

SUPPLEMENTARY MATERIAL

Identification of Compounds with Anti-Human Cytomegalovirus Activity that
Inhibit Production of IE2 Proteins

Rooksarr Beelontally¹, Gavin S Wilkie², Betty Lau², Charles J Goodmaker¹,
Catherine M K Ho¹, Chad M Swanson³, Xianming Deng^{4,5}, Jinhua Wang^{4,5},
Nathanael S Gray^{4,5}, Andrew J Davison² & Blair L Strang^{1,4}

Institute of Infection & Immunity, St George's, University of London, London, UK¹;
MRC-University of Glasgow Centre for Virus Research, Glasgow, UK²;
Department of Infectious Diseases, King's College London, London, UK³;
Department of Biological Chemistry & Molecular Pharmacology, Harvard Medical
School, Boston, MA, USA⁴; Department of Cancer Biology, Dana-Farber Cancer
Institute, Boston, MA, USA⁵.

Compound treatment and infection of cells for high throughput screening. The Gray Kinase Inhibitor library (stock concentration of 3.3 mM of each compound in DMSO) was screened in duplicate. Twenty four hours before infection 2×10^3 HFF cells were seeded in each well of each Corning 384 plate. Unless stated otherwise, liquid was added to wells using a WellMate apparatus. At the time of infection, medium was removed with a suction manifold and 30 μ l of complete medium was added to each well. Compounds were added to the

plate containing HFF cells using a 100 nl pin transfer on a liquid handling robot. Negative and positive controls (water+0.3% DMSO or heparan sulfate (5 µg/ml) + 0.3% DMSO, respectively) were added to plates by hand (12 wells of each). Cells were then infected with HCMV strain AD169 (multiplicity of infection (MOI) of 1 plaque-forming unit (p.f.u.) per cell) in a total volume of 5 µl. Thus, the final concentration of compound in each well was 9.4 µM. Infected cells were incubated for 72 hours at 37°C and then prepared for analysis.

Preparation of screening plates for high throughput microscopy analysis. Cell culture medium was removed from infected cells and replaced with 20 µl Hoechst 33342 (SIGMA) diluted in PBS to a final concentration of 10 µg/ml. After incubation for 1 hour at 37°C, 20 µl of Deep Red Cell Mask (Invitrogen) (diluted in PBS to a concentration of 5 µg/ml) was added to each well. Cells were incubated for a further 5 min at 37°C. Cells were then fixed by removing PBS containing Hoechst and Cell Mask and adding 50 µl of 3.5% Formaldehyde (SIGMA) in PBS to each well. After incubating at room temperature for 10 min, fixative was removed and 50 µl of PBS containing 0.5% TritonX-100 was added per well to permeabilize cells. After 10 min incubation at room temperature, PBS containing detergent was removed, and cells were washed once with PBS. PBS was removed and replaced with 20 µl MAb P207 recognizing pp28 (Virusys) (dilution 1:1000) and anti-mouse secondary antibody conjugated to fluorophore Alexa488 (Molecular Probes) (dilution 1:1000). Plates were incubated at 37°C for 1 hour. After incubation, PBS containing antibodies

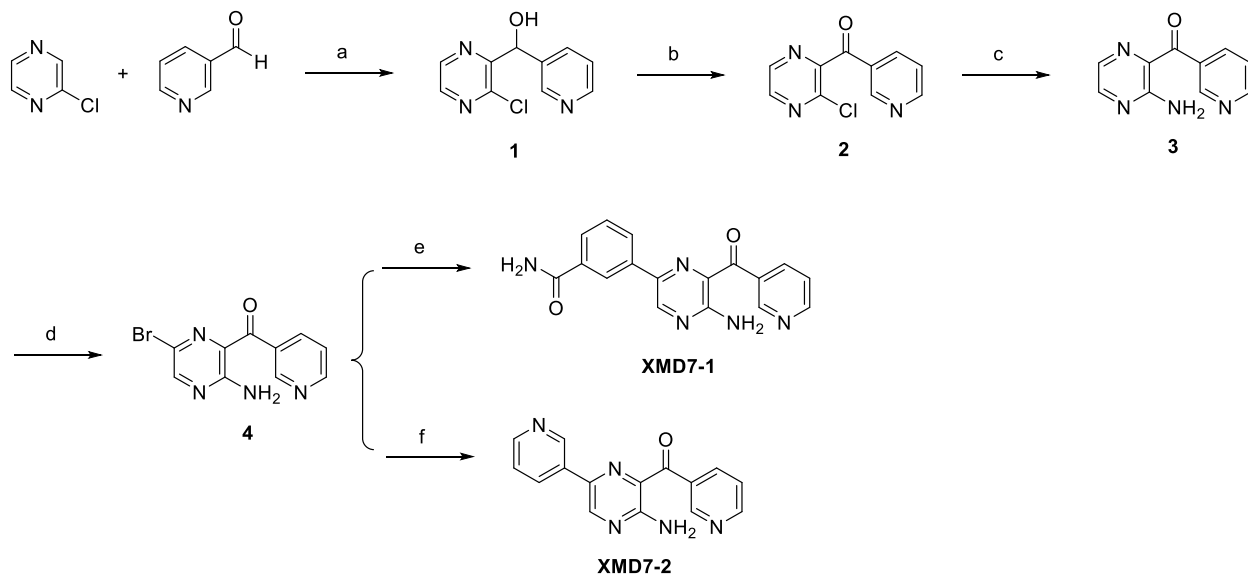
was removed and replaced with 50 μ l of PBS. Plates were then analyzed using automated microscopy for the presence of pp28 protein.

Microscopy analysis of screening plates. Infected cells stained with antibody to detect pp28 were imaged on an Image Express Micro (IXM) microscope (Molecular Devices) at 10x magnification to detect 3 wavelengths; 488 nm to detect antibody recognizing pp28, 568nm to detect Deep Red CellMask and 350 nm to detect Hoescht 33342 stain bound to nuclear DNA. Three images were captured from each wavelength in each well of the 384-well plate. The number of cells positive at all 3 wavelengths and percentage of pp28 positive cells in each well were determined using the Metamorph Multiwavelength Cell Scoring software (Molecular Devices). Typically, approximately 60% of cells were infected in wells treated with negative control, DMSO (data not shown).

Analysis of screening results. To assess the quality of data that could be returned from the screening protocol we calculated the Z'-factor (Birmingham et al., 2009; Zhang et al., 1999) derived from the positive (heparan sulphate treated infected cells) and negative (DMSO treated infected cells) control wells. The screening controls returned Z'-factors of greater than or equal to 0.5, indicating a robust separation of difference in the data derived from positive and negative controls (data not shown). Thus, the screening protocol could be reliably used to screen the compound collection.

After screening of the compound collection, data were discarded from any well in which the number of cells stained with Hoescht 33342 fell below 2-fold of the mean of the number of cells in each well of the plate. The data from the remaining wells from each plate was converted to a z-score (the number of standard deviations from the mean of the data (Birmingham et al., 2009; Zhang et al., 1999)) and the average z-score from data in duplicate plates was determined. Images chosen at random were visually inspected throughout image capture and analysis to ensure raw data were consistent with z-scores.

Chemistry and synthetic procedure for XMD7 compounds. XMD7-1 and XMD7-2 were synthesized based on synthesis of XMD7-27 in patent WO2009115517. Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. ¹H NMR spectra were recorded on 600 MHz (Varian AS600), and chemical shifts are reported in parts per million (ppm, δ) downfield from tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Spin multiplicities are described as s (singlet), d (doublet). Mass spectra were obtained on a Waters Micromass ZQ instrument. Preparative HPLC was performed on a Waters Symmetry C18 column (19 x 50 mm, 5 μ M) using a gradient of 5-95% acetonitrile in water containing 0.05% trifluoacetic acid (TFA) over 8 min (10 min run time) at a flow rate of 30 mL/min. Purities of assayed compounds were in all cases greater than 95%, as determined by reverse-phase HPLC analysis.



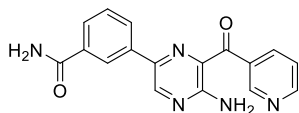
Reagents and conditions: a) 2,2,6,6-Tetramethyl piperidine, n-Butyllithium, THF, -78°C - r.t.; b) Manganese dioxide, Toluene, 110 °C; c) ammonia (2.0 M in isopropanol), 100 °C; d) bromine, acetic acid, sodium carbonate, r.t..

1) Intermediates **1** - **4** were synthesized based on PCT WO2009115517.

2) General synthetic procedure of XMD7-1 and XMD7-2:

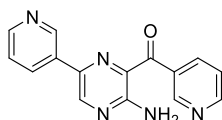
(3-amino-6-bromo-2-pyridinyl)-3-pyridinyl-Methanone (1 eq.), β -[3-(aminocarbonyl)phenyl]-Boronic acid (1.3 eq) or β -3-pyridinyl-Boronic acid (1.3 eq), [1,1'-Bis(diphenylphosphino)ferrocene]dichloropalladium (0.1 eq.), and 1M aqueous Na_2CO_3 (5 eq.) were added into a mixture of toluene and ethanol (6:1). The reaction was stirred at 100 °C for 2h after degas. The reaction mixture was diluted with ethyl acetate and filtered through a Celite pad, washed with water and saturated brine solution, and then dried with anhydrous sodium sulfate. Purification by HPLC provided final product:

3-[5-amino-6-(3-pyridinylcarbonyl)-2-pyrazinyl]-Benzamide (XMD7-1)



^1H NMR (600 MHz, CD_3OD) δ 9.38 (s, 1H), 8.95 (s, 1H), 8.87 (s, 1H), 8.85 (s, 1H), 8.39 (s, 1H), 8.06 (s, 1H), 7.95 (s, 1H), 7.86 (s, 1H), 7.54 (s, 1H). MS (ESI) m/z 320.16 ($\text{M}+\text{H}$) $^+$.

[3-amino-6-(3-pyridinyl)-2-pyrazinyl]-3-pyridinyl-Methanone (XMD7-2)



^1H NMR (600 MHz, CD_3OD) δ 9.38 (s, 2H), 9.15 (s, 1H), 8.94 (s, 1H), 8.83 (s, 1H), 8.77 (d, $J=8.4$ Hz, 1H), 8.67 (d, $J = 7.2$ Hz, 1H), 7.91 (s, 1H), 7.84 (s, 1H). MS (ESI) m/z 278.10 ($\text{M}+\text{H}$) $^+$.

Birmingham, A., et al., 2009. Statistical methods for analysis of high-throughput RNA interference screens. *Nature methods* 6, 569-575.

Zhang, J.H., et al., 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal of biomolecular screening* 4, 67-73.

Table S6. Kinase selectivity profile of XMD7-27

Ambit KINOMEScan: Kinase Target	Ambit KINOMEScan: kinase activity, percentage of control (%)
CLK1	0
GSK3A	0
HIPK1	0
MKNK2	0
PCTK1	0.1
PLK4	0.3
GSK3B	0.45
CLK2	0.5
CLK4	0.85
CDK7	1.4
DYRK1B	1.4
TAOK1	1.4
TYK2(Kin.Dom.1)	2.2
TGFBR2	2.3
FLT3(D835Y)	2.8
RPS6KA4(Kin.Dom.2)	3.1
MEK3	3.7
PIK3CG	4.4
ACVR1	4.9
AXL	4.9
ABL1(T315I)	5
MINK	5
GCN2(Kin.Dom.2,S808G)	5.6
FLT3(D835H)	6.4
ANKK1	7.6
AAK1	7.8
BIKE	7.9
RIOK3	8.2
JNK1	9.8
ARK5	10
BMPR2	10
STK16	11
CLK3	12
RIOK1	12
FLT3(N841I)	13
KIT(V559D,T670I)	13
SNARK	13
SRPK3	13
BMPR1B	14
GAK	14

IKK-alpha	14
MST4	15
SLK	15
FLT3(ITD)	16
KIT(V559D)	16
RPS6KA2(Kin.Dom.2)	16
CAMKK2	17
PIM2	17
PIP5K1A	17
SYK	18
CAMKK1	19
ZAK	19
ACVRL1	20
JAK1(Kin.Dom.1)	20
ACVR2B	21
BTK	22
KIT	22
TAOK3	22
MEK4	23
MERTK	23
MYLK	23
ACVR2A	24
PIK3CD	24
TNIK	24
ERK8	25
FLT3(K663Q)	25
IRAK3	25
MAP4K4	25
ULK2	25
ULK3	25
CSNK1D	26
DMPK	26
AURKC	27
CSNK1G1	27
JNK3	27
FLT3	28
SRPK1	28
CAMK1	30
DCAMKL3	30
PIM1	30
RIOK2	30
TTK	31

MAP4K2	32
RPS6KA5(Kin.Dom.2)	32
TAK1	32
CSNK1E	33
DAPK1	33
DAPK2	33
FGFR4	33
CSNK1G2	34
MAP4K5	34
NEK5	34
NEK7	34
PIM3	34
PRKCD	34
Sgk085	34
ABL1	100
ABL1(E255K)	100
ABL1(F317I)	100
ABL1(F317L)	100
ABL1(H396P)	100
ABL1(M351T)	100
ABL1(Q252H)	100
ABL1(Y253F)	100
ABL2	100
ACVR1B	100
ADCK3	100
ADCK4	100
AKT1	100
AKT2	100
AKT3	100
ALK	100
AMPK-alpha1	100
AMPK-alpha2	100
AURKA	100
AURKB	100
BLK	100
BMPR1A	100
BMX	100
BRAF	100
BRAF(V600E)	100
BRSK1	100
BRSK2	100
CAMK1D	100

CAMK1G	100
CAMK2A	100
CAMK2B	100
CAMK2D	100
CAMK2G	100
CAMK4	100
CDC2L1	100
CDC2L2	100
CDK11	100
CDK2	100
CDK3	100
CDK5	100
CDK8	100
CDK9	100
CDKL2	100
CHEK1	100
CHEK2	100
CIT	100
CSF1R	100
CSK	100
CSNK1A1L	100
CSNK1G3	100
CSNK2A1	100
CSNK2A2	100
DAPK3	100
DCAMKL1	100
DCAMKL2	100
DDR1	100
DDR2	100
DLK	100
DMPK2	100
DRAK1	100
DRAK2	100
EGFR	100
EGFR(E746-A750del)	100
EGFR(G719C)	100
EGFR(G719S)	100
EGFR(L747-E749del, A750P)	100
EGFR(L747-S752del, P753S)	100
EGFR(L747-T751del,Sins)	100
EGFR(L858R)	100
EGFR(L861Q)	100

EGFR(S752-I759del)	100
EPHA1	100
EPHA2	100
EPHA3	100
EPHA4	100
EPHA5	100
EPHA6	100
EPHA7	100
EPHA8	100
EPHB1	100
EPHB2	100
EPHB3	100
EPHB4	100
ERBB2	100
ERBB4	100
ERK1	100
ERK2	100
ERK3	100
ERK4	100
ERK5	100
FER	100
FES	100
FGFR1	100
FGFR2	100
FGFR3	100
FGFR3(G697C)	100
FGR	100
FLT1	100
FLT4	100
FRK	100
FYN	100
HCK	100
IGF1R	100
IKK-beta	100
IKK-epsilon	100
INSR	100
INSRR	100
ITK	100
JAK1(Kin.Dom.2)	100
JAK2(Kin.Dom.2)	100
JAK3(Kin.Dom.2)	100
JNK2	100

KIT(D816V)	100
KIT(V559D,V654A)	100
LATS1	100
LATS2	100
LCK	100
LIMK1	100
LIMK2	100
LKB1	100
LOK	100
LTK	100
LYN	100
MAP3K3	100
MAP3K4	100
MAP3K5	100
MAP4K1	100
MAP4K3	100
MAPKAPK2	100
MAPKAPK5	100
MARK1	100
MARK2	100
MARK3	100
MARK4	100
MEK1	100
MEK2	100
MEK6	100
MELK	100
MET	100
MKNK1	100
MLCK	100
MLK1	100
MLK2	100
MLK3	100
MRCKA	100
MRCKB	100
MST1	100
MST1R	100
MST2	100
MST3	100
MUSK	100
MYLK2	100
MYO3A	100
MYO3B	100

NDR2	100
NEK1	100
NEK2	100
NEK6	100
NEK9	100
NLK	100
p38-alpha	100
p38-beta	100
p38-delta	100
p38-gamma	100
PAK1	100
PAK2	100
PAK3	100
PAK4	100
PAK6	100
PAK7/PAK5	100
PCK2	100
PCK3	100
PDGFRA	100
PDGFRB	100
PDPK1	100
PFTAIRE2	100
PFTK1	100
PHKG1	100
PHKG2	100
PIK3C2B	100
PIK3CA	100
PIK3CA(E545K)	100
PIK3CB	100
PIP5K2B	100
PKAC-alpha	100
PKAC-beta	100
PKMYT1	100
PKN1	100
PKN2	100
PLK1	100
PLK3	100
PRKCE	100
PRKCH	100
PRKCQ	100
PRKD1	100
PRKD2	100

PRKD3	100
PRKG1	100
PRKG2	100
PRKR	100
PRKX	100
PTK2	100
PTK2B	100
PTK6	100
RAF1	100
RET	100
RET(M918T)	100
RET(V804L)	100
RET(V804M)	100
RIPK1	100
RIPK2	100
RIPK4	100
ROCK2	100
ROS1	100
RPS6KA1(Kin.Dom.1)	100
RPS6KA1(Kin.Dom.2)	100
RPS6KA2(Kin.Dom.1)	100
RPS6KA3(Kin.Dom.1)	100
RPS6KA4(Kin.Dom.1)	100
RPS6KA5(Kin.Dom.1)	100
RPS6KA6(Kin.Dom.1)	100
RPS6KA6(Kin.Dom.2)	100
SgK110	100
SNF1LK	100
SNF1LK2	100
SRC	100
SRMS	100
SRPK2	100
STK33	100
STK35	100
STK36	100
TEC	100
TESK1	100
TGFBR1	100
TIE1	100
TIE2	100
TLK1	100
TLK2	100

TNK1	100
TNK2	100
TNNI3K	100
TRKA	100
TRKB	100
TRKC	100
TSSK1	100
TXK	100
TYK2(Kin.Dom.2)	100
TYRO3	100
ULK1	100
VEGFR2	100
WEE1	100
WEE2	100
YANK2	100
YANK3	100
YES	100
YSK1	100
ZAP70	100



