phenyl)ethanoate carried forward from the previous step was added methyl suberate (4.1 g, 21.8 mmol) and EDC (4.6 g, 23.9 mmol) and the reaction stirred at RT for 16 h. After this time additional methyl suberate (0.4 g, 2.1 mmol) and EDC (0.8 g, 4.2 mmol) were added and the reaction stirred for a further 4 h at RT. The reaction was
partitioned with water (50 mL) and the aqueous layer extracted with EtOAc (2 x 50 mL). The combined organic layers were washed with 0.5N NaOH (2 x 40 mL) and water (50 mL), dried (MgSO₄) and concentrated under reduced pressure to give methyl 8-[[3-(((1S)-2-(cyclopentyl)oxy)-2-oxo-1-phenylethyl)amino]methyl)phenyl]amino]-8-oxooctanoate (4.84 g, 83%) which was used in the next step without purification.

Cyclopentyl(2S)-[[3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl] amino] (phenyl)ethanoate Hydrochloride (CHR-2875): Methyl 8-[[3-(((1S)-2-(cyclopentyl)oxy)-2-oxo-1-phenylethyl)amino]methyl)phenyl]amino]-8-oxooctanoate (8.8 g, 17.8 mmol) was dissolved in MeOH (100 mL) and hydroxylamine hydrochloride (3.71 g, 53.3 mmol) and the resulting solution cooled to < 5 °C. To this cooled solution was added dropwise lithium hydroxide (4.48 g, 106.8 mmol) dissolved in water (10 mL) over 20 min and the reaction stirred at 4 °C for 0.5 h. The reaction was brought to pH 7-8 by the addition of 4N HCl and the MeOH removed under reduced pressure. The resulting aqueous solution was extracted with EtOAc (3 x 20 mL) and the combined organic layers were washed with water (3 x 20 mL), dried (MgSO₄) and evaporated. The residue was purified by chromatography [silica gel, EtOAc] to give the free base as a colourless oil (4. g, 53%). Of this material 1.1 g was dissolved in EtOAc (40 mL) and 2N HCl in Et₂O (2 mL) was added. The resulting solid was collected by filtration, washed with EtOAc and dried under reduced pressure to give cyclopentyl (2S)-[[3-[[8-(hydroxyamino)-8-oxooctanoyl] amino]benzyl]amino] (phenyl)ethanoate hydrochloride CHR-2875 (1.06 g). m/z 496 [M+H]+.

t-Butyl(2S)-[[3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl] amino] (phenyl)ethanoate (CHR-2908): t-Butyl (2S)-[[3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl] amino] (phenyl)ethanoate was prepared in a route analogous to compound (CHR-2875) starting from t-butyl (2S)-amino(phenyl) ethanoate. m/z 484 [M+H]+. ¹H NMR (400MHz, CDCl₃) δ: 7.75 (1H, s), 7.50-7.35 (5H, m), 7.33-7.25 (2H, m), 7.05 (1H, d), 4.95-4.65 (1H, m), 3.99 (2H, q), 2.40-2.20 (2H, m), 2.05-1.93 (2H, m), 1.65-1.45 (4H, m), 1.30 (13H, m).
**Scheme 2:** Preparation of CHR-2880

**Reagents and conditions:**  
- a) O-Benzylhydroxylamine hydrochloride, HOBT, NMM, EDC, RT, 103%.  
- b) LiOH, H$_2$O, THF, RT, 77%.  
- c) 3-aminobenzyl alcohol, HOBT, NMM, EDC, DMF, RT, 50%.  
- d) MnO$_2$, DCM, RT, 63%.  
- e) (S)-Phenylglycine ester tosylate salt, MgSO$_4$, NMM, NaBH$_3$CN, MeOH, 37%.  
- f) 10% Pd-C, H$_2$, EtOH, THF, RT, 49%.

**Methyl 8-[(benzyloxy)amino]-8-oxooctanoate 6:** Suberic acid, monomethyl ester 5 (2.0 g, 10 mmol) was dissolved in DMF (10 mL). O-Benzylhydroxylamine hydrochloride (2.0 g, 13 mmol) was added, followed by HOBT (1.95 g, 13 mmol), NMM (3.5 mL, 32 mmol) and
EDC (2.44 g, 13 mmol) and the reaction was stirred at RT overnight. The reaction was partitioned between EtOAc (25 mL) and water (25 mL), and the water layer extracted with EtOAc (2 x 25 mL). The combined organic layers were washed with 2N HCl (2 x 25 mL), NaHCO₃ (2 x 25 mL) and brine (2 x 25 mL), dried (MgSO₄) and evaporated under reduced pressure to give methyl 8-[(benzyloxy)amino]-8-oxooctanoate 6 (3.22 g, 103%) as a colourless oil which was used in the next step without purification. m/z 294 [M+H]⁺.

8-[(benzyloxy)amino]-8-oxooctanoic acid 7: Methyl 8-[(benzyloxy)amino]-8-oxooctanoate 6 (3.2 g, 11 mmol) was dissolved in THF (30 mL) and a solution of lithium hydroxide (0.77 g, 33 mmol) in water (30 mL) was added. The reaction was then stirred at RT for 18 hr. EtOAc (50 mL) was added to the reaction and the layers separated. The aqueous layer was acidified to pH 3-4 with 10% HCl (aq) and extracted with EtOAc (3 x 25 mL). The combined organic layers were combined, dried (MgSO₄) and evaporated under reduced pressure to give 8-[(benzyloxy)amino]-8-oxooctanoic acid 7 (2.36 g, 77%) which was used without purification. m/z 281 [M+H]⁺.

N-(Benzyloxy)-N'-(3-hydroxymethyl)phenyl)octanediamide 8: 8-[(Benzyloxy) amino]-8-oxooctanoic acid 7 (500 mg, 1.79 mmol), 3-aminobenzyl alcohol (265 mg, 21.5 mmol), HOBt (329 mg, 2.15 mmol) and NMM (229 mg, 2.15 mmol) were dissolved in DMF (5 mL) and EDC (612 mg, 2.15 mmol) was added. The mixture was stirred at RT for 72 h and then partitioned between EtOAc (2 x 25 mL) and water (25 mL). The aqueous layer was extracted with EtOAc (2 x 25 mL). The combined organic layers were washed with 10% HCl (aq) (2 x 15 mL), sat. NaHCO₃ (2 x 15 mL) and brine (2 x 15 mL), dried (MgSO₄), and evaporated under reduced pressure. The residue was purified by column chromatography (5-10% MeOH:DCM) to give N-(benzyloxy)-N'-(3-(hydroxymethyl)phenyl)octane diamide 8 (337 mg, 50%) as a colourless oil. m/z 385 [M+H]⁺.

N-(Benzyloxy)-N'-(3-formylphenyl)octanediamide 9: N-(Benzyloxy)-N'-(3-(hydroxylmethyl)phenyl)octanediamide 8 (330 mg, 0.86 mmol) was dissolved in DCM (5 mL) and MnO₂ (373 mg, 4.29 mmol) added. The resulting suspension was stirred at RT for 16 h and then filtered through Celite, washing through with additional DCM (50 mL) and MeOH (20 mL). The combined filtrates were evaporated under reduced pressure and the residue purified by column chromatography [silica gel, 0-5% MeOH:DCM] to give N-(benzyloxy)-N'-(3-formylphenyl)octanediamide 9 (208 mg, 63%) as a colourless oil. m/z 383 [M+H]⁺.

Benzyl (2S)-[3-[[8-[(benzyloxy)amino]-8-oxooctanoyl] amino]benzyl] amino)(phenyl)ethanoate 10: N-(Benzyloxy)-N'-(3-formylphenyl) octanediamide (450 mg, 1.2
mmol) and (S)-phenylglycine benzyl ester tosylate (536 mg, 1.3 mmol) were dissolved in MeOH (15 mL) and MgSO₄ (200 mg) was added. The pH of the solution was adjusted to 5.5 by the addition of NMM (2 drops) and sodium cyanoborohydride (185 mg, 2.9 mmol) was added. The resultant mixture was stirred at RT for 72 h and then partitioned between water (50 mL) and EtOAc (50 mL). The aqueous layer was extracted with EtOAc (2 x 50 mL) and the combined organic layers were dried (MgSO₄) and evaporated under reduced pressure. The residue was purified by column chromatography [silica gel, 0-2% MeOH : DCM] to give benzyl (2S)-[[3-[(8-[(benzyloxy)amino]-8-oxooctanoyl]amino]benzyl]amino}(phenyl)ethanoate 10 (268 mg, 37%) as a colourless oil. m/z 609 [M+H]+.

(2S)-[[3-[(8-(Hydroxyamino)-8-oxooctanoyl]amino]benzyl]amino](phenyl)ethanoic acid (CHR-2880): Benzyl (2S)-[[3-[(8-[(benzyloxy)amino]-8-oxooctanoyl]amino]benzyl]amino](phenyl)ethanoate (250 mg, 0.11 mmol) was dissolved in 1:1 v/v EtOH/THF (5 mL) and the flask purged with argon. To the solution was added 10% palladium on charcoal (25 mg) and the mixture was then stirred under an atmosphere of hydrogen (balloon pressure) at RT for 2 h. The reaction was filtered through Celite washing with additional MeOH. The combined filtrates were evaporated under reduced pressure and the residue purified by chromatography [C18 silica, 0-100% MeOH : water] to give (2S)-[[3-[(8-(Hydroxyamino)-8-oxooctanoyl]amino]benzyl]amino](phenyl)ethanoic acid CHR-2880 (86 mg, 49%) as a colourless solid. m/z 428 [M+H]+. ¹H NMR (400MHz, CDC₁₃) δ: 7.70 (1H, m), 7.50-7.25 (7H, m), 7.05 (1H, m), 4.90 (1H, m), 4.00 (2H, dd), 2.30 (2H, m), 2.00 (2H, m), 1.65-1.50 (4H, m), 1.35-1.20 (4H, m).

Scheme 3: Preparation of CHR-3111 and CHR-3112
Reagents and conditions: a) 3-Nitrobenzyl bromide, K$_2$CO$_3$, AcCN, 60°C, 94%. b) Raney Ni, EtOH, H$_2$ (balloon pressure), RT, 65%. c) Suberic acid monomethyl ester, EDC, DCM, RT, 86%. d) NH$_2$OH.HCl, LiOH, H$_2$O, 0°C, 10%. e) KOTMS, THF, RT then O-(1-isobutoxyethyl)hydroxylamine, EDC, DMAP, DMF, RT followed by KOTMS, THF.

Cyclopentyl N-(3-nitrobenzyl)-L-leucinate 12: Cyclopentyl L-leucinate tosylate (1.41 g, 4.63 mmol), 3-nitrobenzyl bromide (1.0 g, 4.63 mmol) and K$_2$CO$_3$ (1.92 g, 13.89 mmol) were added to acetonitrile (30 mL) and the suspension was heated at 60 °C under a N$_2$ atmosphere for 16 h. On cooling, the reaction was partitioned between water (100 mL) and EtOAc (50 mL), the layers separated and the aqueous layer extracted with EtOAc (50 mL). The combined organic layers were dried (Na$_2$SO$_4$) and concentrated in vacuo. The residue was purified by column chromatography [silica gel, 0-100% EtOAc : heptanes] to give cyclopentyl N-(3-nitrobenzyl)-L-leucinate (0.95 g, 94%) as a colourless oil. m/z 335 [M+H]$^+$.  

Cyclopentyl N-(3-aminobenzyl)-L-leucinate 13: Cyclopentyl N-(3-nitrobenzyl)-L-leucinate 12 (1.04 g, 3.1 mmol) was dissolved in EtOH (50 mL) and Raney nickel [slurry in water] (~4 mL) was added and the reaction stirred under a hydrogen atmosphere (balloon pressure) for 4 h at RT. The reaction was filtered through Celite and the filtrate concentrated under reduced pressure to give cyclopentyl N-(3-aminobenzyl)-L-leucinate 13 (900mg, 95%) as a colourless oil which was used without purification. m/z 304 [M+H]$^+$.  

Methyl 8-[[3-({[(2S)-1-(cyclopentyoxy)-4-methyl-1-oxopentan-2-yl]amino}methyl)phenyl]amino]-8-oxooctanoate 14: Cyclopentyl N-(3-aminobenzyl)-L-leucinate 13 (900 mg, 2.97 mmol), suberic acid, monomethyl ester (534 mg, 2.97 mmol) and EDC (627 mg, 3.27 mmol) were added to DCM (20 mL) and the reaction stirred at RT for 2 h. The reaction was then partitioned with water (50 mL), the layers separated and the aqueous layer extracted with EtOAc (3 x 100 mL). The combined organic layers were dried (Na$_2$SO$_4$) and evaporated under reduced pressure. The residue was purified by column chromatography [silica gel, 0-100% EtOAc : heptanes] to give methyl 8-[[3-({[(2S)-1-(cyclopentyoxy)-4-methyl-1-oxopentan-2-yl]amino}methyl)phenyl]amino]-8-oxooctanoate 14 (500 mg, 86%) as a yellow oil. m/z 475 [M+H]$^+$.  

Cyclopentyl N-3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl-L-leucinate trifluoroacetate (CHR-3111): To a solution of methyl 8-[[3-({[(2S)-1-(cyclopentyoxy)-4-methyl-1-oxopentan-2-yl]amino}methyl)phenyl]amino]-8-oxooctanoate 14 (730 mg, 1.54
mmol) in MeOH (7.3 mL) at 0 °C was added hydroxylamine hydrochloride (321 mg, 4.62 mmol) and then a solution of lithium hydroxide (221 mg, 9.24 mmol) in water (1.8 mL) was added slowly. The reaction was continued at 0 °C for 5 h and then was acidified to pH 5.0 using 1M HCl. The solvent was removed under reduced pressure and the residue purified by HPLC to give cyclopentyl N-(3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl)-L-leucinate trifluoroacetate CHR-3111 (58 mg, 10%) as a colourless solid. m/z 476 [M+H]+ 1H NMR (400MHz, CDCl₃) δ: 7.80 (1H, s), 7.30 (2H, m), 7.10 (1H, d), 5.25 (1H, m), 4.10 (2H, q), 3.90 (1H, s), 2.30 (2H, t), 2.00 (2H, t), 1.75 (2H, m), 1.70-1.50 (13H, m), 1.30 (4H, m), 0.95-0.85 (6H, d).

**t-Butyl N-(3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl)-L-leucinate trifluoroacetate (CHR-6511):** t-Butyl N-(3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl)-L-leucinate trifluoroacetate was synthesized by a route analogous to compound CHR-3111 starting from t-butyl L-leucinate. m/z 464 [M+H]+ 1H NMR (300 MHz, CD₃OD) δ: 7.92 (1H, m), 7.43 (2H, m), 7.23 (1H, m), 4.21 (2H, m), 3.93 (1H, m), 2.41 (2H, m), 2.12 (2H, m), 1.81 (7H, m), 1.56 (9H, s), 1.42 (4H, m), 1.00 (6H, m).

**N-(3-[[8-(Hydroxyamino)-8-oxooctanoyl]amino]benzyl)-L-leucine trifluoroacetate (CHR-3112):** Methyl 8-[[3-(((2S)-1-(cyclopentyloxy)-4-methyl-1-oxopentan-2-yl)amino)methyl]phenyl]amino]-8-oxooctanoate CHR-3111 (100 mg, 0.21 mmol) and KOTMS (27 mg, 0.21 mmol) were dissolved in THF (10 mL) and stirred at RT for 4 h. The solvent was then removed under reduced pressure and the residue was re-dissolved in DMF (10 mL). To this solution was added EDC (61 mg, 0.32 mmol), DMAP (1 mg), and O-(1-isobutoxyethyl)hydroxylamine (87 mL, 0.63 mmol) and the reaction stirred at RT for 4 h. Water (100 mL) was added to the reaction which was then extracted with DCM (2 x 100 mL). The combined organic layers were dried (Na₂SO₄) and then evaporated under reduced pressure. The residue was dissolved in THF (5 mL) and KOTMS (135 mg, 1.05 mmol) was added and the reaction stirred at RT for 3h. The reaction was brought to pH 3 by the addition of 1M HCl and stirring was continued for 1 h. The solvent was removed under reduced pressure and the residue purified by preparative HPLC to give N-(3-[[8-(hydroxyamino)-8-oxooctanoyl]amino]benzyl)-L-leucine CHR-3112 (3 mg, 3% over three steps). m/z 408 [M+H]+
Scheme 4: Preparation of CHR-4487 and CHR-4495

**Reagents and conditions:**
- a) Trimethyl phosphonoacetate, K₂CO₃, H₂O, RT
- b) BH₃-Me₂S, THF, RT followed by 2N HCl
- c) 1M NaOH, MeOH, RT 50% over three steps
- d) Pd(OAc)₂, H₂ (balloon pressure), 0.5N NaOH, RT, 94%
- e) O-(1-Isobutoxyethyl)hydroxylamine, EDC, HOBT, Et₃N, DCM, RT 57%
- f) MnO₂, DCM, RT, 94%
- g) cyclopentyl L-cyclohexylglycinate, NaBH₃CN, 1,2-dichloroethane, RT
- h) 4N HCl in dioxane, DCM, RT, 4%
- i) 1N NaOH, MeOH, 50°C, 6 days then 10% HCl (aq), 7%

**4-[(1E)-3-methoxy-3-oxoprop-1-en-1-yl]benzoic acid 16:**
4-Carboxy benzaldehyde 15 (50 g, 0.33 mol) and K₂CO₃ (138.2 g, 1.00 mol) were added to water (500 mL) and cooled to 0-5 °C. Trimethyl phosphonoacetate (64.0 mL, 0.40 mol) was charged dropwise maintaining the reaction temperature below 15 °C. The reaction was warmed and stirred at RT for 20 h, filtered and the filter cake retained. The filtrate was acidified to pH 1 with 2N hydrochloric acid, filtered and the combined filter cakes dried *in vacuo* to afford the product 16 as a white solid (82.2 g). ¹H NMR (300MHz, d₆-DMSO) δ: 7.89-7.52 (5H, m), 6.59 (1H, d, J=16.2Hz), 3.7 (3H, s).
Methyl (2E)-3-[4-(hydroxymethyl)phenyl]prop-2-enoate 17: 4-[(1E)-3-methoxy-3-oxoprop-1-en-1-yl]benzoic acid 16 (82.2 g, 0.39 mol) was added to THF (600 mL) and cooled to 0-5°C. Borane-DMS complex, ~10M in THF (78 mL, 0.78 mol) was added dropwise and the reaction allowed to warm to RT and stirred for 20 h. The reaction was quenched with 2N hydrochloric acid then the organics removed in vacuo. The residue was extracted with EtOAc (2 x 200 mL) and the combined organics washed with saturated NaHCO₃ solution (200 mL), dried (MgSO₄) and concentrated in vacuo to afford the product as a yellow oil (49.1 g). ¹H NMR (300MHz, CD₃OD) δ: 7.75-7.70 (5H, m), 6.54 (1H, d, J=16.2Hz), 4.64 (2H, s), 3.80 (3H, s).

(2E)-3-[4-(Hydroxymethyl)phenyl]prop-2-enolic acid 18: Methyl (2E)-3-[4-(hydroxymethyl)phenyl]prop-2-enoate 17 (49.1 g, 0.26 mol) was added to 1M NaOH aq (500 mL) and MeOH (750 mL) and stirred at rt for 19 h. The MeOH was removed in vacuo and the residue washed with EtOAc. The aqueous phase was acidified to pH ~ 1 and the resulting precipitate was filtered and dried in vacuo to afford the product as an off-white solid (29.2 g, 49.7% over three steps). ¹H NMR (300MHz, d₆-DMSO) δ: 7.75-7.07 (5H, m), 6.50 (1H, d, J=15.9Hz), 4.59 (2H, s).

3-[4-(Hydroxymethyl)phenyl]propanoic acid 19: (2E)-3-[4-(Hydroxymethyl) phenyl]prop-2-enolic acid 18 (3 g, 16.9 mmol), palladium acetate (0.3 g, 1.3 mmol) and 0.5N NaOH solution (50 mL) were charged to a flask, purged with and stirred under hydrogen for 4.5 h. The reaction was filtered, washed with DCM (30 mL), acidified to pH = 1 with 10% hydrochloric acid and extracted with EtOAc (2 x 30 mL). The combined organics were dried (MgSO₄) and concentrated in vacuo to afford the product as a pale yellow solid (2.85 g, 94%). ¹H NMR (300MHz, CDCl₃) δ: 7.25 (4H, q), 4.57 (2H, s), 2.92 (2H, t), 2.60 (2H, t).

3-[4-(Hydroxymethyl)phenyl]-N-[1-(2-methylpropoxyethoxy] propanamide 20: 3-[4-(Hydroxymethyl)phenyl]propanoic acid (2.85 g, 15.8 mmol), EDC (3.64 g, 19.0 mmol) and HOBt (2.57 g, 19.0 mmol) were added to DCM (35 mL) and stirred at RT for 0.5 h. O-(1-Isobutoxyethyl)hydroxylamine (6.5 mL, 47 mmol) and Et₃N (6.6 mL, 47 mmol) were charged and the reaction stirred at RT for 17 h. The reaction was separated with water, the aqueous phase extracted with DCM (25 mL) and the combined organics dried (MgSO₄) concentrated in vacuo. The crude material was purified by flash chromatography [silica gel, 0-10% EtOAc-heptanes] to afford the product as an off-white solid (2.65 g, 57%). ¹H NMR (300MHz, CDCI₃) δ: 7.78 (1H, s), 7.35-7.17 (4H, m), 4.90 (1H, m), 4.68 (2H, d, J=5.7Hz),
3.49 (1H, m), 3.26 (1H, m), 3.00 (2H, t), 2.43 (1H, m), 1.86 (1H, m), 1.71 (1H, t), 1.34 (3H, d, J=5.1Hz), 0.91 (6H, d, J=6.9Hz).

3-(4-Formylphenyl)-N-[1-(2-methylpropoxy)ethoxy]propanamide 21: 3-[4-(Hydroxymethyl)phenyl]-N-[1-(2-methylpropoxy)ethoxy]propanamide 20 (2.65 g, 9.0 mmol) and MnO₂ (3.91 g, 45.0 mmol) were stirred in DCM (40 mL) at RT for 23 h. The reaction was filtered through celite and the filtrate concentrated to dryness to afford the product as a yellow oil (2.48 g, 94%). LC/MS purity 96%, m/z = 292 [M – H].

Cyclopentyl (2S)-cyclohexyl{(4-[3-(hydroxyamino)-3-oxopropyl]benzyl)amino}ethanoate 22: (4-Formylphenyl)-N-[1-(2-methylpropoxy)ethoxy]propanamide 21 (0.4 g, 1.37 mmol) and cyclopentyl (S)-cyclohexylglycinate (0.36 g, 1.38 mmol) were dissolved in 1,2-dichloroethane (10 mL). NaBH₃CN (0.17 g, 2.70 mmol) was charged and the reaction stirred under nitrogen for 1 h. The reaction was then quenched with saturated NaHCO₃ solution (10 mL) then separated. The aqueous phase was extracted with DCM (2 x 10 mL) and the combined organics dried (MgSO₄) and concentrated to dryness in vacuo to afford the product as a yellow oil (1.36 g). LCMS purity 67%, m/z 503 [M+H].

Cyclopentyl (2S)-cyclohexyl{(4-[3-(hydroxyamino)-3-oxopropyl]benzyl)}amino)ethanoic acid (CHR-4495): 1N Sodium hydroxide (3 mL) was charged to a solution of the protected hydroxamate 22 (0.45 g, 0.89 mmol) in MeOH (5 mL) and stirred at 50°C for 6 days. The reaction was acidified to pH 1 with 10% hydrochloric acid then concentrated to dryness in vacuo to afford the crude product as a yellow solid. Purification by preparative HPLC gave the desired product as an off-white solid (7 mg, 2%). ¹H NMR (300MHz, CDCl₃) δ: 7.45-
7.22 (4H, m), 4.12 (2H, q), 2.95 (2H, t), 2.39 (2H, t), 1.91-1.04 (11H, m). m/z = 335 [M + H]^+.

**Scheme 5:** Preparation of CHR-5355

Reagents and conditions: a) Conc. H$_2$SO$_4$, EtOH, RT, 83%. b) MnO$_2$, DCM, RT, 97%. c) t-butyl L-cyclohexylglycinate, STAB, Et$_3$N, DCE, RT, 60%. d) NH$_2$OH.HCl, KOH, H$_2$O, EtOH, RT, 6%.

**Ethyl 3-[4-(hydroxymethyl)phenyl]propanoate 23:** 3-[4-(hydroxymethyl)phenyl]propanoic acid 19 (2.0 g, 11.09 mmol) was dissolved in EtOH, treated with conc. H$_2$SO$_4$ (20 mL) and stirred for 4 h at RT. The solvent was removed in vacuo and the residue re-dissolved in EtOAc (50 mL) and washed with sat. NaHCO$_3$ (1 x 50 mL). The organic layer was dried (MgSO$_4$), filtered and concentrated to give 23 as an orange oil (1.91 g, 83 %). m/z 231 [M+Na]^+.

**Ethyl 3-(4-formylphenyl)propanoate 24:** Ethyl 3-[4-(hydroxymethyl)phenyl]propanoate 23 (1.91 g, 9.17 mmol) was dissolved in anhydrous DCM (20 mL), treated with MnO$_2$ (3.98 g, 45.85 mmol) and stirred at RT for 18 h. The spent MnO$_2$ was removed by filtration through Celite and this was further washed with EtOAc (50 mL). The combined filtrates were concentrated in vacuo to give 24 as a yellow oil which solidified on standing to form yellow crystals (1.83g, 97%). m/z 207 [M+H]^+.
Ethyl 3-[4-([(1S)-2-tert-butoxy-1-cyclohexyl-2-oxoethyl]amino)methyl)phenyl]propanoate 25: To ethyl 3-(4-formylphenyl)propanoate 24 (535 mg, 2.60 mmol), in anhydrous dichloroethane (50 mL) was added tert-butyl (2S)-amino(cyclohexyl)ethanoate (554 mg, 2.60 mmol), STAB (1102 mg, 5.20 mmol) and Et$_3$N (362 µL, 2.60 mmol). The mixture was stirred at RT for 72 h. The mixture was diluted with de-ionised water (100 mL) and extracted with DCM (2 x 100 mL). The combined organic phase was dried (MgSO$_4$), filtered and concentrated in vacuo. The residue was purified by column chromatography [silica gel, 0-100% EtOAc : heptane) to provide 25 as a colourless oil (630 mg, 60%). m/z 404 [M+H]$^+$. 

tert-Butyl (2S)-cyclohexyl{[4-3-(hydroxyamino)-3-oxopropyl] benzyl} amino)ethanoate trifluoroacetate (CHR-5355): A stirred solution of ethyl 3-[4-([(1S)-2-tert-butoxy-1-cyclohexyl-2-oxoethyl]amino)methyl)phenyl]propanoate 25 (630 mg, 1.56 mmol) in ethanol (6.3 mL) was cooled to -5 °C. A solution of KOH (700 mg, 12.48 mmol) in de-ionised water (1.5 mL) was added and the mixture allowed to warm to RT over 1 h. The bulk of the solvent was then removed in vacuo and the residue purified by preparative HPLC to provide the title compound as a white solid (57 mg, 6%). m/z 391 [M+H]$^+$. $^1$H NMR (300 MHz, $d_6$-DMSO) δ: 10.40 (1H, m), 9.18 (2H, m), 8.74 (1H, m), 7.36 (2H, d, J=7.8 Hz), 7.26 (2H, d, J=7.8Hz), 4.07 (2H, m), 3.73 (1H, m), 2.82 (2H, t, J=8.1 Hz), 2.26 (2H, t, J=8.1Hz), 1.71 (4H, m), 1.91 (1H, m), 1.43 (9H, s), 1.15 (4H, m), 0.87 (1H, m),
Scheme 6: Preparation of p38 MAP kinase inhibitor intermediates 27 and 31

Reagents and conditions:  
a) Br$_2$, AcOH, 15 °C, 97%.  
b) NaCN, ethanol/water, 5 °C, 72%.  
c) 4-chloro benzenethiol, ethanol, diethyl ether, HCl(g), RT, 52%.  
d) Potassium tert-butoxide, NMP, tert-butyl chloroacetate, -20 °C, 53%.  
e) TFA, DCM, 0 °C, 86%.  
f) BH$_3$Me$_2$S, THF, 0°C, 89%;  
g) Pd/C, EtOAc, H$_2$, 100%.

4-Chlorophenyl 3-(2,4-difluorophenyl)-3-oxopropanimidothioate 27: The synthesis of 27 is reported in WO03076405 (2003).

tert-Butyl (3, 5-difluoro-4-nitrophenyl)acetate 28: A mixture of potassium tert-butoxide (12.3 g, 111.0 mmol) in 1-methyl-2-pyrrolidinone (100 mL) was cooled to -20 °C under N$_2$. A mixture of 2, 6-difluoronitrobenzene (5.0 g, 31.43 mmol) and tert-butylchloroacetate (7.6 mL, 53.11 mmol) in 1-methyl-2-pyrrolidinone (100 mL) was added slowly at -10 °C to -20 °C over 1.5h. After 1.5h the reaction was quenched by pouring into 2M HCl (120 mL) and ice, then heptane (300 mL) was added. The mixture was stirred for 10 mins, separated and the aqueous extracted with heptane (2 x 400 mL). The organic layer was washed with brine twice, dried (MgSO$_4$), filtered and washed with heptane. The solution was
concentrated in vacuo and the residue purified by column chromatography [silica gel 3-4% EtOAc/Heptane] to provide the title compound as an orange oil (4.34 g, 53%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 7.06 (2H, d, J=8.7Hz), 3.59 (2H, s), 1.48 (9H, s).

(3,5-Difluoro-4-nitrophenyl) acetic acid 29: To a solution of tert-butyl (3,5-difluoro-4-nitrophenyl)acetate 28 (4.34 g, 15.88 mmol) in DCM (10 mL), at 0 °C, was added TFA (10 mL). The reaction was warmed to RT and stirred for 1.5 h. The reaction was concentrated in vacuo, slurried in heptane (10 mL), filtered and dried to provide 29 as an orange solid (2.95 g, 86%). $^1$H NMR (300 MHz, $d_6$-DMSO) $\delta$: 7.45 (2H, d, J=9.6Hz), 3.79 (2H, s).

2-(3,5-Difluoro-4-nitrophenyl)ethanol 30: A solution of (3,5-difluoro-4-nitrophenyl)acetic acid 29 (2.95 g, 13.59 mmol) in THF (30 mL), under N$_2$, was cooled to 0 °C and a solution of BH$_3$Me$_2$S in THF (10.2 mL, 20.38 mmol) was added dropwise over 5 minutes. The mixture was warmed to RT and stirred for 4.5 hrs. The reaction was cooled to 0 °C and quenched with MeOH (10 mL). The mixture was concentrated in vacuo and the residue purified by column chromatography [silica gel, 30-60% EtOAc:heptanes] to provide the compound 30 as an oil (2.45 g, 89%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 7.03 (2H, d, J=9.3 Hz), 3.97-3.91 (2H, q, J=5.4, 5.7 Hz), 2.93 (2H, t, J=6.2 Hz), 1.52 (1H, t, J=5.0 Hz).

2-(4-Amino-3,5-difluorophenyl)ethanol 31: To a solution of 2-(3,5-difluoro-4-nitrophenyl)ethanol 30 (2.45 g, 12.06 mmol) in EtOAc (50 mL) was added Pd/C (0.8 g). The mixture was stirred under an atmosphere of H$_2$ for 19h, filtered and concentrated in vacuo to provide the title compound as a pale brown solid (2.15 g, 100%). $^1$H NMR (300 MHz, CDCl$_3$) $\delta$: 6.70-6.67 (2H, m), 3.82 (2H, t, J=6.5 Hz), 2.76 (2H, t, J=6.5 Hz).

Scheme 7: Preparation of CHR-3464 and CHR-5623
Reagents and conditions: a) 2,6-difluoroaniline, AcOH, 80 °C, 49%. b) CDI, THF, propiolic acid, 0 °C – 80 °C, 59%. c) Compound 31, acetic acid, 80 °C, 67%; d) CDI, THF, propiolic acid, 0 °C – 80 °C, 37%. e) HCl solution, 100°C, 100%. f) Dess-Martin periodinane, DCM, RT, 100%. g) Cyclopentyl L-leucinate, THF, sodium triacetoxyborohydride, RT, 31%.

3-(2,4-difluorophenyl)-N-(2,6-difluorophenyl)-3-oxopropanimidamide 32: To a mixture of 4-chlorophenyl 3-(2,4-difluorophenyl)-3-oxopropanimidothioate hydrochloride 27 (5.0 g, 13.8 mmol) in AcOH (50 mL) was added 2,6-difluoroaniline (1.97 mL, 14.5 mmol) and the mixture heated at 80 °C for 20 h. The mixture was cooled, concentrated in vacuo and the residue triturated in diethyl ether to provide a solid. The solid was partitioned between EtOAc and sat NaHCO₃, washed with brine, dried (MgSO₄) and concentrated in vacuo to provide the title compound as an off-white solid (2.1 g, 49% yield). m/z 311 [M+H]⁺.
6-Amino-1-(2,6-difluorophenyl)-5-[(2,4-difluorophenyl)carbonyl]pyridin-2(1H)-one (CHR-3464): To a solution of CDI (1.64 g, 10.1 mmol) in THF (50 mL), under N₂ at 0 °C, was added dropwise propionic acid (620 µl, 10.1 mmol). The mixture was warmed to RT and stirred for 1 h. A solution of 3-(2,4-difluorophenyl)-N-(2,6-difluorophenyl)-3-oxopropanimidamide 32 (2.1 g, 6.7 mmol) in THF (25 mL) was added dropwise and the mixture heated at 80 °C for 18hrs. The mixture was cooled, concentrated in vacuo and the residue purified by column chromatography [silica gel, 5% MeOH/DCM] and further trituration with diethyl ether to provide the title compound as a pale yellow solid (1.43 g, 59% yield). ¹H NMR (300 MHz, d₆-DMSO), δ: 7.78-7.68 (1H, m), 7.63-7.55 (1H, m), 7.46-7.35 (4H, m), 7.24 (1H, td, J=2.1, 8.5 Hz), 5.76 (1H, d, J=9.8 Hz).

2-(4-{[3-(2,4-Difluorophenyl)-3-oxopropanimidoyl]amino}-3,5-difluoro phenyl)ethyl acetate 33: To a mixture of 4-chlorophenyl 3-(2,4-difluorophenyl)-3-oxopropanimidothioate hydrochloride 27 (3.99 g, 11.1 mmol) in acetic acid (20 mL) was added 2-(4-amino-3,5-difluorophenyl)ethanol 31 (2.00 g, 11.6 mmol) and the mixture heated at 80 °C for 20h. The mixture was cooled, concentrated in vacuo and the residue triturated in Et₂O to provide a solid. The solid was partitioned between EtOAc and sat NaHCO₃, washed with brine, dried (MgSO₄) and concentrated in vacuo to provide the title compound as a solid (2.91 g, 67 % yield). m/z 397 [M+H]⁺.

2-(4-{6-Amino-5-[2,4-difluorophenyl]carbonyl-2-oxopyridin-1(2H)-yl}-3,5-difluorophenyl)ethyl acetate 34: To a solution of CDI (1.78 g, 10.98 mmol) in THF (36 mL), under N₂ at 0 °C, was added dropwise propionic acid (675 µl, 10.98 mmol). The mixture was warmed to RT and stirred for 1.5hrs. A solution of 2-(4-{[3-(2,4-difluorophenyl)-3-oxopropanimidoyl]amino}-3,5-difluorophenyl)ethyl acetate 33 (2.9 g, 7.32 mmol) in THF (18 mL) was added dropwise and the mixture heated at 80 °C for 5hrs. The mixture was cooled, concentrated in vacuo and the residue purified twice by column chromatography [silica gel, 0.7-1% MeOH:DCM) to provide compound 34 as a solid (1.20 g, 37 % yield). ¹H NMR (300 MHz, CDCl₃) δ: 7.49-7.39 (2H, m), 7.09-6.90 (4H, m), 5.93 (1H, d, J=9.9Hz), 4.37 (2H, t, J=6.4Hz), 3.06 (2H, t, J=6.6 Hz), 2.10 (3H, s).

6-Amino-1-[2,6-difluoro-4-(2-hydroxyethyl)phenyl]-5-[2,4-difluorophenyl)carbonyl]pyridin-2(1H)-one 35: A mixture of 2-(4-{6-amino-5-[(2,4-difluorophenyl)carbonyl]-2-oxopyridin-1(2H)-yl}-3,5-difluorophenyl)ethyl acetate 34 (1.1 g, 2.45 mmol) in 6N aq HCl (50 mL) was heated at reflux for 24 h. The mixture was cooled, filtered and washed with water. The precipitate was partitioned between EtOAc and sat aq
NaHCO₃, the organic layer further washed with brine, dried (MgSO₄) and concentrated in vacuo to provide the compound 35 as a solid (993 mg, 100% yield). ¹H NMR (300 MHz, CDCl₃) δ: 7.49-7.39 (2H, m), 7.15-6.90 (4H, m), 5.92 (1H, d, J=9.6Hz), 4.00-3.85 (2H, m), 2.95 (2H, t, J=6.0Hz).

(4-{6-Amino-5-{[2,4-difluorophenyl]carbonyl}-2-oxopyridin-1(2H)-yl}-3,5-difluorophenyl)acetaldehyde 36: To a mixture of 6-amino-1-[2,6-difluoro-4-(2-hydroxyethyl)phenyl]-5-{[2,4-difluorophenyl]carbonyl}pyridin-2(1H)-one 35 (500 mg, 1.23 mmol) in DCM (20 mL) was added Dess-Martin periodinane (783 mg, 1.85 mmol). The mixture was stirred for 3.5 h, sat Na₂S₂O₃ (20 mL) and sat NaHCO₃ (20 mL) was added and the mixture stirred vigorously for 30 minutes. The organic layer was separated and the aqueous extracted with DCM. The organic layer was washed with brine, dried (MgSO₄) and concentrated to provide 36 as a solid (497 mg, 100% yield). ¹H NMR (300 MHz, CDCl₃) δ: 9.88 (1H, s), 7.49-7.40 (2H, m), 7.12-6.91 (4H, m), 5.93 (1H, d, J=9.9Hz), 3.89 (2H, s).

Cyclopentyl N-(2-{4-[6-amino-5-(2,4-difluorobenzoyl)-2-oxopyridin-1(2H)-yl]-3,5-difluorophenyl}ethyl)-L-leucinate (CHR-5623): To a solution of (4-{6-amino-5-{[2,4-difluorophenyl]carbonyl}-2-oxopyridin-1(2H)-yl}-3,5-difluorophenyl)acetaldehyde 36 (46 mg, 0.114 mmol) in THF (2 mL) was added cyclopentyl L-leucinate (40 mg, 0.201 mmol), stirred for 30 mins, and then sodium triacetoxyborohydride (80 mg, 0.377 mmol). The reaction stirred for 24 h, diluted with EtOAc and the organic washed with sat NaHCO₃, brine, dried (MgSO₄) and concentrated in vacuo. The residue was purified by column chromatography [silica gel, 0.75-1.25% MeOH:DCM], and then purified by preparative HPLC to provide the title compound (29 mg, 31% yield). m/z 588 [M+H]⁺. ¹H NMR (300MHz, CDCl₃) δ: 7.57-7.48 (2H, m), 7.32-7.10 (4H, m), 5.84 (1H, d, J=9.6 Hz), 5.41-5.30 (1H, m), 4.10-4.03 (1H, m), 3.45-3.30 (2H, m), 3.20-3.14 (2H, m), 2.05-1.60 (11H, m), 1.10-0.95 (6H, m).
Scheme 8: Synthesis of CHR-5803 and CHR-5250

Reagents and conditions: 

a) Cyclopentyl L-leucinate tosylate salt, (3-formylphenyl) boronic acid, NaBH(OAc)_3, DCM, RT, 66%.  
b) Et_3N, 2-cyanoacetamide, ethanol, RT, 88%.  
c) Trichloroacetyl isocyanate, pyridine, 0 °C, 99%.  
d) N-bromosuccinimide, acetic acid, chloroform, RT, 97%.  
e) Na_2CO_3 solution, ethanol, 89%.  
f) Phenylboronic acid, palladium tetrakis(triphenylphosphine), DME, 80 °C, 24%.  
g) Compound 37, palladium tetrakis(triphenylphosphine), DME, 90 °C, 67%.  

5-Bromo-2-(carbamoylamino)thiophene-3-carboxamide 38: The synthesis of compound 38 is detailed reported in WO03104218 (2003).
2-(Carbamoylamino)-5-phenylthiophene-3-carboxamide (CHR-5803): A suspension of 38 (0.5 g, 1.89 mmol), phenylboronic acid (0.462 g, 3.79 mmol) and palladium tetrakis(triphenylphosphine) (0.219 g, 0.19 mmol) in DME (6 mL) was purged with nitrogen. Saturated aqueous Na₂CO₃ (2 mL) was added and the reaction mixture was placed in a preheated oil bath at 80 ºC before being left to stir for 18hrs. The reaction mixture was cooled to RT and then partitioned between 1N NaOH solution (50 mL) and EtOAc (100 mL). After shaking vigorously, the organic layer was collected and washed with brine (100 mL), dried (MsSO₄) and reduced to dryness under vacuum. The residue was purified by column chromatography [silica gel, 5% MeOH] in DCM. The desired product (123mg, 0.461mmol, 24% yield) was isolated as an off-white solid. m/z 262 [M+H]+, 260 [M-H]-. ¹H NMR (300 MHz, d6-DMSO) : 10.9 (1H, s), 7.8 (1H, s), 7.69 (2H, br s), 7.52 (2H, d, J=7.2Hz), 7.39 (2H, m), 7.32 (1H, br s), 7.24 (1H, m), 6.98 (2H, br s).

Cyclopentyl N-[3-(dihydroxyboryl)benzyl]-L-leucinate 37: To a solution of cyclopentyl (S)-leucinate (244.6 mg, 1.227 mmol) and (3-formylphenyl) boronic acid (184 mg, 1.227 mmol) in DCM (10 mL) was added NaBH(OAc)₃ (780 mg, 3.68 mmol) in portions over 20 minutes. The reaction was stirred at RT for 2 h after which time the reaction mixture was poured into 1M HCl (50 mL) and was washed with DCM (50 mL). The aqueous phase was neutralised to pH 7 with NaHCO₃ and extracted with DCM (2 x 50 mL). The combined organic extracts were dried over magnesiu m sulphate and the solvent removed. The product (270.3 mg, 0.811 mmol, 66.1% yield) was isolated as a colourless foamy solid and was used without further purification. m/z 334 [M+H]+.

Cyclopentyl N-{3-[4-carbamoyl-5-(carbamoylamino)-2-thienyl]benzyl}-L-leucinate (CHR-5250): A vial containing tetrakis(triphenylphosphine)palladium (99 mg, 0.085 mmol), 38 (225 mg, 0.854 mmol) and 37 (313 mg, 0.939 mmol) was purged with N₂ and DME (dry, 12 mL) was added. To the mixture was added a saturated aqueous solution of sodium bicarbonate (2.5 mL) and the reaction mixture was heated to 90 ºC in an oil bath for 4h. The mixture was cooled to RT and poured into water (50 mL) and extracted with EtOAc (100 mL). The combined organic extracts were washed with brine, dried (MgSO₄) and reduced to dryness under vacuum. The residue was purified by column chromatography [silica gel, 0-3% MeOH] in DCM. The desired product (291 mg, 67% yield) was isolated as a pale yellow solid. m/z 473 [M+H]+, 472 [M-H]-. ¹H NMR (400 MHz, d6-DMSO) : 10.9 (1H, s), 7.7 (1H, s), 7.62 (2H, br s), 7.46 (1H, s), 7.25 (3H, m), 7.0(2H, br s), 5.1 (1H, m), 3.72 (1H, d, J=13.7Hz), 3.53 (2H, d, J=12.9Hz), 3.10 (1H, t, J=7.1Hz), 1.80-1.71 (2H, m), 1.68-1.33 (6H, m), 0.81 (3H, d, J=8.7Hz), 0.78 (2H, d, J=8.8Hz).
**Scheme 9: Synthesis of CHR-6907 and 6576**

![Scheme 9](image)

**Reagents and conditions:**

a) N-methyl-N-benzylamine, HATU, DCM, DIPEA, RT.  
b) Pd/C, H\_2, K\_2CO\_3, EtOAc, RT, 13%.  
c) Cyclopentyl L-leucinate tosylate, sodium triacetoxyborohydride, DCE, RT, 83%.  
d) methylamine hydrochloride, NaHCO\_3, EtOH, RT, 9%.  
e) 2,4-bis(benzyloxy)-5-(prop-1-en-2-yl)benzoic acid, EDC, HOBt, DCM, DIPEA, RT, 64%.

**N-benzyl-2,4-dihydroxy-N-methyl-5-(propan-2-yl)benzamide (CHR-6907):** To a solution of 2,4-bis(benzyloxy)-5-(prop-1-en-2-yl)benzoic acid (0.080g, 0.21 mmol) (prepared as described in WO 2006/109075) in DCM (5 mL) was added DIPEA (1 mL, 5.7 mmol), N-methyl-N-benzylamine (1 mL, 7.70 mmol) and HATU (0.145 g, 0.38 mmol). The mixture was stirred at RT for 20 mins, then poured into EtOAc (50 mL) and washed with 2M HCl solution (3 x 25 mL) and brine (50 mL). The organic extracts were dried and concentrated. The residue obtained (0.143 g) was dissolved in EtOAc, and K\_2CO\_3 (0.220 g, 1.59 mmol) and palladium on carbon were added. The reaction vessel was evacuated, filled with hydrogen and stirred overnight. The flask was purged with nitrogen, filtered through Celite, and washed through with EtOAc (50 mL). The filtrate was concentrated and purified by flash column chromatography (silica gel, 1:1 heptane:EtOAc) to yield the desired product (8.6 mg, 13% yield over two steps). m/z 300.25 [M+H]\(^+\). \(^{1}\)H NMR (300 MHz, d\textsubscript{6}-DMSO), \(\delta\): 9.61 (1H, s), 9.47 (1H, s), 7.19-7.39 (5H, m), 6.86 (1H, s), 6.36 (1H, s), 4.54 (2H, s), 3.04 (1H, sep, J=6.9 Hz), 2.80 (3H, s), 1.06 (6H, d, J=7.0 Hz).
Cyclopentyl \(N\{-4\-\text{[(methylamino)methyl]benzyl}\}-L\)-leucinate 39: To a solution of 4-(bromomethyl)-benzaldehyde (0.940 g, 4.72 mmol) in dichloroethane (20 mL) was added cyclopentyl L-leucinate tosylate (2.40 g, 6.4 mmol) and sodium triacetoxyborohydride (2.53 g, 11.9 mmol). The reaction mixture was stirred for 1 h then poured into EtOAc (300 mL). The organic fraction was washed with saturated sodium hydrogen carbonate (100 mL, 3 x 50 mL) then dried (MgSO\(_4\)), concentrated and purified by flash column chromatography [silica gel, 0-3% MeOH:DCM] to yield the desired intermediate product (1.504 g, 83% yield). \(m/z\) 382/384 [M+H]\(^+\). To a solution of this product (1.504 g, 3.9 mmol) in ethanol (30 mL) was added methylamine hydrochloride (1.98 g, 29 mmol) and sodium hydrogen carbonate (1.69 g, 20.1 mmol). The mixture was stirred at RT for 18 h, concentrated under vacuum and purified by column chromatography, [silica gel, 1:9 MeOH:DCM] to yield the desired product 39 (0.120 g, 9.2% yield). \(m/z\) 333.25 [M+H]\(^+\).

Cyclopentyl \(N\{-4\-\text{[[2,4-dihydroxy-5-(propan-2-yl)phenyl]carbonyl}\-\text{(methyl)amino}]-\text{methyl]benzyl}\}-L\)-leucinate (CHR-6576): To a solution of 2,4-bis(benzyloxy)-5-(prop-1-en-2-yl)benzoic acid (0.138 g, 0.37 mmol) in DCM (10 mL) was added 39 (0.120 g, 0.36 mmol), DIPEA (1 mL, 5 mmol), HOBt (0.010 g, 0.074 mmol) and EDC (0.094 g, 0.49 mmol). The solution was stirred at RT for 18 h, then loaded directly onto a silica gel column and eluted with 2:98 MeOH:DCM to yield the desired intermediate product (0.164 g, 64% yield). \(m/z\) 689.25 [M+H]\(^+\). To a solution of this product (0.081 g, 0.12 mmol) in EtOAc (5 mL) was added K\(_2\)CO\(_3\) (0.057 g, 0.41 mmol) and palladium on carbon (0.046 g, 0.04 mmol). The reaction vessel was evacuated and filled with hydrogen twice and stirred for 90 minutes. The flask was purged with nitrogen, filtered through Celite and washed with EtOAc. The filtrate was concentrated to yield the desired product (40.0 mg, 66% yield). \(m/z\) 511.25 [M+H]\(^+\). \(^1\)H NMR (300 MHz, CDCl\(_3\)) : 7.36 (2H, d, J=8.1 Hz), 7.24 (2H, d, J=8.1 Hz), 7.09 (1H, s), 6.36 (1H, s), 5.23-5.30 (1H, m), 3.84 (1H, d, J=12.8 Hz), 3.67 (1H, d, J=12.8 Hz), 3.30 (1H, t, J=7.3 Hz), 3.30-3.20 (4H, m), 1.57-1.99 (9H, m), 1.50 (2H, t, J=7.1 Hz), 0.97 (6H, d, J=6.9 Hz), 0.92 (3H, d, J=6.6 Hz), 0.86 (3H, d, J=6.6 Hz).

Scheme 10: Synthesis of CHR-7296
Reagents and conditions: a) 4-hydroxypiperidine, DCM, DIPEA, EDC, RT, 62%.  b) O-t-buty1 serine cyclopentyl ester, STAB, DCE, RT.  c) Pd/C, H₂, K₂CO₃, EtOAc, RT.

[2,4-bis(benzyloxy)-5-(prop-1-en-2-yl)phenyl][4-hydroxypiperidin-1-yl] methanone 40: To a solution of 2,4-bis(benzyloxy)-5-(prop-1-en-2-yl)benzoic acid (2.313 g, 6.2 mmol) in DCM (20 mL) was added 4-hydroxypiperidine (1.11 g, 10.97 mmol), DIPEA (3 mL) and EDC (2.62 g, 13.7 mmol). The solution was stirred at RT for 23hrs, then purified directly by dry flash chromatography (EtOAc) to yield the desired product 40 (1.764 g, 62% yield). m/z 458.25 [M+H]+

1-[[2,4-bis(benzyloxy)-5-(prop-1-en-2-yl)phenyl]carbonyl]piperidin-4-one 41: To a solution of 40 (0.850 g, 1.85 mmol) in DCM (30 mL) was added Dess-Martin periodinane (1.42 g, 3.34 mmol). The reaction was stirred at RT until consumption of the starting material was complete. The reaction was quenched by addition of 1:1 saturated sodium hydrogen carbonate:sodium thiosulfate (30 mL), then extracted with EtOAc (20 mL, 10 mL). The combined organic extracts were dried (MgSO₄) and concentrated to yield the desired product 41 which was used without further purification (containing some excess oxidant). m/z 456 [M+H]+

Cyclopentyl N-1-[[2,4-dihydroxy-5-(propan-2-yl)phenyl]carbonyl] piperidin-4-yl)-L-serinate (CHR-7296): To a solution of 41 (0.150 g, 0.33 mmol) in DCE (10 mL) was added cyclopentyl O-tert-butyl-L-serinate (0.069 g, 0.30 mmol) and STAB (0.127 g, 0.60 mmol). The solution was stirred at RT for 2hrs. An aqueous solution of sodium hydrogen bicarbonate (10 mL) was added and the desired product extracted into EtOAc (3 times 10
mL). The organic layers were combined, dried (MgSO₄) and concentrated. The residue was purified by flash chromatography (100% heptane to 100% EtOAc), to give the product as a clear oil (90.0 mg, 45% yield). m/z 669 [M+H]+

To a solution of the above product (90.0 mg, 0.13 mmol) in EtOAc (12 mL) was added palladium on carbon (9 mg, 10%, 0.8 mmol). The reaction vessel was evacuated and filled with hydrogen twice. The mixture was stirred for 20hrs then purged with nitrogen. Celite was added, and the mixture filtered through Celite, washing with additional EtOAc (3 x 15 ml). The filtrate was then concentrated and purified by HPLC to yield the desired product (18.6 mg, 28% yield). m/z 435 [M+H]+. ¹H NMR (300MHz, CDCl₃) δ: 6.98 (1H, s), 6.35 (1H, s), 5.42-5.29 (1H, m), 4.34 (1H, br s), 4.29 (2H, t, J=3.3Hz), 4.08-4.01 (2H, m), 3.61-3.46 (1H, m), 3.18 (1H, dt, J=6.8, 13.8Hz), 3.01 (2H, t, J=13.0Hz), 2.18 (2H, br s), 2.04-1.93 (2H, m), 1.93-1.60 (8H, m), 1.19 (6H, d, J=7.0Hz).

Biology

Enzyme assays

HSP-90:
A homogeneous time resolved fluorescence assay was used to measure the interaction of the compounds with HSP-90. The assay measures binding of biotinylated geldanamycin (bio-GM) to human recombinant His-tagged HSP-90α in the presence or absence of a competing ligand (added compounds). A signal is generated by fluorescence resonance energy transfer from a Europium-cryptate-labeled anti-His antibody via the HSP-90-bio-GM complex to a fluorescence acceptor (allo-phycocyanin) linked to streptavidin. A preformed complex of HSP90 with the anti-His is added to the compound solution in a 384-well microplate and incubated for 15 minutes. A preformed complex of bio-GM with streptavidin is added to the wells and incubated for 20 hours at room temperature. The final concentrations in the assay are: 50mM Hepes pH 7.3, 50mM NaCl, 100mM KF, 1mM EDTA, 1mM DTT, 0.1% Triton-X-100, 1nM anti-His, 40nM HSP-90, 40nM streptavidin, 40nM bio-GM. Compounds are tested at a final concentration between 0.3-5000nM. IC₅₀ values are calculated by non-linear least squares fitting to the standard dose-response model.

IKKβ:
The ability of compounds to inhibit IKKβ activity was measured in a Z'™-LYTE biochemical assay performed by Invitrogen (Paisley, UK). The final kinase reaction consisted of 0.9-
8.0 ng IKKβ, 2 μM peptide substrate and 5 μM ATP (~km for ATP) in 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl₂ and 1 mM EGTA, plus test compounds. After 60 mins at room temperature, the development reagent was added and the assay plate was incubated for a further 60 minutes at room temperature before fluorescence was measured on a plate reader.

Duplicate data points (10 per experiment) were generated from a 1/3 log dilution series of a stock solution of test compound in DMSO. Data were collected and analysed using XLfit software from IDBS. The dose-response curve was curve fitted to model number 205 (sigmoidal dose-response model). From the curve generated, the concentration giving 50% inhibition was determined. The experiment was repeated twice.

**HDAC:**
The ability of compounds to inhibit HDAC activity was monitored using the Fluor de Lys kit (Fluorogenic Histone Deacetylase Lysyl Substrate/Developer) according to the manufacturer’s instructions (Biomol). The enzyme preparation used was a Hela cell nuclear extract from Cilbiotech S.A. (Mons, Belgium). Briefly, compounds were equilibrated with the nuclear extract for 5 min at room temperature prior to addition of 62.5 μM Fluor de Lys substrate for 15 min. After stopping the reaction by adding 2 μM trichostatin A and Fluor de Lys Developer, fluorescence was read on a Wallac Victor II plate reader using the manufacturer’s protocol. The assay was conducted in duplicate using 8 (3-fold) compound dilutions to construct a concentration-response curve. It was repeated at least twice.

**p38 MAP kinase α:**
The ability of compounds to inhibit p38 MAP kinase α activity was determined in a coupled reaction using Invitrogen’s Z'-lyte® system at Invitrogen (Paisley, UK). A mixture of p38 MAP kinase α (0.003 - 0.01 ng), inactive MAPKAPK-2 (5 ng) and MAPKAPK-2 substrate peptide (2 μM) in 50 mM HEPES pH 7.5, 0.01% BRIJ-35, 10 mM MgCl₂ and 1 mM EGTA is incubated with compound plus 100 μM ATP for 1 hour at 37°C prior to detection of the phosphorylated peptide product. The assay, which was repeated at least twice, was conducted in duplicate using 10 (3-fold) compound dilutions to construct a concentration-response curve.

**Cytokine gene primers used for qPCR**

| GAPDH forward | GAA GGT GAA GGT CGG AGT C |
Assessment of the effect of drugs on anisomycin-stimulated MAPKAPK-2 phosphorylation in intact U-937 and HuT-78 cells

U-937 and HuT-78, grown in RPMI1640, with 10% foetal calf serum, 1% glutamine and 1% penicillin/streptomycin were treated with vehicle/compound for 4 hours before adding anisomycin (10μM, 30 min) or vehicle. Cells were harvested on ice, washed twice and lysed in SDS lysis buffer (62.5mM Tris, pH 6.8, 2% SDS, 10% glycerol, 50mM DTT supplemented with protease and phosphatase inhibitors according to the manufacturer’s instructions). Following sonication and centrifugation to remove cell debris, samples were run on 10% BT MOPS gels (Novex/Invitrogen). Phosphorylation of MAPKAPK-2 was measured from the resulting nitrocellulose membranes following the manufacturer’s instructions for the specific primary antibody (Cell Signalling Technology, Danvers, USA). GAPDH was used as a loading control. Visualisation of the signal was carried out using ECL reagent and film (GE Healthcare, Amersham, UK).

Human blood cell hCE-1 expression: FACS determination of cellular hCE-1 immunoreactivity

Human blood samples were collected into sodium heparin. Following dilution into Phosflow buffer (BD Biosciences), and centrifugation, pelleted cells were washed with
phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin and 0.1% (w/v) sodium azide. After re-centrifugation, the Fc receptor on pelleted cells was blocked by incubation with human IgG (100μg/ml) for 10 minutes at room temperature. Following surface staining with CD14-APC and CD66-PE (both BD Biosciences) by incubation for 30 minutes at room temperature in the dark, cells were washed twice, permeabilised with BD PhosFlow Permeabilisation buffer (ice, 30mins) and incubated either with a pre-bleed control or an hCE-1-specific rabbit polyclonal antibody preparation. Following 45 minutes at room temperature in the dark, an FITC-conjugated secondary antibody (Goat anti-rabbit IgG, Jackson ImmunoResearch) was added, incubated for the same time and cells washed as before. Lastly, cells were resuspended in phosphate-buffered saline and analysed in a BD FACS Canto.

**Experimental animals**

The hCE1 knock-in transgenic mouse was generated by Genoway (Lyon, France) by targeted insertion of the expression cassette into the expression permissive hprt locus on the X-chromosome, by homologous recombination. Expression of the hCE1 transgene was driven by the human CD68 promoter (provided by Dr David Greaves, Oxford University, UK), which has previously been shown to direct transgene expression in macrophages of transgenic mice (24). Briefly, a targeting vector consisting of the CD68 promoter plus hCE1 transgene was introduced into 129ola E14 embryonic stem (ES) cells by electroporation. Recombinant ES cell clones, selected by growth on HAT medium and validated by Southern blot, were injected into C57BL/6J blastocysts which were subsequently re-implanted into pseudo-pregnant females. Male chimeras were selected by coat colour and crossed with wild-type DBA/1J females. The F1 progeny were backcrossed to DBA/1J animals, establishing a colony generating hemizygous transgenic and matched wild-type male controls for study. The genetic background of the colony was approximately 95% DBA/1J, 5% 129ola.

**Pharmacokinetic/pharmacodynamic studies in transgenic animals**

Wild-type or hemizygous hCE-1 ‘knock-in’ transgenic male mice (littermate animals), three animals per time-point, were treated with CHR-4487 for a range of times between 2 and 420 minutes. At the appropriate time points, animals were euthanized by cervical dislocation or CO₂ inhalation, 0.5ml blood taken by cardiac puncture and added to chilled tubes containing a dried mixture of sodium fluoride/EDTA (free acid) to give a final concentration of 10% (w/v) of each in the blood. After gentle mixing, 0.1 ml samples were removed and added to separate pre-chilled cryovials containing 0.1 ml of cold water
supplemented with 10% (w/v) sodium fluoride and EDTA, which were then snap-frozen. Upon thawing, 0.05ml samples of blood extract were added to an equal volume of 0.1% (v/v) formic acid, mixed and 0.3ml acetonitrile added. Samples were then centrifuged and the content of ester (CHR-4487) and its product acid (CHR-4495) measured by LC/MS/MS (Sciex API3000). Chromatography was based on an AceCN (75*21mm) column with a 5-95% (v/v) acetonitrile, 0.1% (v/v) formic acid mobile phase.

**Mouse blood cell acetylation studies following i.p. dosing in hCE-1 ‘knock-in’ transgenic mice**

Hemizygous male hCE-1 transgenic animals (3 animals per time-point; 6 untreated controls) were treated with CHR-4487 (10mg/kg, i.p.), as described above, for a variety of times. At the appropriate time-points, animals were euthanized by cervical dislocation or CO₂ inhalation, 0.3ml blood taken by cardiac puncture and added to lithium heparin tubes. After blocking Fc receptors (10 mins, room temperature, mouse Fc block antibodies (BD Biosciences), surface antigens were stained by incubation for 15mins with CD11b-A647 (Serotec) and LY6G-PE (BD Biosciences). 5ml of Phosflow lyse/fix buffer was added and the samples were incubated for 10mins at 37°C. After mixing, samples were centrifuged (400g; 8mins; 10°C), supernatants decanted and 2ml of stain/wash buffer added. Samples were then permeabilised with Phosflow II buffer (BD Biosciences) for 30mins on ice and then incubated with primary antibody (rabbit polyclonal anti-lysine (Cell Signalling 9441) for 45mins at room temperature, washed and then incubated with secondary antibody (goat anti-rabbit IgG-FITC, Jackson Labs). Following washing, samples were analysed using a BD FACS Canto. Monocytes were detected as CD11b⁺Ly6G⁻; Granulocytes were detected as CD11b⁺Ly6G⁺; Lymphocytes were detected as CD11b⁻Ly6G⁻.

**In vitro mouse blood cell acetylation studies**

Blood was obtained from either wild-type or hemizygous hCE-1 ‘knock-in’ transgenic male mice (littermate animals). It was diluted 1:1 with RPMI-1640 and incubated with compounds (SAHA or CHR-4487) at the appropriate concentrations for 6hrs, with rotation at 10 rpm. Protein acetylation was determined in the monocyte, granulocyte and lymphocyte populations as described immediately above.

**Mouse anti-collagen antibody arthritis model**

An anti-collagen antibody-induced arthritis model was conducted in littermate wild-type or hCE-1 transgenic mice. The disease was initiated on day 0 by injection of the monoclonal antibody cocktail (4 mg/mouse, i.v.). Three days later, LPS (50μg/mouse) was injected
(i.p.) to stimulate clinical arthritis development. Drugs were administered once-daily i.p. from day 0 until day 11. Animals (10 per group) were scored on day 0 and from day 3 onwards for signs on inflammation in one or more joints and body weights were monitored throughout. All paws (front left & right and rear left & right) of each animal were examined for signs of arthritogenic responses prior to arthritis induction on study day 0 (study commencement) and, subsequently, once daily from day 3 until day 12 (study termination). Arthritis reactions were scored and recorded according to a 0-4 scale in ascending order of severity as shown below:

**Arthritis Score**

<table>
<thead>
<tr>
<th>Arthritis Score</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reaction, normal</td>
<td>0</td>
</tr>
<tr>
<td>Mild, but definite redness and swelling of the ankle/wrist or apparent redness and swelling limited to individual digits, regardless of the number of affected digits</td>
<td>1</td>
</tr>
<tr>
<td>Moderate to severe redness and swelling of the ankle/wrist</td>
<td>2</td>
</tr>
<tr>
<td>Redness and swelling of the entire paw including digits</td>
<td>3</td>
</tr>
<tr>
<td>Maximally inflamed limb with involvement of multiple joints</td>
<td>4</td>
</tr>
</tbody>
</table>

**Histology**

Front and rear paws were collected from each mouse into neutral formalin buffer (10%). Following fixation for at least a week, the left fore paws were transferred into decalcification buffer. Each left wrist joint complex was trimmed and embedded in paraffin. A single slide of 6 μm thickness was cut and stained with Hematoxylin/Eosin (H&E). Histopathological changes in the joints were described and graded using the following scoring method:

**Infiltrate**

0: No infiltrate detected
1: Modest leukocyte infiltrate in synovial tissue, no fluid leukocytes
2: Moderate leukocyte infiltrate in synovial tissue and in fluid phase with loss of synovial architecture
3: Gross leukocyte infiltrate in synovial membrane and fluid space with significant loss of synovial and articular architecture.

Hyperplasia
0: No abnormalities are detected
1: Synovial lining layer 2-4 cells thick
2: Synovial lining layer >5 cells thick associated with moderate expansion of the sublining layer zone.
3: Synovial lining layer >5 cells thick associated with significant expansion of the sublining layer zone and potentially with loss of synovial architecture.

Erosion of cartilage / bone
0: No abnormalities are detected
1: Fibrillation of cartilage and / or mild erosive infiltration of periosteal and subchondral bone. Nuclei intact within lacunae.
2: Moderate fibrillation and loss of cartilage and / or moderate erosive infiltration of periosteal and subchondral bone. Nuclei may show apoptosis within lacunae.
3: Significant loss of cartilage and / or erosive infiltration of periosteal and subchondral bone. Nuclei show apoptosis within lacunae across a wide area of cartilage / bone.