

CHR-2797: An Antiproliferative Aminopeptidase Inhibitor that Leads to Amino Acid Deprivation in Human Leukemic Cells

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Abstract

CHR-2797 is a novel metalloenzyme inhibitor that is converted into a pharmacologically active acid product (CHR-79888) inside cells. CHR-79888 is a potent inhibitor of a number of intracellular aminopeptidases, including leucine aminopeptidase. CHR-2797 exerts antiproliferative effects against a range of tumor cell lines *in vitro* and *in vivo* and shows selectivity for transformed over nontransformed cells. Its antiproliferative effects are at least 300 times more potent than the prototypical aminopeptidase inhibitor, bestatin. However, the mechanism by which inhibition of these enzymes leads to proliferative changes is not understood. Gene expression microarrays were used to profile changes in mRNA expression levels in the human promyelocytic leukemia cell line HL-60 treated with CHR-2797. This analysis showed that CHR-2797 treatment induced a transcriptional response indicative of amino acid depletion, the amino acid deprivation response, which involves up-regulation of amino acid synthetic genes, transporters, and tRNA synthetases. These changes were confirmed in other leukemic cell lines sensitive to the antiproliferative effects of CHR-2797. Furthermore, CHR-2797 treatment inhibited phosphorylation of mTOR substrates and reduced protein synthesis in HL-60 cells, both also indicative of amino acid depletion. Treatment with CHR-2797 led to an increase in the concentration of intracellular small peptides, the substrates of aminopeptidases. It is suggested that aminopeptidase inhibitors, such as CHR-2797 and bestatin, deplete sensitive tumor cells of amino acids by blocking protein recycling, and this generates an antiproliferative effect. CHR-2797 is orally bioavailable and currently undergoing phase II clinical investigation in the treatment of myeloid leukemia. [Cancer Res 2008;68(16):6669–79]

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Introduction

A number of classes of Zn²⁺-metalloenzyme [e.g., matrix metalloproteinases (MMP), aminopeptidases] have been implicated in the regulation of tumor growth (1–3). As a class, MMP inhibitors have not shown direct antiproliferative effects against human cancer cells. One exception, however, is batimastat (BB-94), which has been shown to exert an antiproliferative effect on the human melanoma cell line RPMI-7951 (4). This observation led to the discovery of a novel class of aminopeptidase inhibitor exemplified by CHR-2797, which shows a powerful antiproliferative effect on both hematopoietic and epithelial tumor cell lines.

Aminopeptidases catalyze the sequential removal of amino acids from the amino-terminus of peptide substrates (5). Functions of these enzymes include the regulation of biologically active peptides, removal of the NH₂ terminal methionine of newly synthesized proteins, trimming of antigens for MHC class I presentation, and protein recycling (5–8). The aminopeptidase inhibitor, bestatin, is a potent inhibitor of a number of metalloproteinase (M) family aminopeptidases (9) and exerts antiproliferative effects across a range of human tumor cell lines (10). Furthermore, it has shown clinical efficacy in nonlymphocytic leukemia and squamous cell lung carcinoma (3, 11).

The mechanism by which inhibition of aminopeptidases leads to anticancer effects is not understood. In this study, gene expression microarrays were used to profile changes in mRNA expression in a human promyelocytic leukemia cell line (HL-60) treated with CHR-2797. By comparing the changes seen with those induced by treatment with the chemically dissimilar aminopeptidase inhibitor, bestatin, and with an inactive analogue of CHR-2797, we sought to discriminate between on-target and off-target effects of the compound.

We provide evidence that agents, such as CHR-2797, exert their cellular effects through depletion of intracellular amino acids. Aminopeptidase inhibition led to the appearance of intracellular small peptides, the substrates of aminopeptidases, and, in sensitive cells, to the induction of a well-defined transcriptional response, the amino acid deprivation response (AADR), which involves up-regulation of amino acid synthetic genes, transporters, and tRNA synthetases. Aminopeptidase inhibition also reduced phosphorylation of mTOR substrates and rates of protein synthesis, both indicative of amino acid depletion. Taken together, these data suggest that drugs such as CHR-2797 and bestatin are able to deplete many leukemic cells of intracellular amino acids, despite their presence at high concentrations outside the cell. The control of amino acid provision is emerging as a powerful physiologic and pharmacologic mechanism for limiting cell proliferation.

Materials and Methods

Materials. CHR-2797 (2S-[2R-(S-hydroxy-hydroxycarbonyl-methyl)-4-methylpentanoyl-amino]-2-phenylethanoic acid cyclopentyl ester), CHR-79888 ((2S-[2R-(S-hydroxy-hydroxycarbonyl-methyl)-4-methylpentanoylamino]-2-phenyl-ethanoic acid)), CHR-5346 (2S-[2R-(S-hydroxy-hydroxycarbonyl-methyl)-4-methylpentanoylamino]-3,3-dimethylbutanoic acid cyclopentyl ester), CHR-3204 (2S-[2R-(S-hydroxy-carbamoylmethyl)-4-methylpentanoylamino]-2-phenylethanoic acid cyclopentyl ester), and CHR-5515 (2S-[2R-(S-hydroxy-hydroxycarbonyl-methyl)-4-methylpentanoyl-amino]-3,3-dimethylbutanoic acid) were synthesized by published methods (12) at Chroma Therapeutics Ltd. Batimastat was synthesized at British Biotech Pharmaceuticals Ltd. All reagents were from Sigma, unless otherwise stated. Bestatin hydrochloride was from Cayman Chemical. Bromodeoxyuridine (BrdUrd) and antimouse BrdUrd-FITC were from Becton Dickinson.

Full-length recombinant human leukotriene A₄ (LTA₄) hydrolase and methionine aminopeptidase-2 (MetAP-2) were expressed in baculovirus-infected insect cells and affinity purified via a COOH terminal FLAG-tag. Full-length recombinant human puromycin-sensitive aminopeptidase (PuSA) was expressed in COS cells and affinity purified via a COOH terminal FLAG-tag. Recombinant human aminopeptidase N and leucine aminopeptidase (LAP) from porcine kidney were obtained from Sigma. Full-length recombinant human puromycin-insensitive leucine aminopeptidase (PILSAP) was obtained from R&D Systems. Aminopeptidase B was a partially purified preparation obtained by Mono-Q fractionation of Jurkat cell cytoplasm.

In vitro enzyme assays. *In vitro* enzyme assays were conducted as described in Supplementary Information.

Cell culture. Cell lines were from the American Type Culture Collection (LGC Promochem). Cell culture media and supplements were from Sigma.

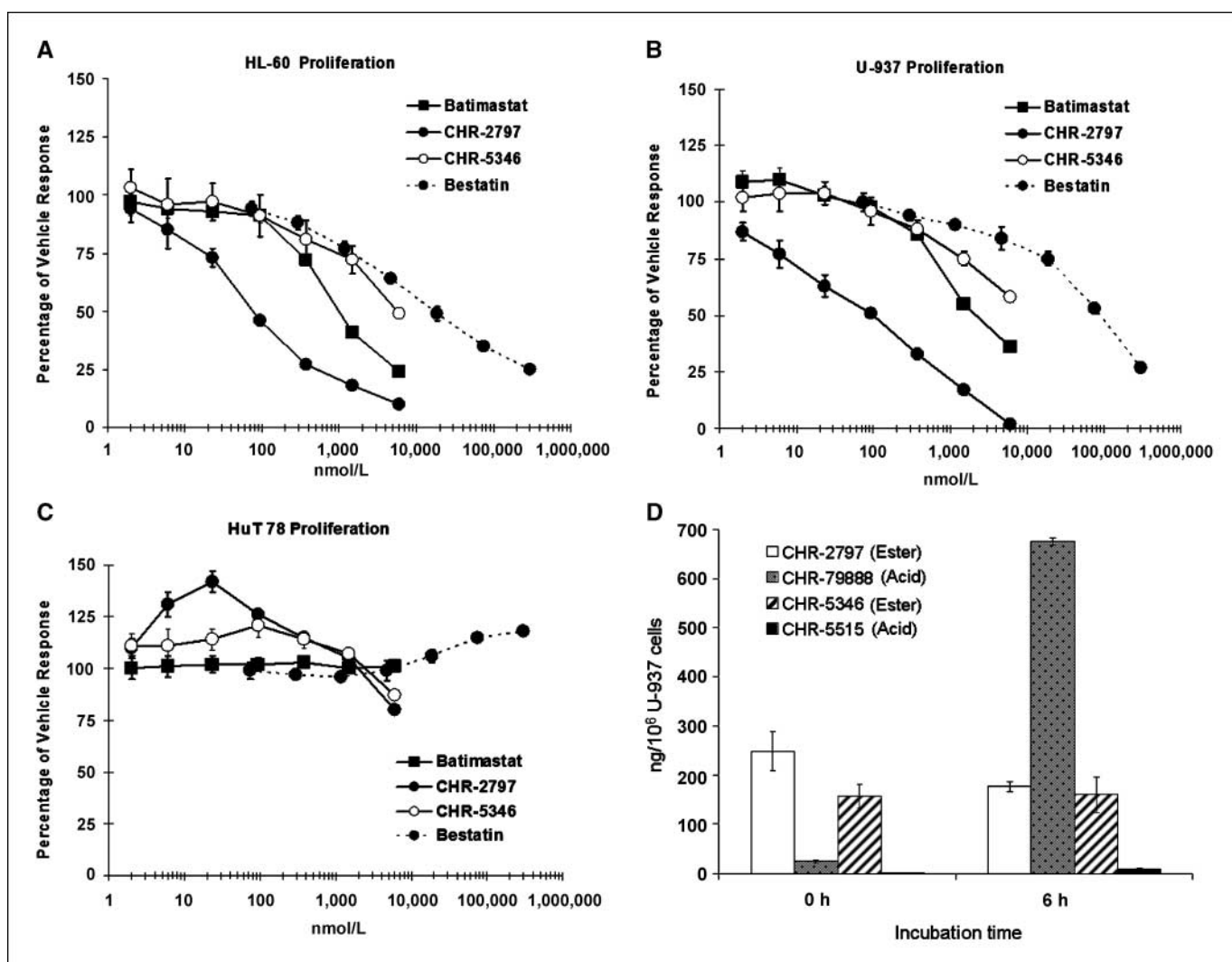
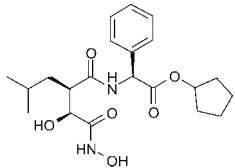
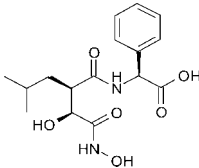
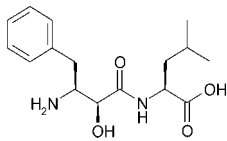


Figure 1. Inhibition of the proliferation (A, B, C) of U-937 and other human tumor cells by CHR-2797 and related agents in relation to intracellular acid formation in U-937 cells (D). Data shown are log concentration-response curves for inhibition of cell proliferation, as measured by [³H]thymidine incorporation, for CHR-2797 (●-●), Batimastat (■-■), CHR-5346 (○-○), and Bestatin (●---●). A, top left, HL-60 cells; B, top right, U-937 cells; C, bottom left, HuT 78 cells; D, amount of esters (open column, CHR-2797; striped column, CHR-5346) and acids (stippled column, CHR-79888; filled column, CHR-5515) associated with U-937 cells after 0 or 6-h incubation of the cells with the two esters. Statistically significant increases at 6 h in cell-associated CHR-79888 were seen (unpaired *t* test, *P* < 0.0001). Cells (4×10^6 /mL) were incubated at 37°C in culture medium containing 6 μmol/L compound in a 5% (v/v) CO₂-humidified atmosphere. Incubations were terminated after 6 h by centrifugation of 25 mL aliquots of the cell suspension at $300 \times g$ for 5 min at 4°C. 0.2-mL samples of the culture media supernatants were added to four volumes of acetonitrile. After decanting the supernatant, the residual cell pellet (10^6 cells) was extracted into 1 mL of acetonitrile. Samples were then analyzed for the ester and acid metabolite at room temperature by LC/MS/MS (Sciex API3000). Chromatography was based on an AceCN (75 × 21 mm) column with a 5% to 95% (v/v) acetonitrile, 0.1% (v/v) formic acid mobile phase. For the zero time samples, the cell suspension was chilled and centrifuged as soon as the ester had been added and then extracted into acetonitrile as described. The small amount of acid (CHR-79888) associated with the U-937 cells at zero time reflects acid formed within the cell during the separation of cells and medium.

Table 1. Chemical structures, aminopeptidase inhibition, and inhibition of tumor cell growth by CHR-2797

A					
Aminopeptidase	CHR-2797	CHR-79888	Bestatin		
					
PuSA	150	850	350		
LTA ₄ hydrolase	>10,000	8	200		
Aminopeptidase N	220	190	300		
PILSAP	>5,000	>5,000	>5,000		
Aminopeptidase B	>1,000	>1,000	1,000		
LAP	100	30	4		
MetAP-2	>30,000	>30,000	—		
B					
Cell line	Tumor type	IC ₅₀ (nmol/L)	K-Ras	PTEN	TP53
U-937	Histiocytic lymphoma	10		mut	mut
HL-60	Promyelocytic leukemia	30	wt	wt	mut
KG-1	Acute myelogenous leukemia	15	wt	wt	mut
RPMI 8226	Myeloma	330	mut	wt	mut
MEG-01	Megakaryoblastic leukemia	400	wt	wt	wt
HNT-34	Acute myelogenous leukemia	35			
AML-193	Acute monocytic leukemia	770	wt	wt	wt
KU812	Chronic myelogenous leukemia	100	wt	wt	wt
MV-4-11	Biphenotypic myelomonocytic leukemia	305	wt	wt	wt
GDM-1	Myelomonoblastic leukemia	15	wt	wt	wt
HEL 92.1.7	Erythroleukemia	10,000			
CCRF-CEM	Acute lymphoblastic leukemia	3,800	mut	mut	mut
MOLT-4	Acute lymphoblastic leukemia	1,450	wt	mut	mut
HuT 78	Cutaneous T cell lymphoma	>10,000	wt	wt	mut
Jurkat E6-1	Acute T cell leukemia	>10,000		mut	mut

NOTE: (A) Aminopeptidase assays were conducted in the presence of the compounds shown. IC₅₀ values (nmol/L) represent the mean of two to three independent experiments involving at least six concentrations of drug per experiment. (B) Inhibition of the proliferation of human cancer cell lines was measured by [³H]thymidine incorporation. Plates were formatted so that there were six replicates for each concentration of compound to be tested. The data presented are the means of at least two separate experiments. Abbreviations: wt, wild type; mut, mutant.

Fetal bovine serum (FBS) was from HyClone. For amino acid deprivation, cells were grown in amino acid-free RPMI 1640 supplemented with 10% (v/v) dialyzed FBS.

Cellular proliferation assays. Cells were seeded in 96-well BD-Falcon plates (Becton Dickinson) at a density of 1 to 5 × 10³ cells per well in the appropriate serum-containing culture medium and cultured at 37°C in a humidified 5% (v/v) CO₂ incubator for 24 h. Compounds were diluted in the relevant culture medium and added to the wells for a further 72 h. During the final 4 h of this incubation, cells were pulsed with 0.4 μCi/well of [³H]thymidine (specific activity, 5 mCi/mmol; Amersham Biosciences), harvested onto GF/C glass fiber filter mats (Perkin-Elmer) using a Tomtec harvester, and counted on a 1450 MicroBeta scintillation counter (Perkin-Elmer) to determine the amount of [³H]thymidine incorporated into cellular DNA.

In vitro studies of drug de-esterification and acid accumulation. Intracellular hydrolysis of CHR-2797 or CHR-5346 was measured by LC/MS/MS as described in the legend to Fig. 1D.

RNA extraction and gene expression microarray studies. Biotin-labeled cRNA was synthesized from purified total RNA (Qiagen) and fragmented before hybridization to Affymetrix Human Genome U133 Plus 2.0 arrays (Almac Diagnostics Ltd.) using manufacturer's standard protocols.

Real-time quantitative PCR. cDNA was synthesized from total RNA using Superscript III reverse transcriptase (Invitrogen) and anchored oligo-dT primers (Abgene). For all genes (except *TRIB3*; TaqMan Gene Expression Assay), SYBR Green quantitative PCR was performed in triplicate using the 7300 Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were 95°C for 10min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as the endogenous control gene. PCR primer sequences are detailed in Supplementary Table S5.

Protein extraction, SDS-PAGE, and immunoblotting. Cell pellets were lysed in radioimmunoprecipitation assay buffer [50mmol/L Tris-HCl (pH 7.5), 300 mmol/L NaCl, 1% NP-40 Alternative (Calbiochem), 0.1%

SDS, 0.5% deoxycholate, 1mmol/L EDTA, 1mmol/L DTT, protease, and phosphatase inhibitors (Roche)] on ice for 15 min, briefly sonicated and centrifuged at $16,000 \times g$ for 15 min at 4°C . Protein extracts (10 μg) were resolved by SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline/0.1% Tween (TBS-T) for 1 h, incubated with primary antibody overnight at 4°C , washed in TBS-T, and incubated with horseradish peroxidase-labeled secondary antibodies (Cell Signaling Technology) for 1 h at room temperature. Detection was achieved using an enhanced chemiluminescence reagent (Amersham Biosciences).

Protein synthesis. Cells were seeded at a density of $4 \times 10^4/\text{mL}$, cultured for 24 h, then treated with 0.06 to 6 $\mu\text{mol/L}$ CHR-2797 for 24 h or 20 $\mu\text{g/mL}$ cycloheximide for 2 h. After treatment, 5×10^4 cells were washed with PBS and seeded in 100 μL Cys/Met-free RPMI 1640 containing compound, supplemented with 10% dialyzed FBS. 1.5 μCi [^{35}S]Cys/Met (Amersham Biosciences; $>1,000$ Ci/mmol) was added, and incubation continued for 1 h at 37°C . Cells were captured onto 96-well GF/B filter plates (Millipore) and washed twice with PBS before precipitation with 10% ice-cold trichloroacetic acid (TCA) for 1 h at 4°C . Precipitated proteins were washed four times with ice-cold 10% TCA and air-dried for 1 h. UltimaGold scintillation cocktail (25 μL ; PerkinElmer) was added and allowed to mix for 1 h before scintillation counting using a 1450 MicroBeta scintillation counter.

Results

In vitro studies. The MMP inhibitor batimastat (Fig. 1A–C) inhibited the proliferation of a number of human tumor cell lines *in vitro* (4). This was surprising as MMP inhibitors, in general, have not been reported to exhibit antiproliferative effects (13). Because, despite considerable effort, the antiproliferative effect of batimastat and related agents could not initially be linked to inhibition of any one intracellular metalloenzyme, compounds were optimized using a cell-based assay: inhibition of U-937 cell proliferation. This led to the synthesis of CHR-2797, an hydroxamate-containing ester which was a potent inhibitor of tumor cell proliferation (Table 1A and B; Figs. 1A–C; Supplementary Table S1A).

The acid derived from CHR-2797, CHR-79888 (Table 1A), was a much weaker antiproliferative agent (IC_{50} versus U-937, HL-60, and HuT 78 cells, >10 $\mu\text{mol/L}$; data not shown). Because charged acids often have low ability to penetrate cells, this observation suggested that an intracellular target might be responsible for the pharmacologic action of these agents. This hypothesis was substantiated by studies investigating the fate of CHR-2797 upon incubation with human cells. Incubation of CHR-2797 with U-937 cells leads to its de-esterification and a time-dependent cell accumulation of the acid CHR-79888 derived from this agent (Fig. 1D). Certain related esters, such as CHR-5346, in which the phenylglycine side chain of CHR-2797 is replaced by a *t*-butylglycine group, are not hydrolyzed by intracellular esterases and generate no charged acid (CHR-5515) inside cells (Fig. 1D). As shown in Fig. 1A–C, CHR-5346 was a much weaker antiproliferative agent than CHR-2797. For a large number of related esters, generation of ester-derived acids inside the cell was essential for potent *in vitro* antiproliferative activity (data not shown).

A range of intracellular metalloenzymes have been described, including the aminopeptidases, thimet oligopeptidase, and dipeptidyl peptidase III. Attention was drawn to the aminopeptidase family as a potential source of the CHR-2797 antiproliferative target(s) by data on the aminopeptidase inhibitors, bestatin and puromycin. These agents have been shown to exert an antiproliferative effect on a number of human tumor cell lines *in vitro* (10, 14). When CHR-2797 was tested in aminopeptidase assays, it

was found to inhibit a number of these enzymes (Table 1A). In particular, the intracellular aminopeptidases, LTA_4 hydrolase, LAP, and PuSA, were all potently inhibited by the intracellularly accumulating acid CHR-79888 and are, therefore, potential drug targets. All antiproliferative agents related to CHR-2797 retained the ability to block these aminopeptidases. Other enzymes that have been reported to be involved in tumor growth, such as aminopeptidase N, PILSAP, and MetAP-2, were either insensitive to CHR-2797 and its acid (PILSAP, MetAP-2) or exhibit an inappropriate cellular location (AP-N; extracellular) to be considered target candidates (Table 1A).

In general, myeloid leukaemic breast and lung cancer cell lines were the most sensitive to CHR-2797 (Table 1B; Supplementary Table S1A). Lymphoma lines, such as HuT 78, were very insensitive whether proliferation was measured by thymidine incorporation (Fig. 1C), WST-1 assay, or cell counting (Supplementary Tables S1B and S1C). However, in this cell line [^3H]thymidine incorporation is uniquely and reliably enhanced by exposure to CHR-2797 and related analogues (Fig. 1C). Although the reasons for this effect remain unclear, exposure to bestatin produces a similar stimulatory effect (Fig. 1C), emphasizing the qualitative, but not quantitative, similarity between these agents (bestatin is usually 300–1,000 times weaker than CHR-2797 as an antiproliferative agent; Fig. 1A–C). There was no obvious correlation between sensitivity to CHR-2797 and the mutational status of p53, PTEN, or K-Ras in cells (Table 1B and Supplementary Table S1A). Nontransformed stromal cells, such as fibroblasts (e.g., MrC5 or NRK), were found to be more resistant to the inhibitory effects of CHR-2797 than their oncogenically transformed counterparts MrC5-SV2 or K-ras NRK, an effect not seen with chemotherapeutic agents (Supplementary Table S2).

Flow cytometric analyses of the DNA profile of sensitive and insensitive leukemic cells after incubation with CHR-2797 or its nonhydrolyzable analogue CHR-5346 have been conducted. Results of bivariate propidium iodide (PI)–BrdUrd analysis are summarized in Supplementary Table S3 and Supplementary Fig. S1. Pulsed BrdUrd labeling of drug-treated cells, in combination with PI staining, allowed an estimate of the active (BrdUrd⁺) and inactive (BrdUrd[−]) S-phase fraction and the rate of DNA synthesis across the entire S phase (including the $\text{G}_1\text{-S}$ and $\text{S-G}_2\text{M}$ boundaries). In agreement with [^3H]thymidine incorporation data, HL-60 and U-937 cells showed a dramatic decrease in the active S-phase fraction with no corresponding increase in the inactive S-phase compartment (Supplementary Table S3). In HL-60 cells, this was accompanied by a modest accumulation in G_1 . U-937 cells showed only minimal G_1 accumulation but showed a significant apoptotic sub- G_1 fraction. The absence of a significant increase in the inactive S-phase fraction in either cell type suggests that the decline in active S is a consequence of reduced feed-through from G_1 . In the case of HL-60 cells, this may explain the G_1 -specific accumulation (Supplementary Table S3). In U-937 cells, apoptosis from G_1 phase could explain the sub- G_1 DNA profile and the absence of any appreciable G_1 -specific cytokinetic effect. Apoptosis induced by CHR-2797 in U-937 cells was also readily apparent when assessed by Annexin V labeling (Supplementary Fig. S2). No appreciable cytokinetic effects or apoptosis were seen in the insensitive HuT 78 cell line (Supplementary Table S3 and Supplementary Fig. S1).

In vivo studies. The efficacy of CHR-2797 has been investigated in a range of *in vivo* tumor models, including syngeneic rat and human tumor xenografts. CHR-2797 is active as an anticancer agent *in vivo* in rodent cancer models, and a dose-response

Table 2. Selected gene expression changes in HL-60 cells treated with 6 $\mu\text{mol/L}$ CHR-2797 or deprived of extracellular amino acids

Gene symbol	Description	CHR-2797 6 h	CHR-2797 24 h	w/o AAs 6 h
<i>CLEC7A</i>	C-type lectin domain family 7, member A	6.7	29.5	6.3
<i>STC2</i>	Stanniocalcin 2	13.3	15.3	3.7
<i>RETN</i>	Resistin	1.4	15.0	0.7
<i>CCNG2</i>	Cyclin G2	5.1	13.9	4.5
<i>DDIT4</i>	DNA-damage-inducible transcript 4 (REDD1)	18.7	13.8	4.0
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.7	11.7	3.0
<i>CTH</i>	Cystathionine γ -lyase	17.9	11.3	1.7
<i>SES2</i>	Sestrin 2	14.0	10.0	3.9
<i>PPP1R15A</i>	Protein phosphatase 1, regulatory (inhibitor) subunit 15A (GADD34)	4.5	7.5	5.9
<i>ATF5</i>	Activating transcription factor 5	4.2	7.1	1.9
<i>TRIB3</i>	Tribbles homologue 3	4.7	6.7	2.6
<i>MGC4504</i>	Hypothetical protein	11.9	6.0	5.1
<i>ATF3</i>	Activating transcription factor 3	8.1	5.9	3.9
<i>VEGF</i>	Vascular endothelial growth factor	5.8	5.4	2.3
<i>FYN</i>	Protooncogene tyrosine protein kinase Fyn	4.6	5.1	2.4
<i>ASNS</i>	Asparagine synthetase	4.4	5.1	1.8
<i>DDIT3</i>	DNA-damage-inducible transcript 3 (CHOP, GADD153)	5.2	4.8	3.8
<i>WARS</i>	Tryptophanyl-tRNA synthetase	1.9	4.2	1.2
<i>SLC7A11</i>	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	7.5	3.8	4.8
<i>ASS</i>	Argininosuccinate synthetase	1.6	3.7	0.7
<i>CBS</i>	Cystathionine β -synthase	3.8	3.4	1.6
<i>SLC38A2</i>	Solute carrier family 38, member 2 (SNAT2)	4.0	3.1	3.4
<i>IL-8</i>	Interleukin 8	3.8	3.0	4.6
<i>GADD45A</i>	Growth arrest and DNA-damage-inducible, α (DDIT1)	3.5	2.9	1.0
<i>PSAT1</i>	Phosphoserine aminotransferase 1	2.9	2.2	1.3
<i>CARS</i>	Cysteinyl-tRNA synthetase	2.2	2.0	2.8
<i>CEBPB</i>	CCAAT/enhancer binding protein (C/EBP), β	1.2	1.9	2.9
<i>SARS</i>	Seryl-tRNA synthetase	1.6	1.9	1.1
<i>MARS</i>	Methionine-tRNA synthetase	2.1	1.4	1.4

NOTE: HL-60 cells were treated with 6 $\mu\text{mol/L}$ CHR-2797 for 6 and 24 h or deprived of extracellular amino acids for 6 h. Gene expression was measured by real-time quantitative PCR and compared with cells treated with 0.06% (v/v) DMSO (for CHR-2797 treatment) or cells grown in normal growth medium supplemented with dialyzed FBS (for amino acid deprivation). Results for CHR-2797 were generated from the same pooled triplicate samples used for the gene expression microarray experiments, and those for amino acid deprivation are representative of at least three independent experiments. Abbreviation: w/o AAs, deprived of extracellular amino acids.

relationship has been shown in two models. These data are summarized in the Supplementary Section of this paper (Supplementary Fig. S3). The effect of CHR-2797 is less apparent when the tumor burden is higher before treatment (Supplementary Fig. S4).

Gene expression studies in cells treated with CHR-2797. The mechanistic link between aminopeptidase inhibition and anti-proliferative effects was investigated by gene expression profiling in HL-60 cells treated with CHR-2797. Cells were treated with vehicle or 6 $\mu\text{mol/L}$ CHR-2797, equivalent to 200 \times the IC_{50} for its inhibition of proliferation (30 nmol/L; Fig. 1A), for 6 and 24 h, and gene expression changes were analyzed using Affymetrix Whole Genome U133 Plus 2.0 microarrays. This concentration is comparable with peak plasma levels seen in human subjects dosed with CHR-2797 in on-going clinical trials.⁴ The complete list of gene expression changes is included in Supplementary Table S4.

Approximately, 40% of the genes represented on the microarray were expressed in HL-60 cells, and the expression of 2461 of these

were increased or decreased by ≥ 2 -fold after treatment with CHR-2797. Many of the up-regulated genes function in amino acid transport (e.g., *SLC7A11*, cationic amino acid transporter, y+ system; *SLC38A2*, solute carrier family 38, member 2) and metabolic pathways (e.g., *ASNS*, *CBS*, *CTH*; Table 2). Genes in these pathways have been shown to be induced as part of an AADR, a transcriptional response mounted by cells deprived of amino acids whose primary purpose is to increase intracellular amino acid levels (15, 16). Not all of the genes previously described as being induced during this response function in amino acid transport or metabolism (16–18), and a number of these genes were also up-regulated by CHR-2797 treatment, including *TRIB3*, *ATF5*, *DDIT3*, *CDKN1A*, *VEGF*, *ATF3*, *CEBPB*, and *IL-8*. These genes were also up-regulated by amino acid deprivation in HL-60 cells (Table 2).

CHR-2797 treatment also led to the up-regulation of a number of genes which have not previously been described as AADR genes. Amino acid deprivation in HL-60 cells also led to the up-regulation of these genes (Table 2), confirming them as novel AADR genes. Some of these encode proteins involved in amino acid transport and metabolism pathways, including *CTH*, *SLC7A11*, and *CBS*,

⁴ Unpublished data.

whereas others have a diverse range of other functions (e.g., *CLEC7A*, *SESN2*, *DDIT4*, *CCNG2*, *STC2*, *MGC4504*, *FYN*, and *PPP1R15A*). Stanniocalcin 2 (*STC2*), one of the most highly up-regulated of these genes, is a secreted protein and, therefore, of interest as a potential plasma-based pharmacodynamic marker. Treatment of HL-60 cells with CHR-2797 led to an increase in the secretion of *STC2* protein into the growth medium (Supplementary Fig. S5).

Because cells were treated with 6 $\mu\text{mol/L}$ CHR-2797 (200 \times the proliferation IC_{50}) in the microarray study, gene expression induced by lower concentrations of the compound was also examined.

Figure 2A shows a time-response and dose-response for two representative genes, *SLC7A11* and *TRIB3*. Increases in *SLC7A11* expression were detectable at 60 nmol/L CHR-2797 (equivalent to twice the IC_{50} for inhibition of proliferation) and as early as 2 h posttreatment.

To investigate the cell selectivity of the transcriptional response to CHR-2797, two further leukemic cell lines were treated with the compound: U-937 and HuT 78. The IC_{50} s for inhibition of proliferation by CHR-2797 in these cell lines are 10 nmol/L and >10 $\mu\text{mol/L}$, respectively (Fig. 1B and C). CHR-2797 treatment increased expression of AADR genes in U-937 cells but not in HuT

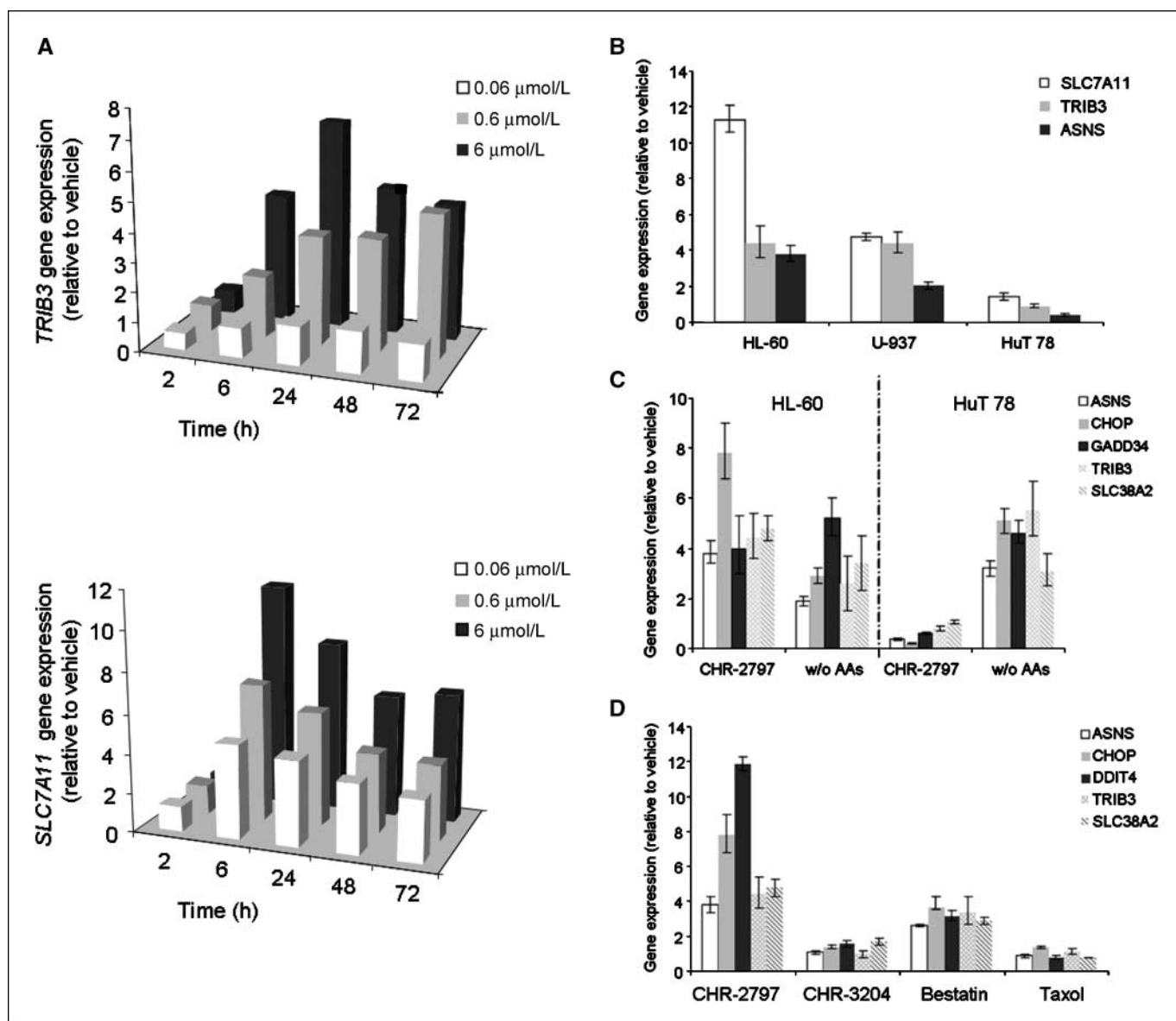


Figure 2. CHR-2797 treatment induces a transcriptional AADR. **A**, expression of *TRIB3* (top) and *SLC7A11* (bottom) in HL-60 cells treated with 0.06, 0.6, and 6 $\mu\text{mol/L}$ CHR-2797 over a 72-h time course. **B**, expression of AADR genes in various cell lines treated with 6 $\mu\text{mol/L}$ CHR-2797 for 6 h. **C**, expression of AADR genes in HL-60 and HuT 78 cell lines treated with 6 $\mu\text{mol/L}$ CHR-2797 or deprived of extracellular amino acids (w/o AAs) for 6 h. **D**, expression of AADR genes in HL-60 cells treated with 6 $\mu\text{mol/L}$ CHR-2797, 6 $\mu\text{mol/L}$ CHR-3204, 200 $\mu\text{mol/L}$ bestatin or 200 ng/mL Taxol for 6 h. Gene expression was measured by real-time quantitative PCR and compared with cells treated with 0.06% (v/v) DMSO (for compound treatments) or cells grown in normal growth medium supplemented with dialyzed FBS (for amino acid deprivation). Statistically significant differences compared with control were seen for gene expression changes beyond 2 h (A, *SLC7A11*; unpaired *t* test, $P < 0.05$), beyond 2 h and >0.06 $\mu\text{mol/L}$ CHR-2797 (A, *TRIB3*; $P < 0.05$), in HL-60 and U-937 cells treated with CHR-2797 (B; $P < 0.01$), for all genes in HL-60 cells treated with CHR-2797 and HL-60 and HuT 78 cells deprived of extracellular amino acids (C; $P < 0.05$), and for all genes in HL-60 cells treated with CHR-2797 and bestatin (D; $P < 0.01$). Data for CHR-2797 treatment are duplicated in B to D. Representative of at least three independent experiments.

78 cells, which are insensitive to the antiproliferative effects (Fig. 2B). Interestingly, although HuT 78 cells did not respond transcriptionally to CHR-2797, the cell line did respond to amino acid deprivation (Fig. 2C).

The aminopeptidase inhibitor, bestatin, is less active as an antiproliferative agent than CHR-2797 but in HL-60 cells treated with 200 $\mu\text{mol/L}$ bestatin ($\sim 10\times$ the IC_{50} for inhibition of proliferation), the expression of AADR genes was induced (Fig. 2D), suggesting that the effects of CHR-2797 treatment result from aminopeptidase inhibition. HL-60 cells, treated with CHR-3204, a close analogue of CHR-2797 in which the hydroxamate residue has been replaced with a non-metal-binding carboxamide residue, is inactive as an antiproliferative agent and did not show any AADR response (Fig. 2D). To determine if the transcriptional response to CHR-2797 treatment was a nonspecific consequence of cell death, HL-60 cells were treated with 200 ng/mL paclitaxel (equivalent to $100\times$ the IC_{50} for cell proliferation). No changes in the expression of any of the AADR genes were noted (Fig. 2D).

The patterns of gene expression in the endoplasmic reticulum (ER) stress response are somewhat similar to the AADR, although the expression of the ER chaperone *GRP78* is specific to ER stress (19). Treatment of HL-60 cells with the ER stress inducer thapsigargin (0.9 $\mu\text{mol/L}$; equivalent to $300\times$ the IC_{50} for cell proliferation, 3 nmol/L), but not deprivation of amino acids or treatment with aminopeptidase inhibitors, led to the up-regulation of *GRP78* expression (Supplementary Fig. S6).

CHR-2797 treatment leads to phosphorylation of eukaryotic initiation factor 2 α . Under conditions of amino acid deprivation, the translation initiation factor eukaryotic initiation factor (eIF) 2 α is phosphorylated, inhibiting general protein synthesis but allowing the translation of a few genes (20). One of these, the transcription factor ATF4, is a regulator of the AADR. To determine whether the transcriptional effects of CHR-2797 were mediated by this signaling pathway, the levels of phosphorylated (Ser⁵¹) eIF2 α were measured in HL-60 cells treated with the compound. Amino acid deprivation and treatment with CHR-2797 or bestatin caused an increase in phosphorylated eIF2 α (Fig. 3A). In agreement with the observed transcriptional effects (Fig. 2C), amino acid deprivation, but not CHR-2797 treatment, increased levels of phosphorylated eIF2 α in HuT 78 cells (Fig. 3A).

Aminopeptidase inhibition regulates mTOR activity. The mTOR signaling pathway controls the rate of protein synthesis in response to intracellular amino acid levels by phosphorylation of its substrates, p70 ribosomal S6 kinase, and the translational repressor protein eIF 4E-binding protein 1 (4E-BP1; refs. 21, 22). In HL-60 cells, amino acid deprivation and treatment with bestatin or CHR-2797, but not its inactive analogue, CHR-3204, caused a decrease in the phosphorylation of both S6 kinase (Thr³⁸⁹) and 4E-BP1 (Thr^{37/46}) at sites controlled by mTOR signaling (Fig. 3B and C). In agreement with the transcriptional AADR effects (Fig. 2C), amino acid deprivation, but not CHR-2797 treatment, caused a decrease in the phosphorylation of S6 kinase and 4E-BP1 in HuT 78 cells (Fig. 3B and C).

CHR-2797 treatment inhibits protein synthesis. As CHR-2797 treatment inhibited the phosphorylation of key mTOR substrates, the effects of the compound on protein synthesis were measured. As expected, CHR-2797 treatment inhibited protein synthesis in HL-60 cells at concentrations as low as 0.06 $\mu\text{mol/L}$ (Fig. 3D). These effects were much less pronounced in HuT 78 cells.

Aminopeptidase inhibition up-regulates expression of the proapoptotic protein, NOXA. Because CHR-2797 causes death by

apoptosis in a number of cell types, the gene expression microarray data were analyzed for changes in the expression of genes associated with apoptosis. The gene exhibiting the most marked change in cells treated with CHR-2797 was *NOXA*. *NOXA*, designated *PMAIP1* on the microarray, a BH3-only proapoptotic protein, was up-regulated by CHR-2797 treatment in HL-60 cells. Like other AADR genes described, *NOXA* was also up-regulated by amino acid deprivation and bestatin treatment, but not by the inactive analogue of CHR-2797 (CHR-3204) or Taxol (Fig. 4A). *NOXA* acts by sequestering the antiapoptotic protein, MCL1, and the relative levels of these two proteins are finely controlled (23). Neither amino acid deprivation, CHR-2797, nor bestatin treatment increased *MCL1* gene expression in HL-60 cells (data not shown). As expected from the transcriptional effects, CHR-2797 treatment increased *NOXA* protein levels without affecting levels of MCL1 (Fig. 4B).

Aminopeptidase inhibition leads to the appearance of intracellular peptides. It seemed possible that inhibition of aminopeptidases may deprive the cell of amino acids due to impaired recycling of amino acids derived from postproteosomal peptides. The effects of aminopeptidase inhibition on intracellular peptide concentrations were therefore examined. Treatment of HL-60 cells with 6 $\mu\text{mol/L}$ CHR-2797 for 2 to 24 hours, followed by mass spectroscopic analysis of cell lysates, led to the discovery of a number of dipeptides and tripeptides in drug-treated cells (Supplementary Fig. S7). These peptides were also detectable in HuT 78 cells treated with the compound (data not shown). The proliferation of the HuT 78 cell line may be less susceptible to aminopeptidase inhibition because it can access amino acids from other sources, such as transmembrane influx. As a result, no sign of an AADR response or antiproliferative effect is seen in this cell line despite intracellular aminopeptidase inhibition.

Discussion

The discovery of CHR-2797 stemmed from the observation that certain membrane-permeant compounds within a metalloenzyme inhibitor-focused chemical library inhibited the proliferation of a subset of tumor cell lines. The belief that the target enzyme is located intracellularly is substantiated by the findings that only esters that drive the accumulation of an acid inside the cell exert a powerful antiproliferative action. In contrast, related compounds which are poorly lipophilic are only weakly active in cell proliferation assays despite good activity against the same class of aminopeptidases, e.g., CHR-79888. The evidence that the target for these effects lay within the cell focused attention on aminopeptidases, histone deacetylases, MetAP-2, or peptide deformylase as intracellular metalloenzyme targets (2, 10, 24–26). CHR-2797 has been shown to be inactive against a number of these targets (histone deacetylase, MetAP-2) and/or to affect the proliferation of a different spectrum of tumor cell lines than inhibitors of some of these targets (histone deacetylase, peptide deformylase; refs. 25, 26). That the target is most likely to be one or more members of the aminopeptidase class of metalloenzyme is indicated by the findings that (a) all active antiproliferative agents related to CHR-2797 were aminopeptidase inhibitors and (b) tumor cells that were sensitive to CHR-2797 were also sensitive to high concentrations of bestatin, a well-established, structurally distinct, aminopeptidase inhibitor (9). This agent is also thought to act within the cell: (a) intracellularly hydrolysable bestatin esters are more potent

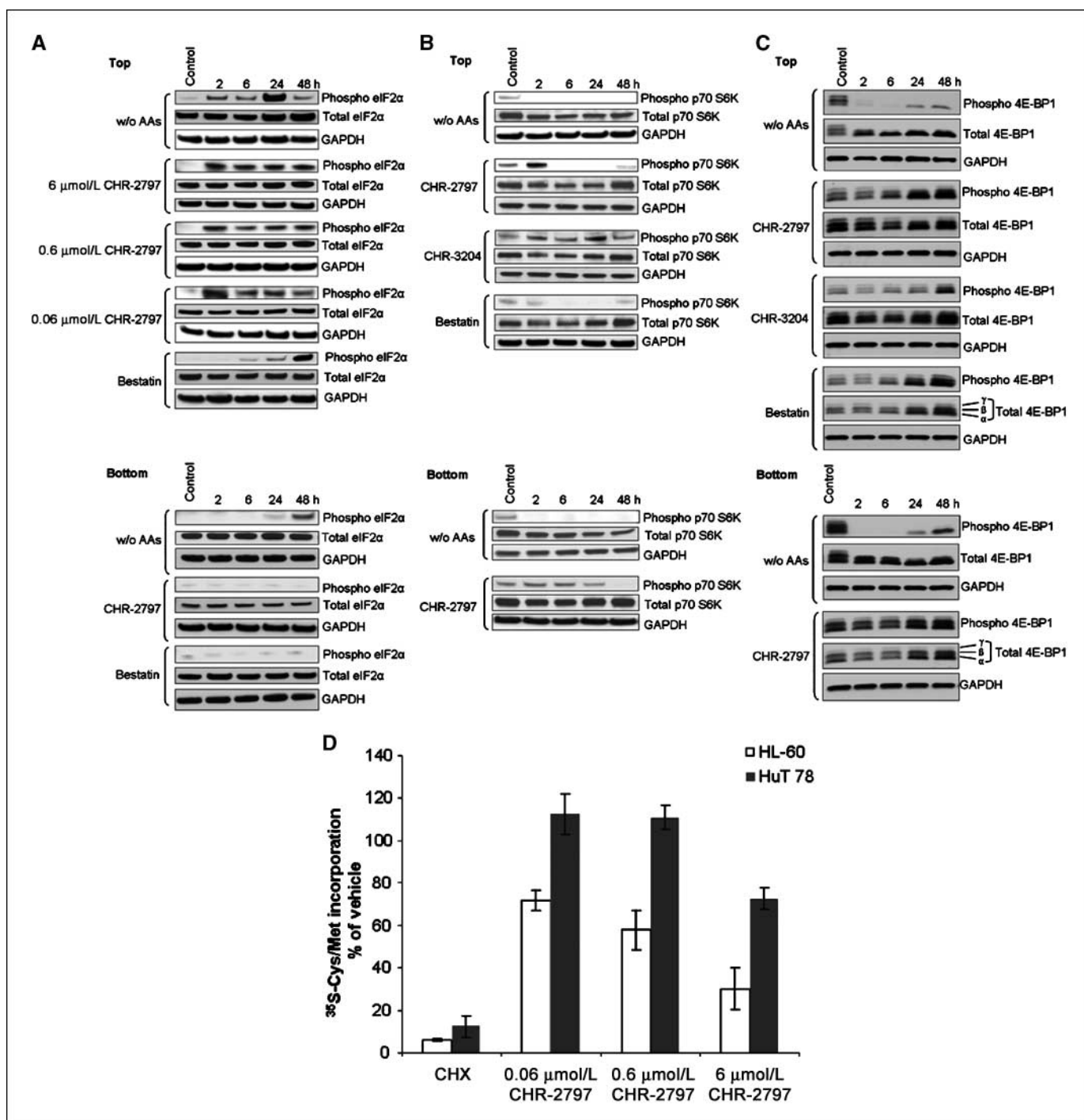


Figure 3. CHR-2797 treatment affects phosphorylation of eIF2 α , S6 kinase, and 4E-BP1. *A, top*, eIF2 α phosphorylation in HL-60 cells treated with 0.06 to 6 μ mol/L CHR-2797, 200 μ mol/L bestatin, or deprived of extracellular amino acids (*w/o AAs*) over a 48-h time course. *Bottom*, eIF2 α phosphorylation in HuT 78 cells treated with 6 μ mol/L CHR-2797, 200 μ mol/L bestatin, or deprived of extracellular amino acids (*w/o AAs*) over a 48-h time course. *B, top*, S6 kinase phosphorylation in HL-60 cells treated with 6 μ mol/L CHR-2797, 6 μ mol/L CHR-3204, 200 μ mol/L bestatin, or deprived of extracellular amino acids (*w/o AAs*) over a 48-h time course. *Bottom*, S6 kinase phosphorylation in HuT 78 cells treated with 6 μ mol/L CHR-2797 or deprived of extracellular amino acids (*w/o AAs*) over a 48-h time course. *C, top*, 4E-BP1 phosphorylation in HL-60 cells treated with 6 μ mol/L CHR-2797, 6 μ mol/L CHR-3204, 200 μ mol/L bestatin, or deprived of extracellular amino acids (*w/o AAs*) over a 48-h time course. *Bottom*, 4E-BP1 phosphorylation in HuT 78 cells treated with 6 μ mol/L CHR-2797 or deprived of extracellular amino acids (*w/o AAs*) over a 48-h time course. Samples were run on 4% to 12% Bis-Tris (eIF2 α , phosphorylated S6 kinase), 7% Tris-acetate (S6 kinase), or 16% Tris-glycine (4E-BP1) gels. Western blots were probed with antibodies to eIF2 α , phosphorylated eIF2 α (Ser⁵¹), S6 kinase, phosphorylated S6 kinase (Thr³⁸⁹), or 4E-BP1, phosphorylated 4E-BP1 (Thr^{37/46}; Cell Signaling Technology). GAPDH was used as a loading control. *Control*, cells treated with 0.06% DMSO (for compound treatments) or cells grown in normal growth medium supplemented with dialyzed FBS (for amino acid deprivation). Representative of at least three independent experiments. *D*, CHR-2797 treatment inhibits protein synthesis. HL-60 and HuT 78 cells were treated with 0.06 to 6 μ mol/L CHR-2797 for 24 h or 20 μ g/mL cycloheximide (CHX) for 2 h. Cells were seeded at 5×10^4 per well of a 96-well filter plate, and newly synthesized proteins were labeled with [³⁵S]Cys/Met for 1 h. Data are expressed as mean (\pm SD) protein synthesis relative to an equal number of DMSO-treated cells in three independent experiments. Statistically significant differences compared with control were seen for both cell lines treated with cycloheximide and for HL-60 cells treated with CHR-2797 at concentrations down to 0.06 μ mol/L and HuT 78 cells treated with 6 μ mol/L CHR-2797 (unpaired *t* test, *P* < 0.05).

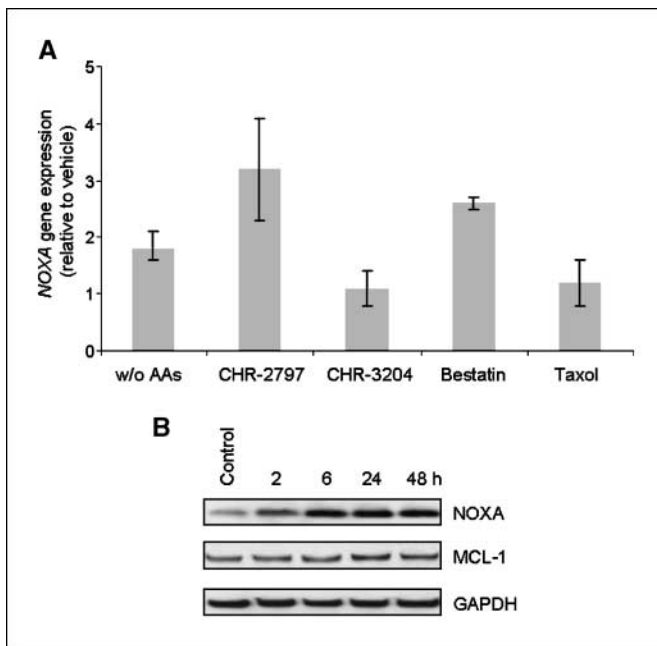


Figure 4. Effects of aminopeptidase inhibition on NOXA expression. *A*, NOXA gene expression in HL-60 cells treated with 6 $\mu\text{mol/L}$ CHR-2797, 6 $\mu\text{mol/L}$ CHR-3204, 200 $\mu\text{mol/L}$ bestatin, 200 ng/mL Taxol, or deprived of extracellular amino acids (*w/o* AAs) for 6 h. Gene expression was measured by real-time quantitative PCR and compared with cells treated with 0.06% DMSO (for compound treatments) or cells grown in normal growth medium supplemented with dialyzed FBS (for amino acid deprivation). Statistically significant differences compared with control were seen for cells deprived of extracellular amino acids and cells treated with CHR-2797 or bestatin (unpaired *t* test, $P < 0.05$). *B*, NOXA and MCL1 protein levels in HL-60 cells treated with 6 $\mu\text{mol/L}$ CHR-2797 over a 48-h time course. Samples were run on 12% Bis-Tris gels, and Western blots were probed with antibodies to NOXA and MCL1 (Calbiochem). GAPDH was used as a loading control. *Control*, cells treated with 0.06% DMSO. Representative of at least three independent experiments.

antiproliferative agents than bestatin itself (27)⁵ and (*b*) agents that inhibit the extrusion of bestatin from the cell can enhance its antiproliferative potency (28). Extensive studies in sensitive leukemic cells using small interfering RNA (siRNA) reagents targeting individual intracellular aminopeptidases have failed to provide convincing evidence that any single enzyme is responsible for the effects of CHR-2797. Aminopeptidases, such as LAP and PuSA, have overlapping substrate preferences, and it seems likely that, for the siRNA approach to replicate the effects of CHR-2797, it would be necessary to knockdown multiple aminopeptidases. The antiproliferative effects of CHR-2797 most likely depend upon the simultaneous inhibition of more than one intracellular aminopeptidase.

CHR-2797 treatment of HL-60 cells up-regulated a number of genes whose primary purpose seems to be to increase intracellular amino acid levels (15, 16). These include, among other genes, amino acid transporters and biosynthetic enzymes, the expression of which are also induced by amino acid deprivation (e.g., *SLC7A11*, *SLC38A2*, *ASNS*, *CBS*, *CTH*). Bestatin, a chemically dissimilar aminopeptidase inhibitor, also induced the up-regulation of AADR genes, whereas CHR-3204, an inactive analogue of CHR-2797, did

not. This suggests that the transcriptional effects of CHR-2797 are a consequence of aminopeptidase inhibition. Other genes up-regulated by CHR-2797 include a number whose protein products are not directly related to amino acid transport or biosynthesis. Some of these have previously been shown to be up-regulated by amino acid deprivation (e.g., *TRIB3*, *ATF5*, *DDIT3*, *CDKN1A*, *VEGF*, *ATF3*, *CEBPB*, and *IL-8*), whereas others seem to be novel AADR genes (e.g., *CLEC-7A*, *SESN2*, *DDIT4*, *CCNG2*, *STC2*, *MGC4504*, *FYN*, and *PPP1R15A*). Interestingly, a number of these genes seem to function in negative feedback loops which attenuate the stress response and ensure that, if and when nutrient levels return to normal, cells are able to successfully recover. For example, the pseudokinase TRIB3 acts as a negative feedback regulator of ATF4-dependent transcription under conditions of amino acid deprivation (29), and ATF3 acts as a transcriptional repressor of AADR genes (30). Furthermore, *PPP1R15A* (*GADD34*) is a cofactor of the eIF2 α phosphatase PP1 (31) acting on eIF2 α phosphorylation in a negative feedback loop, and *DDIT4* (*REDD1*) has been shown to act as a negative regulator of mTOR activity (32).

A small number of genes were up-regulated by CHR-2797 treatment in HL-60 cells but not by amino acid deprivation. *ASS* and *PSAT1* may be expected to be AADR genes, as they are both involved in amino acid metabolism, and *GADD45A* has been shown to respond to amino acid deprivation (33). Resistin (*RETN*), an adipocyte-derived hormone, was significantly up-regulated by CHR-2797 treatment in HL-60 cells but not by amino acid deprivation. The regulation of this gene may, therefore, be a consequence of aminopeptidase inhibition unrelated to intracellular amino acid depletion.

Amino acid deficiency is sensed by the kinase GCN2 (general control nonderepressible kinase 2) by a mechanism that involves uncharged tRNAs binding to a regulatory HisRS domain, homologous to histidyl tRNA synthetase enzymes (34). This leads to autoactivation of its kinase domain and allows phosphorylation of the α -subunit of the translation initiation factor eIF2. Phosphorylated eIF2 α inhibits recycling of eIF2 to its active GTP-bound form and thereby reduces global protein synthesis (35). As expected from the observed transcriptional effects of the compound, treatment of HL-60 cells with CHR-2797, bestatin, or amino acid deprivation increased phosphorylation of eIF2 α .

The ER stress response is coordinated in a manner somewhat similar to the AADR transcriptional response. ER stress is sensed by the kinase PERK, which, like GCN2, phosphorylates eIF2 α , leading to a pattern of gene expression termed the unfolded protein response (36). The expression of the ER chaperone GRP78 is specific to the ER stress response (19). Treatment of HL-60 cells with the ER stress inducer thapsigargin, but not amino acid deprivation or aminopeptidase inhibition, led to the up-regulation of *GRP78* expression, suggesting that aminopeptidase inhibition does not induce an ER stress response.

The mTOR signaling pathway also senses and responds to amino acid levels inside cells, controlling rates of protein synthesis accordingly. Two important substrates of this pathway are S6 kinase and 4E-BP1, and the phosphorylation and activity of both proteins have been shown to be modulated by amino acid levels (21, 22). Phosphorylation of 4E-BP1 releases it from eIF4E, allowing formation of the eIF4F complex critical for cap-dependent translation. In HL-60 cells, amino acid deprivation and treatment with bestatin or CHR-2797, but not its inactive analogue, CHR-3204, caused a decrease in the phosphorylation of both S6 kinase (Thr³⁸⁹) and 4E-BP1 (Thr^{37/46}) at sites controlled via mTOR

⁵ E.A. Bone, A.H. Davidson, L. Hooftman, unpublished observations.

signaling. 4E-BP1 is phosphorylated hierarchically at four sites via mTOR signaling, with phosphorylation at Thr^{37/46} being required for modification of other residues (37). Interestingly, the hyperphosphorylated (slowly migrating) form, whose binding to eIF4E is completely blocked (38), is the form most significantly affected by aminopeptidase inhibition in HL-60 cells (Fig. 3C).

The sensitivity of cells to CHR-2797 and other aminopeptidase inhibitors, such as bestatin, varies considerably. In the case of CHR-2797, this could be due either to variable carboxylesterase activity within cells or to variable sensitivity to the pharmacologic mechanism of the drug. The latter is the more likely because all cell types that we have examined have the ability to accumulate CHR-79888, the acid product of CHR-2797 (data not shown). Moreover, bestatin, which is not an ester drug and is believed to enter cells through a peptide transporter mechanism (39), shows a similar tumor cell selectivity. The most likely explanation for variable tumor cell sensitivity is that, although aminopeptidase inhibition and peptide accumulation occur in all cells, only certain cell types rely on the recycling of intracellular peptides for amino acid provision. In other words, drug-responsive tumor cells run out of amino acids, as judged by the AADR response, eIF2 α phosphorylation, or mTOR inhibition, despite plentiful availability in the extracellular medium. This amino acid-related phenomenon is not unique to cancer cells. The parasite *Plasmodium falciparum* replicates within host erythrocytes and during this part of its life-cycle uses aminopeptidases, such as PFLAP, the *Plasmodium* homologue of LAP, to catabolize large amounts of host hemoglobin to its constituent amino acids (40). Bestatin and other aminopeptidase inhibitors inhibit at least two of the aminopeptidases which are essential for growth and survival of the parasite. Interestingly, the antiparasitic potency of bestatin is not substantially affected by the presence or absence of extracellular amino acids (41), implying, as here, that the parasite is entirely dependent on peptide recycling for provision of adequate amino acid supplies.

These data highlight a further important facet of cell biology, namely that regulation of amino acid provision can be used physiologically or pharmacologically to control cell proliferation. There is now considerable data indicating that control of tryptophan levels in lymphocytes by macrophages and dendritic cells via expression of the tryptophan-metabolizing enzyme, indoleamine 2,3-dioxygenase, serves to limit T-lymphocyte prolif-

eration (42, 43). Previous studies have shown that, in general, human tumor cells are significantly more sensitive to amino acid deprivation than normal cells (44–46). There is widespread up-regulation in human tumors of certain amino acid transporters, such as LAT1 (47, 48), presumably to meet the increased demands for essential amino acids to take part in protein synthesis and cellular metabolism. It may be this increased demand for amino acids in tumor cells that underlies the effects of CHR-2797 and bestatin shown here. HL-60 cells do not increase expression of amino acid transporters, such as LAT1 (SLC7A5), in response to CHR-2797, although other transporters are up-regulated. Decreased amino acid supply, through inhibition of cellular protein recycling by CHR-2797, coupled with an inability to further up-regulate transporters, such as LAT1, may be sufficient, in cell types such as HL-60, to cause fatal nutrient stress, perhaps by imbalancing levels of proapoptotic and antiapoptotic proteins such as NOXA and MCL1. The current data echo findings reported recently with the natural product brasilicardin A, an immunosuppressive and antitumor agent, which is a specific inhibitor of the amino acid transport system L, of which LAT1 is a part (49, 50). This agent stimulates GCN2 activation and, subsequently, eIF2 α phosphorylation in lymphocytes. The potential for synergy between CHR-2797 and agents targeting amino acid transporters is an obvious avenue to be explored.

Further work to establish details of the mechanism of action of CHR-2797 and its selectivity for certain types of tumor cells and to identify biomarkers that may predict a clinical response to the drug is under way. Phase II clinical studies in a number of human cancer types continue.

Disclosure of Potential Conflicts of Interest

L.A. Needham: Ownership interest, British Biotech Pharmaceuticals Ltd. G. Box: Funding for laboratory research. S.A. Eccles: Funding for preclinical studies from British Biotech/Chroma. E.A. Bone: Ownership interest, Chroma Therapeutics Ltd. A.H. Drummond: Ownership interest, Chroma Therapeutics Ltd. and British Biotech Pharmaceuticals Ltd. The other authors disclosed no potential conflicts of interest.

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