Supplemental Information

Cancer-Selective Targeting of the NF-κB Survival Pathway with GADD45β/MKK7 Inhibitors

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SUPPLEMENTAL DATA

A

B

\[ p = 0.03 \]

\[ p = 0.027 \]
Figure S1, related to Figure 1. Correlation between high GADD45B expression and poor clinical outcome in two independent datasets of newly diagnosed MM patients

(A, B) OS in MM patients from the intensive arm of the MRC Myeloma IX trial (GSE15695; n=155) (Dickens et al., 2010; Wu et al., 2011) (A) and the VAD treatment arm of the HOVON-65/GMMG-HD4 trial (GSE19784; n=149) (Broyl et al., 2010, Sonneveld et al., 2008) (B) stratified in each case at diagnosis using k-mean clustering according to the GADD45B expression value (Hartigan and Wong, 1979).
Figure S2, related to Figure 2. NF-κB promotes the survival of MM cells by inhibiting JNK signalling and apoptosis by means of GADD45β

(A) Trypan blue exclusion assays showing the survival of MM cell lines expressing non-specific (sh-ns) or RelA-specific (sh-RelA) sh-RNAs.

(B) Propidium iodide (PI) nuclear staining assays showing apoptotic cells (i.e. cells exhibiting sub-G₁ DNA content) in representative cell lines from (A) at day 6.

(C) Western blots showing RelA, GADD45β and total and phosphorylated (P) JNK in MM cell lines. Also shown are the knockdown efficiency (RelA) and specificity (total JNK, β-actin) of the sh-RNAs.

(D) Trypan blue exclusion assays showing the survival of representative MM cell lines expressing sh-RelA and cultured in the presence (+) or absence (-) of SP600125 for 6 days. UT, untreated.

(E) PI nuclear staining assays showing apoptotic cells in representative cell lines from (D).

(F) [³H]Thymidine incorporation assays showing the survival of MM cell lines expressing sh-ns or sh-MKK7 after treatment for 6 days with the IKKβ inhibitors, SC-514 (top) or PS-1145 (bottom). Values express the percentage of the counts per minute (cpm) measured with the treated cultures relative to the cpm measured with the respective untreated cultures.

(G) Shown are the relative GADD45B mRNA expression levels (qRT-PCR assays; top) and the IC₅₀s of DTP3 at 144 hr ([³H]thymidine incorporation assays; bottom) in a panel of tumor cell lines of different tissues of origin. Values express the logarithm to base 10 (log₁₀) of the IC₅₀ (bottom). A similar correlation between D-peptide cellular activity and GADD45B mRNA expression in cancer cells was observed with the active precursors of DTP3, z-DTP2 and z-DTP1.

(H) PI nuclear staining assays showing apoptotic cells in representative MM cell lines (day 8). Also shown are the results with uninfected cells. The experiment with the ARH-77 B lymphoblastoid cell line further shows the results obtained with one additional non-specific sh-RNA (sh-ns-2), one additional MKK7-specific sh-RNA (sh-MKK7-2), and two additional GADD45β-specific sh-RNAs (sh-GADD45β-2, sh-GADD45β-3).

(I) Western blots showing total and phosphorylated (P) proteins in representative MM cell lines, expressing sh-ns, sh-MKK7 or sh-GADD45β sh-RNAs.

(J) Western blots showing total and phosphorylated (P) proteins in U266 MM cells expressing sh-ns, sh-GADD45β-2 or sh-GADD45β-3.
(K) Western blots showing the expression of GADD45β and MKK7 in representative GADD45β-dependent (KMS-11, KMS-12, U266) and GADD45β-independent (RPMI-8226, KMM-1) MM cell lines.

(L) Trypan blue exclusion assays showing the survival of ARH-77 B lymphoblastoid cells expressing different non-specific (sh-ns, sh-ns-2), GADD45β-specific (sh-GADD45β, sh-GADD45β-2, sh-GADD45β-3) or MKK7-specific (sh-MKK7, sh-MKK7-2) sh-RNAs.

(M) qRT-PCR assays showing the relative GADD45B mRNA expression levels in the MM (NCI-H929, RPMI-8226) and non-MM (ARH-77) cell lines expressing sh-ns, sh-GADD45β or sh-MKK7 sh-RNAs (top). JNK1 is shown as specificity control (bottom).

(N) PI nuclear labelling assays showing apoptotic cells in representative MM cell lines co-expressing sh-ns and sh-ns (sh-ns/sh-ns) or sh-ns and sh-JNK1 (sh-ns/sh-JNK1) on day 8 after lentivirus infection.

(O) Trypan blue exclusion assays showing the survival of representative MM cell lines expressing sh-GADD45β and left untreated (UT; -) or treated with SP600125 (+) for 6 days.

(P) PI nuclear labelling assays showing apoptotic cells in representative MM cell lines from (O) expressing sh-GADD45β sh-RNAs and left untreated (-) or treated (+) with SP600125 for 6 days.

(A, C, F) Note that the ARH-77 cell line is an EBV transformed B lymphoblastoid cell line, and not a MM cell line, and is shown for the purpose of establishing the dependence of GADD45β expression on constitutive NF-κB activity also in non-MM tumor cell lines.

(A, D, F, G, M, O) Values denote means ± SD (n=3).

(A, D, L, O) Values express the percentages of live enhanced green fluorescent protein (eGFP)* cells present in the cultures at the times shown (A, L) or at day 6 (D, O) relative to the number of live eGFP* cells present in the respective cultures at day 0.

(B, E, H, N, P) The percentages of apoptotic cells are depicted.

(C, I, J, K) β-actin is shown as control.

(I, J) T/I, 12-O-tetradecanoylphorbol-13-acetate (TPA)/ionomycin.
Figure S3, related to Figure 3. The iterative ELISA screen used for the isolation of the bioactive L-tetrapeptides, Ac-LTP1 and Ac-LTP2

(A) Antagonists of the GADD45β/MKK7 interaction were selected by screening a simplified combinatorial L-tetrapeptide library of general core formula $Y_1$-$X_2$-$X_3$-$X_4$, containing all possible combinations of 12 L-amino acids representing different chemical spaces, at each position from $X_1$ to $X_4$ (see Table S1). This library, consisting of a total of $12^4 = 20,736$ different L-tetrapeptides, harboring a C-terminal amide group and an N-terminal Fmoc-(β-Ala)$_2$ tag, was iteratively deconvoluted in four steps in ELISA GADD45β/MKK7 competition assays, using at each step, coated human (h)MKK7 fused to GST (GST-hMKK7), soluble biotinlabelled His$_6$-hGADD45β, and each of the 12 sub-libraries. Shown are the results from the screen and the core amino acid sequences of the peptide mixtures used in steps 1 to 4, using the standard three-letter amino acid code. $Y_1$, $Y_2$, $Y_3$ and $Y_4$, each of the 12 amino acids used in steps 1-4; $X_2$, $X_3$ and $X_4$, mixtures of the 12 amino acids used in steps 1-3. Arrows denote the peptide mixtures or pure peptides chosen for the subsequent steps of the screen.

(B) The most active peptide resulting from (A), namely Tyr-Asp-His-Phe (step 4, column 9), was used as precursor for the synthesis of an additional set of 24 L-tetrapeptides, in which the N-terminal Fmoc-(β-Ala)$_2$ tag was replaced with an acetyl (Ac) group, and amino acid residues excluded in (A) were also included (Table S2). These second-generation L-tetrapeptides were screened by ELISA GADD45β/MKK7 competition assays as in (A), yielding Ac-Tyr-Asp-His-Phe-NH$_2$ (Ac-LTP1) and Ac-Tyr-Glu-Arg-Phe-NH$_2$ (Ac-LTP2) (columns 1 and 8, respectively; black arrows), which were then used in subsequent studies. Peptide 24 (grey arrow) was excluded from further analyses because of low aqueous solubility (Table S2).

(A, B) Values express the percentage of inhibition of GADD45β binding to GST-MKK7 relative to the binding measured in the absence of peptide (-) and denote means ± SD (n=3).
Table S1, related to Figure 3. List of the tetrapeptide mixtures and individual peptides used for the initial ELISA screen

|        | amino acid sequence | % inhibition (ELISA) |
|--------|---------------------|----------------------|
| **Step 1** |                     |                      |
|        | QX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 3                    |
|        | SX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 4                    |
|        | RX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 44                   |
|        | AX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 40                   |
|        | YX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 100                  |
|        | PX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 48                   |
|        | MX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 35                   |
|        | CX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 49                   |
|        | FX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 58                   |
|        | LX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 55                   |
|        | HX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 56                   |
|        | DX<sub>2</sub>X<sub>3</sub>X<sub>4</sub> | 55                   |
|        | YQX<sub>3</sub>X<sub>4</sub>         | 26                   |
|        | YSX<sub>3</sub>X<sub>4</sub>         | 16                   |
|        | YRX<sub>3</sub>X<sub>4</sub>         | 28                   |
|        | YAX<sub>3</sub>X<sub>4</sub>         | 20                   |
|        | YYX<sub>3</sub>X<sub>4</sub>         | 52                   |
|        | YPX<sub>3</sub>X<sub>4</sub>         | 42                   |
|        | YMX<sub>3</sub>X<sub>4</sub>         | 54                   |
|        | YCX<sub>3</sub>X<sub>4</sub>         | 27                   |
|        | YFX<sub>3</sub>X<sub>4</sub>         | 39                   |
|        | YLX<sub>3</sub>X<sub>4</sub>         | 52                   |
|        | YHX<sub>3</sub>X<sub>4</sub>         | 53                   |
|        | YDX<sub>3</sub>X<sub>4</sub>         | 96                   |
| **Step 2** |                     |                      |
|        | YDQX<sub>4</sub>        | 24                   |
|        | YDSX<sub>4</sub>        | 16                   |
|        | YDRX<sub>4</sub>        | 93                   |
|        | YDAX<sub>4</sub>        | 2                    |
|        | YDYX<sub>4</sub>        | 29                   |
|        | YDPX<sub>4</sub>        | 29                   |
|        | YDMX<sub>4</sub>        | 17                   |
|        | YDCX<sub>4</sub>        | 11                   |
|        | YDFX<sub>4</sub>        | 41                   |
|        | YDLX<sub>4</sub>        | 34                   |
|        | YDHX<sub>4</sub>        | 99                   |
|        | YDDX<sub>4</sub>        | 41                   |
| **Step 3** |                     |                      |
|        | YDHQ                 | 6                    |
|        | YDHS                 | 0                    |
|        | YDHHR           | 8                    |
|        | YDHA                 | 20                   |
|        | YDHY                 | 65                   |
|        | YDHP                 | 21                   |
|        | YDHM                 | 20                   |
|        | YDHC                 | 47                   |
|        | YDHF (LTP1)        | 99                   |
|        | YDHL                 | 37                   |
|        | YDHH                 | 43                   |
|        | YDHD                 | 1                    |
Shown is the core amino acid sequence of each of the peptide mixtures and individual peptides used for the ELISA screen depicted in Figure S3A. The simplified combinatorial library used for this screen consisted of a total of \(12^4 = 20,736\) L-tetrapeptides formed by all possible combinations of the following 12 L-amino acids: Gin (Q), Ser (S), Arg (R), Ala (A), Tyr (Y), Pro (P), Met (M), Cys(Acm) (C), Phe (F), Leu (L), His (H), Asp (D). Amino acids are identified by the standard single-letter amino acid code. X_2, X_3 and X_4 represent mixtures of the 12 amino acids listed above and used in steps 1 to 3 of the iterative screen (Figure S3A). Also shown are the results generated at each step of the library deconvolution process. Values express the percentage of inhibition of GADD45β binding to MKK7 obtained for each of the 12 peptide mixtures or individual peptides at the nominal concentration of 42 nM, relative to the binding measured in the absence of competitor and denote means (n=3).
Table S2, related to Figure 3. List of the tetrapeptides used for secondary screening

|   | amino acid sequence              | MW  | % inhibition (ELISA) |
|---|----------------------------------|-----|-----------------------|
| 1 | Ac-YDHF-NH<sub>2</sub> (Ac-LTP1) | 621 | 94                    |
| 2 | Ac-YEHF-NH<sub>2</sub>           | 635 | 52                    |
| 3 | Ac-WDHF-NH<sub>2</sub>           | 644 | 28                    |
| 4 | Ac-WEHF-NH<sub>2</sub>           | 658 | 35                    |
| 5 | Ac-YDRF-NH<sub>2</sub>           | 640 | 45                    |
| 6 | Ac-YDKF-NH<sub>2</sub>           | 612 | 34                    |
| 7 | Ac-YEKF-NH<sub>2</sub>           | 626 | 64                    |
| 8 | Ac-YERF-NH<sub>2</sub> (Ac-LTP2) | 654 | 93                    |
| 9 | Ac-WEKF-NH<sub>2</sub>           | 649 | 65                    |
| 10| Ac-WERF-NH<sub>2</sub>           | 677 | 28                    |
| 11| Ac-WDKF-NH<sub>2</sub>           | 653 | 25                    |
| 12| Ac-WDRF-NH<sub>2</sub>           | 663 | 45                    |
| 13| Ac-YDHW-NH<sub>2</sub>           | 660 | 57                    |
| 14| Ac-YEHW-NH<sub>2</sub>           | 674 | 64                    |
| 15| Ac-WDHW-NH<sub>2</sub>           | 683 | 50                    |
| 16| Ac-WEHW-NH<sub>2</sub>           | 697 | 75                    |
| 17| Ac-YDRW-NH<sub>2</sub>           | 679 | 43                    |
| 18| Ac-YDKW-NH<sub>2</sub>           | 651 | 43                    |
| 19| Ac-YEKW-NH<sub>2</sub>           | 665 | 22                    |
| 20| Ac-YERW-NH<sub>2</sub>           | 693 | 27                    |
| 21| Ac-WEKW-NH<sub>2</sub>           | 688 | 58                    |
| 22| Ac-WERW-NH<sub>2</sub>           | 716 | 64                    |
| 23| Ac-WDKW-NH<sub>2</sub>           | 674 | 69                    |
| 24| Ac-WDRW-NH<sub>2</sub>           | 702 | 93                    |

Shown are the 24 L-tetrapeptides of second generation used for the ELISA screen reported in Figure S3B. L-peptides were synthesized starting from the most active compound derived from step 4 of the screen shown in Figure S3A (column 9), namely Fmoc-(βAla)<sub>2</sub>-YDHF-NH<sub>2</sub> (referred to as LTP1; Table S1), by replacing the N-terminal Fmoc-(βAla)<sub>2</sub> tag with an acetyl (Ac) group and including additional L-amino acids not used for the synthesis of the L-tetrapeptides shown in Table S1 as follows: Tyr (Y) or Trp (W), in the first position; Asp (D) or Glu (E), in the second position; His (H), Arg (R) or Lys (K), in the third position; Phe (F) or Trp (W), in the fourth position. All peptides were C-terminally amidated and N-terminally acetylated, and were in the L configuration. Also shown are the results from the ELISA competition screen. Values express the percentage of inhibition of GADD45β binding to MKK7 obtained for each of the peptides at the concentration of 42 nM, relative to the binding measured in the absence of peptide and denote means (n=3). The most active L-tetrapeptides resulting from this screen were denoted as Ac-LTP1 (Ac-YDHF-NH<sub>2</sub>) and Ac-LTP2 (Ac-YERF-NH<sub>2</sub>) and were used for subsequent analyses. MW, molecular weight.
Figure S4, related to Figure 4. z-DTP1 and z-DTP2 exhibit high activity and stability, in vitro, and potent and selective capacity to kill MM cells by inducing JNK/MKK7-dependent apoptosis.

(A) ELISA GADD45β/MKK7 competition assays showing the IC$_{50}$ values of the Z-protected D-tetrapeptides, z-DTP1 and z-DTP2, before and after a 48-hr incubation with human serum at 37°C, as indicated. Values express the percentage of inhibition of GADD45β binding to MKK7 relative to the binding measured in the absence of peptide.

(B) Summary of the IC$_{50}$ values of Ac-DTP1, Ac-DTP2, z-DTP1 and z-DTP2 in the ELISA competition assays shown in (A) and Figures 3A-B, in the presence or absence of a pre-incubation with serum (note the similar IC$_{50}$ values given by each of the peptides before and after incubation with serum). Values denote means.

(C, D) [3H]Thymidine incorporation assays showing the survival of representative MM cell lines after treatment with acetylated D-tetrapeptides (Ac-DTP1, Ac-DTP2, Ac-DNC; C) or Z-protected D-tetrapeptides (z-DTP1, z-DTP2, z-DNC; D) for the times indicated. (C) Ac-DNC, acetylated negative control D-tetrapeptide.

(E, F) [3H]Thymidine incorporation assays showing the survival of representative sensitive (E) and resistant (KMM-1; F) MM cell lines after a 6-day treatment with the indicated concentrations of z-DTP1, z-DTP2 or z-DNC.

(G) PI nuclear staining assays showing apoptotic cells in representative MM cell lines that depend on GADD45β for survival after treatment with 10 μM of either z-DTP1, z-DTP2 or z-DNC for 6 days. The percentages of apoptotic cells are depicted. UT, untreated cells.

(H) Western blots showing total and phosphorylated (P) proteins in representative sensitive (blue) and resistant (red) MM cell lines after treatment with z-DTP2 (10 μM), z-DNC (10 μM), or TPA/ionomycin (T/I) for the times indicated. Filled and open arrowheads indicate the unprocessed forms and cleavage products, respectively, of caspase-3 and its proteolytic substrate, PARP-1. β-actin is shown as control.

(I) Shown is the survival of healthy, primary human bone marrow stromal cells (BMSCs), primary human mesenchymal stem cells (MSCs), and primary mouse splenocytes and lymph node (LN) cells after treatment with z-DTP1 or z-DTP2 for 72 hr (splenocytes, LN cells) or 144 hr (BMSCs, MSCs). Cell viability was measured using trypan blue exclusion assays (BMSCs) or [3H]thymidine incorporation assays (MSCs, splenocytes, LN cells). Values express the percentage of live cells present in the
treated cultures relative to the live cells present in the respective untreated cultures, represented as 100%. UT, untreated.

(A, C, D, E, F, I) Values denote means ± SD (n=3).

(A, D, E, F, G) z-DNC, Z-protected negative control D-tetrapeptide.

(C, D, E, F) Values express the percentage of the cpm measured with the treated cultures relative to the cpm measured with the respective untreated cultures.
Table S3, related to Figure 4. List of the D-peptides used to improve cell penetration

| Amino acid sequence          | MW  | % inhibition (ELISA) | IC<sub>50</sub> (ELISA) | IC<sub>50</sub> (U266 cells) |
|------------------------------|-----|----------------------|--------------------------|-----------------------------|
| z-YERF-NH<sub>2</sub> (z-DTP2) | 746 | 95                   | 0.19 nM                  | (†) 40.70 nM                |
| z-YDHF-NH<sub>2</sub> (z-DTP1) | 713 | 84                   | 0.20 nM                  | (‡) 40.70 nM                |
| z-YDHQ-NH<sub>2</sub> (z-DNC)  | 694 | 12                   | > 10.00 nM               | > 10,000.00 nM              |
| z- YD(O-methyl)HF-NH<sub>2</sub> | 727 | 84                   | > 10.00 nM               | N.D.                        |
| z-YD(O-methyl)HQ-NH<sub>2</sub> | 709 | 3                    | > 10.00 nM               | N.D.                        |
| 2CI-z-YERF-NH<sub>2</sub>     | 780 | 38                   | > 10.00 nM               | N.D.                        |
| 2CI-z-YDHF-NH<sub>2</sub>     | 747 | 38                   | > 10.00 nM               | N.D.                        |
| 2CI-z-YDHQ-NH<sub>2</sub>     | 729 | 40                   | > 10.00 nM               | N.D.                        |
| Myr-YERF-NH<sub>2</sub>      | 822 | 37                   | > 10.00 nM               | > 10,000.00 nM             |
| Myr-YDHF-NH<sub>2</sub>      | 788 | 26                   | > 10.00 nM               | > 10,000.00 nM             |
| Myr-YDHQ-NH<sub>2</sub>      | 770 | 28                   | > 10.00 nM               | > 10,000.00 nM             |
| Benzoyl-YERF-NH<sub>2</sub>  | 717 | 24                   | > 10.00 nM               | > 10,000.00 nM             |
| Benzoyl-YDHF-NH<sub>2</sub>  | 684 | 0                    | > 10.00 nM               | > 10,000.00 nM             |
| Benzoyl-YDHQ-NH<sub>2</sub>  | 665 | 1                    | > 10.00 nM               | > 10,000.00 nM             |
| 3-hydroxy-4-methoxy-benzyl-YERF-NH<sub>2</sub> | 755 | 86                   | < 10.00 nM               | < 10,000.00 nM             |
| 3-hydroxy-4-methoxy-benzyl-YDHF-NH<sub>2</sub> | 722 | 81                   | < 10.00 nM               | < 10,000.00 nM             |
| 3-hydroxy-4-methoxy-benzyl-YDHQ-NH<sub>2</sub> | 703 | 7                    | < 10.00 nM               | < 10,000.00 nM             |
| Fmoc-YERF-NH<sub>2</sub>     | 834 | 60                   | < 10.00 nM               | N.D.                        |
| Fmoc-YDHF-NH<sub>2</sub>     | 802 | 58                   | < 10.00 nM               | N.D.                        |
| Fmoc-YDFQ-NH<sub>2</sub>     | 782 | 19                   | > 10.00 nM               | N.D.                        |
| Ac-YERFK(Z)-NH<sub>2</sub>   | 916 | 6                    | > 10.00 nM               | N.D.                        |
| Ac-YDHFK(Z)-NH<sub>2</sub>   | 883 | 8                    | > 10.00 nM               | N.D.                        |
| Ac-YDHQK(Z)-NH<sub>2</sub>   | 865 | 2                    | > 10.00 nM               | N.D.                        |

Shown are the D-peptide derivatives designed to improve cell penetration and activity in myeloma cells. Also shown are the values obtained for each of these peptides in ELISA competition assays (reported both as percentage of GADD45β/MKK7 binding inhibition at the concentration of 10 nM and as IC<sub>50</sub>), and in [3H]thymidine incorporation or trypan blue exclusion assays in U266 myeloma cells (reported as IC<sub>50</sub> at day 6. All peptides are in the D configuration). Z, benzoyloxycarbonyl; 2CI-Z, 2-chloro-benzoxycarbonyl; Myr, myristyl; Fmoc, fluorenylmethoxycarbonyl; Ac, acetyl; MW, molecular weight; N.D., not determined. (†) Values are from Figure 4A.
Figure S5, related to Figure 5. The development of DTP3: a potent and cancer-selective GADD45β/MKK7 inhibitor with improved bioavailability in vivo

(A) Shown is the putative bioactive pharmacophore model derived by FLAP (Baroni et al., 2007; Kozakov et al., 2001) using the structural superposition of 12 bioactive peptides (i.e. peptides with IC\textsubscript{50} ≤10 nM in ELISA competition assays). Regions matching hydrophobic, hydrogen bond acceptor, and hydrogen bond donor features of the peptides are represented by green, red, and blue contours, respectively.

(B) Graph showing the discrimination capacity of the pharmacophore model for an additional set of active (bottom) and inactive peptides that were not used in (A) to generate this pharmacophore model. The plot shows the percentage of true positives (i.e. peptides inhibiting the GADD45β/MKK7 interaction by more than 60% at 42 nM in ELISA competition assays) as a function of the percentage of false positives (i.e. peptides inhibiting the GADD45β/MKK7 interaction by less than 30% at 42 nM in ELISA competition assays). The area under the curve for the plot is 0.92.

(C) Summary of the drug discovery strategy that led to the development of DTP3. cmpd, compounds.

(D) ELISA GADD45β/MKK7 competition assays showing the IC\textsubscript{50} values of DTP3 and the scrambled control D-tripeptide, SCRB, before and after a 48-hr pre-incubation with human serum, at 37°C, as indicated. Values express the percentage of inhibition of GADD45β binding to MKK7 relative to the binding measured in the absence of peptide.

(E) Co-immunoprecipitation assays showing the disruption of the GADD45β/MMK7 complex by DTP3, but not by the SCRB control D-peptide, at the indicated concentrations. Co-immunoprecipitations (IP) were performed using an anti-FLAG antibody, as in Figure 3C; western blots were developed using an anti-HA or an anti-MKK7 antibody, as shown. -, incubation without D-tripeptide.

(F) [\textsuperscript{3}H]Thymidine incorporation assays showing the survival of GADD45β-dependent (blue) and GADD45β-independent (red) MM cell lines after a 6-day treatment with the indicated concentrations of DTP3 or a negative control D-peptide (z-DNC).

(G) Shown are IC\textsubscript{50} values of DTP3 at 144 hr for the experiment reported in (F).

(H) PI nuclear staining assays showing apoptotic cells in representative GADD45β-dependent and GADD45β-independent MM cell lines from (F, G) after a 6-day treatment with 10 μM of either DTP3 or z-DNC. The percentages of apoptotic cells are depicted.
(I) Circular dichroism (CD) analyses showing the effects of DTP3 (left) and the SCRB peptide (right) at increasing concentrations, ranging from 0.1 nM to 10 µM, on the conformation of GADD45β (1.25 µM; top), the kinase domain (KD) of MKK7 (MKK7-KD, 1.25 µM; middle), and the GADD45β/MKK7-KD complex (1.25 µM of each protein; bottom). Insets depict magnifications of the CD spectra of the proteins within the 200-230 nm UV region (note the reduction in the intensity of the spectra of MKK7 [middle, left] and the complex [bottom, left] at the wavelengths of approximately 210 and 220 nm, denoting a conformational rearrangement of the proteins, in the presence of DTP3, but not of the SCRB D-peptide).

(J) MALDI-TOF mass spectrometry (MS) analysis showing the effects of DTP3 (middle) and the SCRB D-peptide (bottom) on the mass peak values of MKK7-KD (left) and GADD45β (right), as indicated. Proteins were incubated at the concentration of 5 µM in the presence or absence of an equimolar concentration (5 µM) of D-tripeptide, as shown. The experimental molecular weight (MW) of each of the proteins was in close agreement with that expected on the basis of the primary structures, which predict a MW of 19,195 Da for GADD45β and of 36,283 Da for MKK7-KD. Shown is the shift induced in the mass peak value of MKK7-KD (top, left) in the presence of DTP3 (middle, left), but not of the SCRB peptide (bottom, left), denoting the binding of this protein to the active D-tripeptide.

(K) Tryptophan fluorescence quenching analysis showing the effects of DTP3 (top, bottom) and the SCRB D-peptide (middle) on the fluorescence emission spectra of GST-hMKK7 (top, middle) and GST (bottom) (note the 2-nm blue shift of the fluorescence maximum of the emission spectrum of GST-MKK7 at the wavelength of 332-334 nm in the presence of DTP3 [top], but not of the SCRB D-peptide [middle]). GST-hMKK7 was used at the concentration of 1.25 µM and incubated in the presence or absence of increasing concentrations of DTP3 (top) or SCRB D-peptide (middle), ranging from 13 nM to 3.3 µM, as indicated. GST (1.25 µM) (bottom) was used as control. The dose-response curve of the -Δfluorescence values of GST-hMKK7 at the wavelength of 333 nm plotted against the concentration values of DTP3 for the experiment in the top panel is shown in Figure 5B. The stoichiometry of the peptide interaction with the protein on the basis of the values reported in the top panel was 1:1. The estimated equilibrium dissociation constant (K_D) value of this interaction was 64.81 ± 6.22 nM (Williamson, 2013).

(L) Shown is the model of GADD45β built by homology, using as template the crystal structure of human GADD45γ (PDB Id: 3FFM), which shares 58% amino acid sequence identity with GADD45β.
The coordinates of MKK7 were rebuilt on the basis of the experimental structure factors deposited in the PDB database (PDB Id: 2DYL). The missing residues were modeled.

Proteins are coloured according to the estimated residue error (Benkert et al., 2011), using a gradient ranging from blue (most reliable regions) to red (least reliable regions).

The model of the interaction between GADD45β and MKK7 was obtained from the coordinates of the models shown in (L) and (M), using a rigid body docking approach and refining the resulting best model in explicit solvent. The final model of the complex is shown. Most of the protein-protein interactions involve the α4-β4 loop (residues 104-117) and β2-α3 connecting region (residues 63-67) of GADD45β and the region of MKK7 including the ATP-binding site and the basic residue-rich region, comprising β2 and β3, previously identified as the GADD45β-binding site (Papa et al., 2004; Papa et al., 2007). As shown, GADD45β occupies most of the cleft between the two lobes of MKK7 hosting the ATP-binding site, consistent with the mechanism by which GADD45β blocks the catalytic activity of the kinase (Papa et al., 2004; Papa et al., 2007). Green, MKK7; beige, GADD45β.

The models of GADD45β (L), MKK7 (M), and the GADD45β/MKK7 complex (N) are shown in ribbon representation.

Shown are the top scoring ligand-binding sites identified by FTSite (O) and FLAP (P) on MKK7 (depicted in blue and pink, respectively). As illustrated, these sites are located within the same region of the protein, near the predicted interface of the kinase with GADD45β within the complex. The orientation of MKK7 is the same as in (M).

Superposition of the molecular interaction fields (MIFs) of the putative binding site of MKK7 and the pharmacophore model as computed by FLAP, using an hydrophobic/hydrophobic (Q), H-bond donor/acceptor (R) and H-bond acceptor/donor (S) probe. Hydrophobic areas are shown in green; H-bond acceptor and donor areas are shown in red and blue, respectively. Lighter colours denote the pharmacophore model; darker ones denote the ligand-binding site of MKK7. The region of MKK7 identified as favourable for interacting with the pharmacophore model is shown with a brown contour (note the complementarities of the pharmacophore model with the cognate energy fields of the predicted binding site of MKK7). The physicochemical properties of the pharmacophore model and the putative ligand-binding pocket of the kinase are complementary in the areas in which there is a match between hydrophobic regions ([Q], dark and light green), and donors and acceptors of H bonds ([R, S], blue and red).
(T) For clarity purposes, the predicted orientation of the structure of DTP3 is shown in place of the pharmacophore model within the context of the putative binding region of M KK7, depicted as in (Q, R, S).

(U) Shown is the correlation plot of the relative GADD45B mRNA expression (qRT-PCR assays) and the log_{10} IC_{50}s of z-DTP2 at 144 hr ([3H]thymidine incorporation assays) in a panel of cancer cell lines of different tissues of origin. r_s, Spearman correlation coefficient (r_s=-0.8562; p<0.0001). Similar results were obtained with z-DTP1 (data not shown).

(V) Western blots showing total and phosphorylated (P) proteins in representative sensitive (U266) and resistant (RPMI-8226) myeloma cell lines after treatment with DTP3 (10 μM), z-DNC (10 μM) or TPA/ionomycin (T/I) for the times indicated. β-actin is shown as control.

(W) [3H]Thymidine incorporation assays showing the survival of sensitive U266 and KMS-12 MM cells expressing sh-ns or sh-JNK1 after a 6-day treatment with increasing concentrations of DTP3. Also depicted are the IC_{50} values of DTP3 in sh-ns- and sh-JNK1-expressing cells.

(X) [3H]Thymidine incorporation assays showing the survival of ARH-77 B lymphoblastoid cells expressing sh-ns or sh-MKK7 sh-RNAs after a 72-hr treatment with the indicated concentrations of z-DTP2. The IC_{50} values of z-DTP2 in sh-ns- and sh-MKK7-expressing cells are also depicted. Similar results were obtained with z-DTP1 (data not shown).

(D, F, W, X) Values denote means ± SD (n=3).

(F, W, X) Values express the percentage of the cpm measured with the treated cultures relative to the cpm measured with the respective untreated cultures.
Table S4, related to Figure 5. List of the peptides used to generate and validate the pharmacophore model

| Amino acid sequence | IC_{50} (ELISA) | % inhibition of (ELISA) |
|---------------------|------------------|-------------------------|
| Ac-YQRF-NH₂         | 5.00 nM          | N.D.                    |
| Ac-YNRF-NH₂         | 10.00 nM         | N.D.                    |
| Ac-YLRF-NH₂         | 5.00 nM          | N.D.                    |
| Ac-YLFR-NH₂         | 1.80 nM          | N.D.                    |
| Ac-VNF-NH₂          | 1.90 nM          | N.D.                    |
| Ac-VPF-NH₂          | 0.65 nM          | N.D.                    |
| Ac-YHF-NH₂          | 0.70 nM          | N.D.                    |
| H-YHF-NH₂           | 0.65 nM          | N.D.                    |
| Ac-VRF-NH₂          | 0.75 nM          | N.D.                    |
| Ac-YKD-NH₂          | 0.85 nM          | N.D.                    |
| Ac-YPFRF-NH₂(DTP3)  | 0.16 nM          | (#) 94                  |
| Ac-YPF-NH₂          | 0.65 nM          | N.D.                    |
| Ac-YERF-NH₂(Ac-LTP2)| (*) 0.40 nM      | (#) 93                  |
| Ac-WDFR-NH₂         | (*) < 42.00 nM   | (#) 75                  |
| Ac-WERF-NH₂         | (*) < 42.00 nM   | (#) 69                  |
| Ac-WDWF-NH₂         | (*) < 42.00 nM   | (#) 69                  |
| Ac-WERF-NH₂         | (*) < 42.00 nM   | (#) 69                  |
| Ac-WERF-NH₂         | (*) < 42.00 nM   | (#) 69                  |
| Ac-WERF-NH₂         | (*) < 42.00 nM   | (#) 69                  |
| Ac-YERF-NH₂         | 10.00 nM         | (#) 28                  |
| Ac-YERF-NH₂         | 10.00 nM         | (#) 28                  |
| Ac-YERF-NH₂         | 10.00 nM         | (#) 22                  |
| Ac-YPFRF-NH₂        | 10.00 nM         | (#) 25                  |
| Ac-YERF-NH₂         | 10.00 nM         | (#) 24                  |
| Ac-YERF-NH₂         | 10.00 nM         | (#) 24                  |
| Ac-YPFRF-NH₂        | 10.00 nM         | (#) 43                  |
| Ac-YPFRF-NH₂        | 10.00 nM         | (#) 17                  |
| Ac-YPFRF-NH₂        | 10.00 nM         | (#) 13                  |
| Ac-YPFRF-NH₂        | 10.00 nM         | (#) 13                  |
| Ac-YPFRF-NH₂        | 10.00 nM         | (#) 13                  |
| Ac-AERF-NH₂         | 10.00 nM         | (#) 9                    |
| Ac-AERF-NH₂         | 10.00 nM         | (#) 7                    |
| Benzoyl-YDF-NH₂     | (***) > 10.00 nM | (#) 1                     |
| Benzoyl-YDF-NH₂     | (***) > 10.00 nM | (#) 0                     |

Shown are the 12 bioactive peptides used in Figure S5A to derive the pharmacophore model (active [top]). Also shown are the 26 active (bottom) and inactive peptides used in Figure S5B to assess the pharmacophore classification ability. Reported are the percentages of GADD45β/MKK7 binding inhibition measured at the peptide concentration of either 10 or 42 nM, as shown, and the IC_{50} values obtained for each of the peptides in ELISA competition assays. Values in the right column express the percentages of inhibition of GADD45β binding to MKK7 obtained with each peptide at the indicated concentration relative to the binding measured in the absence of peptide and denote means. Amino
acids are identified by the standard single-letter amino acid code. N.D., not determined. * Values are from Table S2. ** Values are from Table S3. (#) Peptide tested at a concentration of 42.00 nM. (##) Peptide tested at a concentration of 10.00 nM.
Table S5, related to Figure 5. Broad kinase-panel profiling showing the absence of any significant effect of DTP3 on the activity of 142 human kinases

| kinase ID    | mean at 1 μM SD | mean at 10 μM SD | kinase ID  | mean at 1 μM SD | mean at 10 μM SD | kinase ID  | mean at 1 μM SD | mean at 10 μM SD |
|--------------|-----------------|------------------|------------|-----------------|------------------|------------|-----------------|------------------|
| MKK7         | 104             | 8                | 91         | 6               | 102              | 5          | 102             | 5                |
| MKK1         | 91              | 3                | 96          | 0               | 88               | 101        | 13              | 97               |
| MKK2         | 114             | 4                | 110         | 9               | 100              | 2          | 99              | 13               |
| MKK6         | 97              | 7                | 104         | 7               | 91               | 3          | 99             | 4                |
| ERK1         | 103             | 0                | 93          | 11              | 95               | 2          | 95             | 5                |
| ERK2         | 102             | 3                | 96          | 9               | 98               | 11         | 117            | 1                |
| ERK5         | 105             | 2                | 93          | 3               | 104              | 7          | 106            | 5                |
| JNK1         | 107             | 2                | 100         | 3               | 82               | 8          | 72             | 3                |
| JNK2         | 106             | 2                | 99          | 1               | 95               | 4          | 92             | 11               |
| JNK3         | 98              | 12               | 90          | 19              | 96               | 6          | 103            | 7                |
| p38a MAPK    | 83              | 17               | 83          | 8               | 85               | 2          | 77             | 7                |
| p38β MAPK    | 115             | 4                | 109         | 14              | 101              | 1          | 102            | 1                |
| p38γ MAPK    | 97              | 15               | 109         | 2               | 98               | 3          | 106            | 14               |
| ROCK 2       | 115             | 20               | 74          | 30              | 98               | 2          | 96             | 3                |
| PRK2         | 90              | 11               | 92          | 0               | 97               | 6          | 93             | 8                |
| PKCβ         | 88              | 4                | 90          | 1               | 97               | 13         | 92             | 11               |
| PKCδ         | 105             | 5                | 77          | 33              | 99               | 3          | 101            | 5                |
| SGK1         | 96              | 2                | 92          | 1               | 91               | 0          | 90             | 7                |
| SIK1         | 98              | 2                | 93          | 8               | 94               | 15         | 110            | 10               |
| PKA          | 92              | 4                | 99          | 2               | 103              | 3          | 99             | 1                |
| ROCK 2       | 115             | 20               | 74          | 30              | 98               | 2          | 96             | 3                |
| PRK2         | 90              | 11               | 92          | 0               | 97               | 6          | 93             | 8                |
| PKCβ         | 103             | 0                | 102         | 4               | 104              | 10         | 117            | 13               |
| PKCγ         | 105             | 6                | 114         | 5               | 105              | 2          | 109            | 4                |
| PKCζ         | 101             | 3                | 105         | 7               | 108              | 6          | 101            | 5                |
| MAPKAP-K2    | 94              | 1                | 106         | 6               | 95               | 1          | 93             | 7                |
| MAPKAP-K3    | 104             | 1                | 100         | 4               | 103              | 6          | 106            | 2                |
| PRAK         | 96              | 9                | 102         | 18              | 123              | 2          | 121            | 17               |
| CAMKK1       | 107             | 30               | 110         | 5               | 114              | 13         | 116            | 12               |
| CAMK1        | 108             | 12               | 96          | 3               | 102              | 13         | 93             | 7                |
| SmMLC         | 105             | 3                | 107         | 2               | 105              | 7          | 93             | 18               |
| PKH          | 118             | 15               | 109         | 7               | 59               | 8          | 90             | 14               |
| DAPK1        | 107             | 6                | 100         | 9               | 109              | 11         | 115            | 16               |
| CHK1         | 107             | 4                | 88          | 23              | 89               | 1          | 97             | 6                |
| CHK2         | 99              | 6                | 100         | 0               | 99               | 3          | 105            | 7                |
| GSK3β        | 95              | 0                | 94          | 2               | 92               | 0          | 87             | 5                |
| CDK2-Cyclin A| 94              | 7                | 105         | 3               | 105              | 13         | 88             | 1                |
| CDK2-Cyclin T1| 96             | 3                | 107         | 2               | 85               | 7          | 94             | 6                |
| PLK1         | 101             | 6                | 95          | 15              | 106              | 12         | 93             | 1                |
| Aurora A     | 105             | 3                | 101         | 7               | 100              | 4          | 109            | 1                |
| Aurora B     | 100             | 2                | 113         | 1               | 95               | 1          | 99             | 5                |
| TLK1         | 103             | 11               | 93          | 12              | 104              | 2          | 105            | 6                |
| LKB1         | 104             | 8                | 102         | 3               | 100              | 4          | 104            | 8                |
| AMPK         | 92              | 19               | 103         | 4               | 112              | 7          | 102            | 4                |
| MARK1        | 123             | 0                | 110         | 16              | 102              | 14         | 108            | 4                |
| MARK2        | 109             | 3                | 96          | 13              | 104              | 15         | 100            | 1                |
| MARK3        | 124             | 3                | 108         | 3               | 103              | 2          | 95             | 2                |
| MARK4        | 107             | 4                | 100         | 1               | 83               | 15         | 88             | 7                |

DPT3 was tested in kinase assays at the concentration of either 1 or 10 μM, as indicated, at the International Centre for Kinase Profiling, MRC-Protein Phosphorylation Unit, Dundee (http://www.kinase-screen.mrc.ac.uk/). The kinase ID names are shown. Values express the percentage of kinase activity measured in the presence of DTP3 relative to the kinase activity measured in the absence of peptide and denote means ± SD (n=3).
**A**

![Graph showing % survival for CD138+ cells/IL-6 with MM and WM cell lines in response to UT, DTTP3 (300 nM), and PS-1145 (20,000 µM).]

**B**

![Graph showing % survival for CD138+ cells/GF-1 with MM and WM cell lines in response to UT, DTTP3 (300 nM), and PS-1145 (20,000 µM).]

**C**

![Graph showing relative mRNA levels for MM and WM cell lines.]

**D**

![Graphs showing % survival for U266 and KMS-12 cell lines in response to DTTP3, bortezombib, DTTP3 + bortezombib.]

**E**

| MM cell line | Fraction Affected (FA) | DTTP3 (Concentration) | Bortezomib (Concentration) | Combination Index (CI) | Description |
|--------------|------------------------|------------------------|----------------------------|------------------------|-------------|
| U266         | 0.5                    | 3 nM                   | 16.6 nM                   | 0.19 nM                | 6 nM        | 0.21        | strong synergism            |
| KMS-12       | 0.5                    | 10 nM                  | 25.1 nM                   | 1 nM                   | 6 nM        | 0.56        | synergism                   |

**F**

![Graphs showing % survival for various MM cell lines (MM1.S, MM1.R, AMO-1, AMO-1a) in response to MM1.S peptide (Log M).](image)

**G**

| Drug          | MM cell line          | DTTP3 (IC_{50}) |
|---------------|-----------------------|-----------------|
| Dexamethasone | MM1.S (parental)      | 457.1 nM        |
|               | MM1.R (resistant)     | 246.0 nM        |
| Bortezomib   | AMO-1 (parental)      | 87.1 nM         |
|               | AMO-1a (resistant)    | 77.6 nM         |
| Lenalolamide | U266 (parental)       | 16.6 nM         |
|               | U266/R10R (resistant) | 19.2 nM         |
|               | MM1.S (parental)      | 457.1 nM        |
|               | MM1/R10R (resistant)  | 666.7 nM        |
Figure S6, related to Figure 6. DTP3 exhibits more potent and selective activity than IKKβ inhibitors, synergizes with bortezomib, and kills drug-resistant MM cells

(A, B) Trypan blue exclusion assays showing the survival of primary CD138⁺ cells from MM or Waldenström's macroglobulinemia (WM) patients after treatment with DTP3 (300 nM) or PS-1145 (20 µM) for 48 hr, in the presence of IL-6 (10 ng/mL; A) or IGF-1 (50 ng/mL; B) stimulation. Values express the percentage of live cells present in the treated cultures relative to the live cells present in the respective untreated cultures and denote means ± SD (WM; n=3) or SEM (MM; n=8). UT, untreated.

(C) qRT-PCR assays showing the relative mRNA expression of GADD45B in CD138⁺ cells from MM patients, CD138⁺ cells from WM patients, and healthy human PBMCs. Values represent individual patients and denote means ± SD (n=3).

(D) [³H]Thymidine incorporation assays showing the survival of representative GADD45β-dependent MM cell lines after treatment with the indicated concentrations of DTP3 and bortezomib, used either as single agents or in combination. Treatments with DTP3 were for 6 days; bortezomib was added to the cell cultures 48 hr prior to the measurement of cell viability. For the combination treatment, DTP3 was used at the concentrations of 3 nM in U266 cells and of 10 nM in KMS-12 cells, whereas bortezomib was used at increasing concentrations, as shown. Based on the data shown, the IC₅₀ value of bortezomib as single agent at 48 hr was 6 nM in each of the two MM cell lines. The survival curves of U266 and KMS-12 cells following treatment with DTP3 as single agent are from the experiment shown in Figure S5F.

(E) Shown is the combination index (CI) of DTP3 and bortezomib for the experiment shown in (D). Viability data from (D) were converted into values representing the fraction of cells affected (FA) equaling 0.5 in the drug-treated cultures compared with untreated cultures, and the interaction of DTP3 with bortezomib was analyzed according to the Chou-Talalay method (Chou, 2006). Also shown are the drug concentrations giving the FA value of 0.5 and the description of the CI. Based on the Chou-Talalay equation (Chou, 2006), synergy is present when the CI is less than 1.0 (where values between 0.3 and 0.7 denote synergism, and values between 0.1 and 0.3 denote strong synergism); the combination is additive when CI equals 1.0, and antagonistic when it is more than 1.0 (Chou, 2006).

(F) [³H]Thymidine incorporation assays showing the survival of sensitive and drug-resistant MM cell lines following a 6-day treatment with the indicated concentrations of DTP3.
(G) Shown are IC\textsubscript{50} values of DTP3 for the experiment in (F).

(D, F) Values express the percentage of the cpm measured with the treated cultures relative to the cpm measured with the respective untreated cultures and denote means ± SD (n=3).

(F, G) Matching pairs of sensitive (parental) and drug-resistant MM cell lines were as follows: MM1.S (parental) and MM1.R (dexamethasone-resistant); AMO-1 (parental) and AMO-1a (bortezomib-resistant); MM1.S (parental) and MM1/R10R (lenalidomide-resistant); U266 (parental) and U266/R10R (lenalidomide-resistant). Data with the parental U266 MM cell line were from the experiment shown in Figures S5F-G (5 out 7 DTP3 concentrations only). For clarity purposes, the same data for the parental MM1.S MM cell line are shown as control for the dexamethasone-resistant (MM1.R) MM cell line and the lenalidomide-resistant (MM1/R10R) MM cell line.
Figure S7, related to Figure 7. Treatment with DTP3 potently induces apoptosis in myeloma xenografts, in vivo, at doses that produce no apparent side effects.

(A) Images of representative myeloma-bearing mice (top) and isolated U266 tumors (bottom) at different times after administration of DTP3 at the dose of 14.5 mg/kg/day, yielding a steady-state peptide plasma concentration of approximately 0.7 µM, or PBS.

(B) PI nuclear staining assays showing apoptotic cells in the tumor tissues of representative myeloma-bearing mice from (A). The percentages of apoptotic cells are depicted.

(C) Shown are the volumes of subcutaneous KMS-11 myeloma xenografts in mice treated by continual infusion of DTP3 at the dose of 14.5 mg/kg/day or PBS, as in Figures 7B-E, for the times indicated. Values denote means ± SEM (n=4). ***, p<0.001.

(D) Images of representative myeloma-bearing mice (top) and isolated tumors (bottom) from (C) at day 28 of the start of treatment.
Table S6, related to Figure 7. Summary of the pharmacokinetic profiles of DTP3 and z-DTP2

| PK Parameters                                      | Values                                      | Drug      |
|----------------------------------------------------|---------------------------------------------|-----------|
| **In vitro**                                       |                                             | DTP3      |
| MW                                                 | 525.38 Da                                   |           |
| LogD                                               | 1.19 ± 0.29 (SD)                            |           |
| plasma stability (human)                           | > 48hrs                                     |           |
| PPB (fraction unbound)                             | 0.42 ± 0.01 (SD) (mouse)                    |           |
|                                                   | 0.933 ± 0.004 (SD) (human)                  |           |
| microsomal stability (human)                       | 4.87 ± 1.24 µL/min/mg protein               |           |
|                                                   | $t_{1/2}$ = 285 min (SEM)                    |           |
| thermodynamic solubility (in PBS, pH 7.4, for 14 days at 37 °C) | 169 ± 7.53 mg/ml (SD)                      |           |
| toxicity - single i.v. (10 mg/kg)                  | no apparent toxicity                        |           |
| toxicity - single s.c. (10 mg/kg)                  | no apparent toxicity                        |           |
| toxicity - oral (100 mg/kg)                        | no apparent toxicity                        |           |
| toxicity - continual s.c. dosing (14.5 mg/kg/day for 28 days) | no apparent toxicity                      |           |
| $t_{1/2}$                                          | 2.09 ± 0.07 hrs (SD)                        |           |
| CL                                                 | 78.11 ± 11.67 mL/min/kg (SD)                | z-DTP2    |
| $V_d$                                              | 14.58 ± 2.23 L/kg (SD)                      |           |
| AUC                                                | 2.062 ± 0.25 µg·hr/mL (SD)                  |           |

Shown are the values of the main in vitro pharmacokinetic (PK) parameters of DTP3, including distribution coefficient (LogD), plasma stability, plasma protein binding (PPB), microsomal stability, and thermodynamic solubility. Also shown is the excellent tolerability of DTP3 in mice after either a single intravenous (i.v.) injection, a subcutaneous (s.c.) injection, or a per os administration at the doses indicated. Additionally, shown are the excellent tolerability and lack of any apparent side effects of DTP3 after prolonged administration via osmotic pumps over a period of 28 days at the therapeutic dose of 14.5 mg/kg/day. Reported at the bottom are the values of the main in-vivo pharmacokinetic parameters of z-DTP2 after a single intravenous injection at the dose of 10 mg/kg, including terminal half-life ($t_{1/2}$), plasma clearance (CL), volume of distribution ($V_d$), and area under the plasma concentration versus time curve (AUC). As also shown in Figure 7A, DTP3 exhibited somewhat shorter $t_{1/2}$, but significantly lower CL and much lower $V_d$ values than z-DTP2. As a result of these properties, the predicted doses of DTP3 and z-DTP2 required to achieve the therapeutic plasma concentration of 1 µM at the steady state, on the basis of the measured pharmacokinetic values and using the equation described in the Supplemental Experimental Procedures, were 0.81 mg/kg/hr and 3.61 mg/kg/hr, respectively, thus demonstrating the superior pharmacokinetic profile of DTP3 as compared with z-DTP2. Values denote means ± SD (n=3) or SEM (n=5), as indicated. MW, molecular weight.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Antibodies, Reagents and Western Blots

Western blots were performed as described previously (Papa et al., 2008; Mauro et al., 2011). The antibodies used were: anti-IκBα (sc-1643; 1:5,000), anti-PARP-1 (sc-56197; 1:1,000), anti-β-actin (sc-1616; 1:3,000), anti-MEK-7 (sc-7104; 1:1,000), anti-HA (sc-805; 1:1,000), goat anti-rabbit IgG-HRP (sc-2030; 1:2,500), donkey anti-goat IgG-HRP (sc-2020; 1:5,000), goat anti-mouse IgG-HRP (sc-2005; 1:2,500), anti-GADD45β (sc-133606; 1:500) (Santa Cruz Biotechnology); anti-PARP-1 (5625P; cleaved product; 1:1,000), anti-caspase-3 (9662S; 1:1,000), anti-JNK (9258S 1:1,000), anti-P-JNK (9251S 1:1,000), anti-p38 (9212; 1:1,000), anti-P-p38 (9215S; 1:1,000), anti-ERK1/2 (4695S; 1:1,000), anti-P-ERK1/2 (4377S; 1:1,000), anti-MKK7 (4172S; 1:1,000), anti-MKK4 (9152S; 1:1,000) (Cell Signaling); anti-RelA (ADI-KASTF110; 1:5,000) (ENZO); anti-FLAG (F-7425; 1:2,000) (Sigma-Aldrich). The anti-FLAG M2 Affinity Gel used for immunoprecipitations and kinase assays was from Sigma-Aldrich. The propidium iodide (PI; 50 µg/mL) and ribonuclease A (50 µg/mL) reagents used for the FACS analysis of apoptosis were also from Sigma-Aldrich. The reagents used for the cell treatments were: human TNFα (2,000 U/mL; Peprotech); 12-O-tetradecanoylphorbol-13-acetate (TPA; 50 ng/mL; Sigma-Aldrich); ionomycin (1 µM; Sigma-Aldrich); IL-6 (10 ng/mL; R&D Systems); IGF-1 (50 ng/mL; R&D Systems); PS-1145 dihydrochloride (20 µM; Sigma-Aldrich); SC-514 (30 µM; Calbiochem); SP600125 (10 µM; Calbiochem); Velcade (bortezomib; ranging from 0.01 nM to 10 µM; Janssen-Cilag).

Cell Culture and Primary Cell Purification

The human multiple myeloma (KMS-11, KMS-12, KMS-18, KMS-27, U266, JJN-3, NCI-H929, AMO-1, MM1.S, KMM-1, RPMI-8226 [parental]; U266/R10R, MM1/R10R, MM1.R, AMO-1a [drug resistant]), diffuse large B-cell lymphoma (DLBCL; LY3, SUDHL-6), T-cell lymphoma/leukaemia (CEM, HUT-78, Jurkat, MOLT-4, MT-2, MT-4, MT2-HTLV-I), Burkitt’s lymphoma (BJAB, HS-Sultan, Namalva, RAJI, ST486), EBV-transformed B-lymphoblastoid (ARH-77), B-cell lymphoma (Karpas), chronic myelogenic leukaemia (K564), and promonocytic leukaemia (U937) cell lines were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin), and 1 mM L-glutamine (Invitrogen). The human embryonic kidney (HEK-293T) and breast cancer cell lines (MCF-7, MDA-MD-231, MDA-MD-468) were cultured in high-
glucose Dulbecco’s modified Eagle’s medium (DMEM; with L-glutamine, without sodium pyruvate; Invitrogen) supplemented with 10% FBS (Sigma-Aldrich), antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin), and 1 mM L-glutamine (Invitrogen). Cells were cultured in a humidified incubator in 5% CO₂ at 37°C. The ARH-77, NCI-H929, RPMI-8226, MM1.S, MM1.R, and HEK-293T cell lines were purchased from the ATCC. The U266 and KMS-12 cell lines were purchased from the Istituto Zooprofilattico “Ugo Umbertini” (Brescia, Italy). The KMS-11, KMS-18, KMS-27, JJN-3, KMM-1, LY3, and SUDHL-6 cell lines were obtained from G. Inghirami (Turin, Italy). The lenalidomide-resistant U266/R10R and MM1/R10R cell lines were kindly provided by R. Orlowski (Houston, TX, USA) (Bjorklund et al., 2011; Bjorklund et al., 2014). The AMO-1 and bortezomib-resistant AMO-1a cell lines were from the C. Driessen’s laboratory (Ruckrich et al., 2009). All the other cell lines were from the G. Franzoso’s laboratory.

The human peripheral blood mononuclear cells (PBMCs) were purified from the blood of healthy volunteers by Ficoll-Hypaque (GE Healthcare) density separation, and cultured in RPMI-1640 medium supplemented with 10% FBS (Sigma-Aldrich), antibiotics, and 1 mM L-glutamine, as described previously (Piva et al., 2008). The human mesenchymal stem cells (MSCs) were provided by F. Dazzi (Vianello et al., 2010), and cultured in high-glucose DMEM (with L-glutamine, without sodium pyruvate; Invitrogen) supplemented with 10% FBS (Sigma-Aldrich), antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin), and 1 mM L-glutamine (Invitrogen). The human bone marrow stromal cells (BMSCs) were isolated from the bone marrow aspirates of patients with multiple myeloma, as reported elsewhere (Piva et al., 2008). Briefly, bone marrow cells were incubated in 100 mm² tissue culture dishes at 37°C, and, 24 hr later, non-adherent cells were removed by washing. Adherent cells with fibroblast morphology were cultured in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) supplemented with 20% FBS (Sigma-Aldrich), antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin), and 2 mM L-glutamine (Invitrogen) to confluence, and then stored in aliquots in liquid nitrogen until they were used. CD138⁺ plasma cells (PCs) were purified from the bone marrow aspirates of patients with multiple myeloma (MM), monoclonal gammopathy of undetermined significance (MGUS), Waldenström’s macroglobulinemia (WM) – a lympho-proliferative disorder of B cells (Schuster et al., 2010; Kyle and Rajkumar, 2009) – or an unrelated disease without PC dyscrasias (normal) by Ficoll-Hypaque (GE Healthcare) density separation followed by positive selection with CD138 MicroBeads (Miltenyi Biotech), according to the manufacturer’s instructions. Purified PCs were cultured in RPMI-1640
medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (100 µg/mL penicillin, 100 µg/mL streptomycin), and 1 mM L-glutamine (Invitrogen), and cell purity after MicroBeads separation was verified by flow cytometry using a FITC-conjugated anti-CD138 antibody (BD Pharmingen™).

Primary murine cells were isolated from the spleen or lymph nodes of C57BL/6 mice using standard protocols (Ahmad et al., 2011), after removal of the red blood cells with RBC lysis buffer (Sigma-Aldrich), and then seeded into wells of 96-well plates at a concentration of 6x10⁴ cells/well and cultured in RPMI-1640 medium supplemented with 10% FBS (Sigma-Aldrich), antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin) and 1 mM L-glutamine (Invitrogen).

**Lentiviral Vectors**

The DNA sequences of the targeting small hairpin (sh)-RNAs are listed below:

| Gene and nucleotide position | targeting sequence |
|-----------------------------|--------------------|
| sh-GADD45β (262-280 bp)     | 3’-CAGAAGATGCAGACGTTGGA-5’ |
| sh-GADD45β-2 (352-371 bp)   | 3’-CCAAGTTGATGAAATGTTGAAG-5’ |
| sh-GADD45β-3 (436-454 bp)   | 3’-CAAATCCACCTCACGCTCA-5’ |
| sh-RelA (538-556 bp)        | 3’-GCATCCAGACCAACAAACAA-5’ |
| sh-JNK1 (435669-435689 bp)  | 3’-CCTGACGACGAGTTAGTGGAG-5’ |
| sh-MKK7 (331-349 bp)        | 3’-GCATTGAGATTGACCAGAA-5’ |
| sh-MKK7-2 (1155-1173 bp)    | 3’-GATCAGAGGAAGAGACCGA-5’ |
| sh-ns                       | 3’-CAGTCGCGTTTGCAGACTGG-5’ |
| sh-ns-2                     | 3’-AAGTATGTTGAGCACGCGT-5’ |

The DNA oligonucleotides encoding the sh-RNAs specific for human RelA (sh-RelA), human MKK7 (sh-MKK7, sh-MKK7-2), human GADD45β (sh-GADD45β, sh-GADD45β-2, sh-GADD45β-3), or human JNK1 (sh-JNK1) were introduced between the XhoI and HpaI restriction sites of the lentiviral vector, pLentiLox3.7, as described previously (Mauro et al., 2011; Yang et al., 2006). The pLentiLox3.7 plasmids, sh-ns and sh-ns-2, expressing non-specific (ns) sh-RNAs were also described previously (Mauro et al., 2011).

**Lentivirus Production and Infections**

The production of high-titer lentiviral preparations in HEK-293T cells and lentiviral infections were performed as described previously (Mauro et al., 2011; Yang et al., 2006). Enhanced green
fluorescent protein (eGFP)+ cells from myeloma cell lines infected with pLentiLox.3.7 lentivirus (expressing the targeting sh-RNAs and eGFP) were purified, where necessary, by FACS sorting using a BD FACSAria™ II cell sorter, and then rested in culture for at least 2 days prior to using them for further analysis.

**Cell Proliferation, Cell Death, and Apoptosis Assays**

[³H]Thymidine incorporation assays were performed using standard protocols (Griffiths and Sundaram, 2011). Briefly, cell lines were seeded into wells of 96-well plates at a concentration of 1.0x10⁴ cells/well and then left untreated or treated daily with the indicated concentrations of peptides and maintained in complete RPMI-1640 medium at 37°C in 5% CO₂, splitting them with medium as necessary. At 24, 72 or 144 hr, cells were incubated for an additional 16 hr with 0.037 MBq/well of [³H]thymidine (Amersham), and then harvested onto glass fibre filter mats using a 96-well plate automated Tomtec cell harvester (Receptor Technologies) and analyzed by liquid scintillation spectroscopy with a LKB Wallac Trilux Microbeta 3 β-counter (PerkinElmer). Values were expressed as the percentage of the counts per minute (cpm) measured in the treated cultures relative to the cpm measured in the respective untreated cultures. The half-maximum inhibitory concentration (IC₅₀) values were calculated using either 5 or 7 concentrations of compound and were defined as the mean concentration of compound inducing 50% inhibition of [³H]thymidine uptake relative to the uptake measured in untreated cells. Trypan blue exclusion assays were performed as reported previously (Mauro et al., 2011; Arnold et al., 2008). Briefly, cells from lentivirus-infected cell lines were seeded into wells of 48-well plates in complete medium at a concentration of 2.0x10⁵ cells/well, and then cultured at 37°C in 5% CO₂, splitting them as necessary during the assays. Cell viability was monitored over a period of up to 8 days by cell counting using trypan blue, and the numbers of live infected cells in the cultures were extrapolated, where appropriate, from the cell counts by accounting for the percentages of eGFP+ cells, using flow cytometry. Values were expressed as the percentage of live infected cells present in the cultures at the times indicated relative to the number of live infected cells present in the same cultures at day 0.

Apoptosis analyses were performed using propidium iodide (PI) nuclear labeling, as described previously (Riccardi and Nicoletti, 2006). Briefly, cells were seeded at a density of 2x10⁵ cells/well into wells of either 24- or 48-well plates and cultured in the presence or absence of treatment, as shown.
Cells were then fixed in 70% ice-cold ethanol for 16 hr, washed in PBS, and treated with ribonuclease A (50 μg/mL) for 30 min at room temperature. Samples were finally stained with PI (50 μg/mL) in 0.1% sodium citrate buffer for 2 to 4 hr at 4°C in the dark, and data were acquired using a FACSCalibur automated system (Becton Dickinson) and analyzed with FlowJo.

The viability of primary CD138+ cells from patients was assessed using trypan blue exclusion assays (Mauro et al., 2011; Arnold et al., 2008), performed as described above. Cells were seeded into wells of 96-well plates at a concentration of 3.0x10^4 cells/well in complete RPMI-1640 medium, and then left untreated or treated daily with peptides or bortezomib for 48 hr. The IC_{50} values were calculated using at least 7 concentrations of each compound and defined as the mean concentration of compound inducing 50% of cell killing relative to the extent of cell death observed in the untreated cells (Arnold et al., 2008). For the analyses shown in Figure 6C, purified CD138+ cells were seeded as above at a concentration of 3.0x10^4 cells/well onto a monolayer of 50-60%-confluent BMSCs in 96-well plates and, then, left untreated or treated with DTP3 (300 nM) or PS-1145 (20 μM) for 48 hr (Piva et al., 2008). Where indicated, CD138+ cells were treated with DTP3 or PS-1145 as above in the presence of IL-6 (10 ng/mL) or IGF-1 (50 ng/mL) and in the absence of BMSCs (Yang et al., 2007) (Figures S6A-B). Cell viability in the treated cultures was expressed as the percentage of cell survival relative to the survival observed in the respective untreated cultures, represented as 100%.

Human PBMCs, BMSCs and MSCs were left untreated or treated with D-peptide, PS-1145 or bortezomib and cultured in the manner described above for up to 144 hr. Splenocytes were treated with Ultra-pure lipopolysaccharide (LPS; 1 ng/mL; ENZO) for 16 hr, and, then, both splenocytes and lymph node cells were treated with D-peptide, PS-1145 or bortezomib for 72 hr. Cell viability was measured by using trypan blue exclusion or [3H]thymidine incorporation assays performed in the manner described above (Mauro et al., 2011; Griffiths and Sundaram, 2011; Arnold et al., 2008), as indicated.

Drug Synergism

The activities of DTP3 and bortezomib in the U266 and KMS-12 myeloma cell lines, both as single agents and in combination, were determined using [3H]thymidine incorporation assays, performed as described above in “Cell Proliferation, Cell Death, and Apoptosis Assays”. The IC_{50} values of bortezomib as single agent were determined at 48 hr using 9 concentrations of drug, ranging from 0.1
to 1,000 nM. The IC_{50} values of DTP3 at day 6 as single agent were from the experiment in Figures S5F-G. The concentrations of DTP3 for the drug interaction studies with bortezomib were selected in pilot experiments. For these studies, U266 and KMS-12 MM cells were treated with DTP3 for 6 days at the concentrations of 3 nM and 10 nM, respectively, and bortezomib was added to the cultures at increasing concentrations, ranging from 0.1 to 1,000 nM, 48 hr prior to the measurement of cell viability by [\textsuperscript{3}H]thymidine incorporation. Viability data were converted into values representing the fraction of cells affected (FA) in the drug-treated cultures compared with untreated cultures, and the interaction between DTP3 and bortezomib was analyzed using the Chou-Talalay method (Chou, 2006). The combination index (CI) was calculated based upon this method according to the following equation:

\[
\text{CI} = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2}
\]

Where (D)_1 and (D)_2 are the doses of drug 1 (DTP3) and drug 2 (bortezomib), respectively, having x effect when used in combination, and (Dx)_1 and (Dx)_2 are the doses of drug 1 and drug 2, respectively, having the same x effect when used alone. Synergy is present when the CI is less than 1.0 (where values between 0.3 and 0.7 denote synergism, and values between 0.1 and 0.3 denote strong synergism); the combination is additive when CI equals 1.0, and antagonistic when it is more than 1.0 (Chou, 2006).

**Quantitative Real-Time Polymerase-Chain Reaction (qRT-PCR)**

Total RNA was extracted with Trizol (Invitrogen) and purified using the RNAeasy Mini-Kit (Qiagen), according to the manufacturer’s instructions. 1 µg of RNA was added as template to reverse-transcriptase (RT) reactions, performed using the GeneAmp RNA PCR Kit (Applied Biosystems) (Mauro et al., 2011). qRT-PCRs were carried out with the resulting cDNAs using the TaqMan® Gene Expression Assays kit (Applied Biosystems), appropriate gene-specific primer/probe sets (Applied Biosystems), and an ABI 7900 real-time PCR machine (Applied Biosystems), according the manufacturer’s instructions. Experimental ΔΔCt values were normalized to 18s ribosomal RNA, β-Actin or GUSB β-Glucuronidase, and relative mRNA levels were calculated against a reference sample (i.e. mRNA from HEK-293T cells, for cell lines; mRNA from PBMCs or BMSCs, for primary cells).

**Co-Immunoprecipitation and Kinase Assays**
Co-immunoprecipitation assays were performed as described previously (Papa et al., 2004). Briefly, human HEK-293T cells were seeded at a density of 1.8-2.2x10⁶ cells/dish in 100 mm² tissue-culture dishes and then transfected with pcDNA-FLAG-hMKK7 and pcDNA-HA-hGADD45β by using the calcium phosphate precipitation method (Papa et al., 2004). 48 hr after transfection, cells were washed with PBS and then lysed in lysis buffer supplemented with protease inhibitors (1 mM phenylmethylsulfonylfluoride, 10 µM chymostatin, 2 µg/mL aprtinin, 2 µg/mL leupeptin; Roche) for 30 min in ice. Cell lysates were cleared by centrifugation at 45,000 x g for 40 min and then used for co-immunoprecipitations with anti-FLAG M2 Affinity Gel (Sigma-Aldrich), binding to FLAG-tagged MKK7. Western blots were developed using an anti-HA (binding to HA-hGADD45β) or an anti-MKK7 antibody.

For competition assays (Figure 3C, Figure S5E), the anti-FLAG immunoprecipitates were incubated for 2-4 hr at 4°C in the presence or absence of bioactive or control inactive D-peptides, before loading them onto SDS-PAGE gels.

MKK7 kinase assays were performed as described previously (Papa et al., 2008; Papa et al., 2004) (Figure 3D). Briefly, HEK-293T cells were transfected with pcDNA-FLAG-hMKK7 using the calcium phosphate precipitation method, as above. 36 hr after transfection, cells were treated with 12-O-tetradecanoylphorbol-13-acetate (TPA; 100 ng/mL) and ionomycin (1 µM) for 30 min at 37°C or left untreated and, then, lysed in lysis buffer and used for immunoprecipitations with anti-FLAG M2 Affinity Gel, as above. In order to measure MKK7 activity, immunoprecipitates were washed in lysis buffer and twice more in kinase buffer (10 mM HEPES, 5 mM MgCl₂, 1 mM MnCl₂, 12.5 mM β-glycerophosphate, 2 mM DTT, 4 mM NaF, 0.1 mM Na₃VO₄) and, then, incubated with recombinant GST-JNK1 (2 µM) in kinase buffer for 10 min at 30°C, followed by incubation with 5 µCi [γ-³²P]ATP (kinase reaction). Where indicated, anti-FLAG-MKK7 immunoprecipitates were pre-incubated with D-tetrapeptides for 10 min at 30°C and, then, incubated for another 10 min at 30°C in the presence or absence of recombinant GST-hGADD45β (5 µM), before setting up the kinase reaction as above. Reactions were stopped with Laemmli sample buffer, and ³²P-labelled proteins were resolved by 10% SDS-PAGE and revealed by autoradiography. Inactive MKK7 was immunoprecipitated from untreated HEK-293T cells. The amino acid sequences of the active and inactive control D-peptides used in the co-immunoprecipitation and/or kinase assays were as follows: Ac-DTP1, Ac-Tyr-Asp-His-Phe-NH₂; Ac-DTP2, Ac-Tyr-Glu-Arg-Phe-NH₂; Ac-DNC, Ac-Tyr-Asp-His-Gln-NH₂; Ac-DNC2, Ac-Tyr-Asp-His-Ala-NH₂; Ac-DNC3, Ac-Tyr-Glu-
Lys-Trp-NH₂; Ac-DNC4, Ac-Tyr-Asp-Lys-Trp-NH₂; DTP3, Ac-Tyr-Arg-Phe-NH₂; scrambled (SCRB), Ac-Arg-Phe-Tyr-NH₂. All peptides were in the D configuration.

JNK kinase assays were performed as described previously (Papa et al., 2008) (Figure 5D). MM cells (1.0x10⁶/mL) were left untreated or treated with DTP3 (10 µM) or TNFα (2,000 U/mL) for the times indicated and then lysed in lysis buffer (Papa et al., 2004), as described above. Cell extracts were used for immunoprecipitations with anti-JNK antibody immobilized onto Protein A/G PLUS-agarose beads (sc-2003; Santa Cruz Biotechnology), and kinase assays were performed as described above, using recombinant GST-cJun (2 µM) as substrate.

**Peptide Library Design and Synthesis**

The combinatorial tetrapeptide libraries were synthesised in a simplified format using 12 L-amino acids (GL-Biochem; Novabiochem) occupying different chemical spaces, as reported previously (Sandomenico et al., 2012). The amino acids used were as follows: Asp (D), representing acidic amino acids; R (Arg) and His (H), representing basic amino acids, with His also representing aromatic residues; Gln (Q) and S-acetamidomethyl(Acm)-Cys (referred to as C), representing amidated side chains; Phe (F) and Tyr (Y), representing aromatic amino acids; Ser (S), representing amino acids containing hydroxyl groups; Leu (L) and Met (M), representing bulky hydrophobic amino acids; Ala (A), representing small hydrophobic amino acids. Pro (P) was used to generate peptides with a bent conformation. The L-tetrapeptides were joined to an N-terminal β-Ala₂ spacer linked to a fluorenlymethyloxy carbonyl (Fmoc) tag and were synthesised by the solid phase method using conventional Fmoc/tBu chemistry, as reported previously (Sandomenico et al., 2012). Random amino acid positions were filled in by using equimolar mixtures of the 12 L-amino acids in large excess, in order to suppress preferential acylations. The L-tetrapeptides contained a C-terminal amide group and were cleaved from the resin using trifluoroacetic acid (TFA)/tri-isopropylsilane (TIS)/water (90:5:5, v/v/v; Sigma-Aldrich) and purified to homogeneity by semi-preparative reverse phase (RP)-HPLC. Peptide identity and purity were determined using liquid chromatography-mass spectrometry (LC-MS), as described below. Peptides were then lyophilized and dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich) at a concentration of 10 mg/mL (stock solution) and stored in aliquots at -20°C until they were used.
The initial library contained a total of 20,736 (12⁴) tetrapeptides, formed by all possible combinations of the 12 L-amino acids, and was iteratively deconvoluted in four steps by ELISA GADD45β/MKK7 competition assays, as described below in “Protein Purification and ELISA”. Each of the peptide mixtures used for the subsequent steps of the screen was prepared in the same manner and on the same scale. Step 3 of the screen identified individual L-tetrapeptides (Table S1), the most active of which (i.e. Fmoc-β-Ala₂-Tyr-Asp-His-Phe-NH₂) was used as template for the synthesis of 24 second-generation L-tetrapeptides in which the N-terminal Fmoc-β-Ala₂ tag was replaced with an acetyl (Ac) group (Table S2). All individual peptides were prepared using the same solid-phase Fmoc/tBu methodology and, then, purified and characterized in the same manner as for the tetrapeptide libraries (see below).

N-terminal acetylation was carried out by treatment with 10% acetic anhydride in dimethylformamide (DMF; Sigma-Aldrich) containing 5% diisopropyl-ethylamine (DIEA; Sigma-Aldrich). As part of the structure-activity relationship (SAR) analysis, in order to improve their stability and cellular activity, peptides were synthesized in the D configuration using the same solid-phase method and Fmoc-protected D-amino acids (GL-Biochem; Novabiochem), as described above (Sandomenico et al., 2012). To increase cellular uptake, where indicated, D-peptides were modified by the introduction of various chemical groups, including a benzylloxycarbonyl (Z), O-methyl, 2-chloro-benzylloxycarbonyl (2Cl-Z), myristyl (Myr), benzyol, 3-hydroxy-4-methoxybenzyl, or Fmoc group, as shown (Table S3). The Z group was introduced by on-resin treatment of the peptides with 0.5 M benzylloxycarbonyl-N-hydroxysuccinimide (Z-OSu; GL-Biochem) in DMF with 5% DIEA. The carboxylic acids used to derivatize the N-terminus of other peptides were pre-activated with 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU; GL-Biochem) and DIEA, and subsequently coupled to the resin.

**Peptide Characterisation by Liquid Chromatography-Mass Spectrometry (LC-MS)**

The identity and purity of each of the peptides were determined by liquid chromatography-mass spectrometry (LC-MS) using an ion trap LCQ Deca XP mass spectrometer (ThermoFisher), equipped with an Opton ESI source operated at 5 kV and 300 °C, coupled to an LC system comprising a Surveyor HPLC and a Biobasic 50x2 mm ID C18 column (ThermoFisher). The peptide spectra were acquired in the positive mode between 200 and 2,000 m/z, using approximately 50 ng of peptide and a
flow rate of 0.20 mL/min, by applying a gradient from 5% to 70% of solvent B (CH₃CN, 0.05% TFA) over solvent A (H₂O, 0.08% TFA), within a time window of 10 min. Where necessary, the gradient was changed in order to elute the compounds within a time window suitable for MS and diode array analyses. Peptide purity was assessed on the basis of the HPLC traces at 214 nm, by area integration of all the recorded peaks. Peptide identity was established on the basis of the experimental molecular weight (MW). The amino acid sequence, the calculated and experimental MW, and the purity level of each of the main peptides used are listed below:

| amino acid sequence            | MW\text{calcd.}\text{, }m^* (g/mol) | MW\text{exp.}\text{, }m^* (g/mol) | Purity, LC-MS (%) |
|--------------------------------|-------------------------------------|-----------------------------------|------------------|
| Ac-YDHF-NH₂ (Ac-LTP1)         | 621.25                              | 621.3                             | 97               |
| Ac-YEHF-NH₂                    | 635.27                              | 635.3                             | 95               |
| Ac-WDHF-NH₂                    | 644.27                              | 644.4                             | 97               |
| Ac-WEHF-NH₂                    | 658.29                              | 658.4                             | 96               |
| Ac-YDRF-NH₂                    | 640.30                              | 640.5                             | 95               |
| Ac-YDKF-NH₂                    | 612.29                              | 612.2                             | 95               |
| Ac-YEKF-NH₂                    | 626.31                              | 626.1                             | 95               |
| Ac-YERF-NH₂ (Ac-LTP2)          | 654.31                              | 654.3                             | 96               |
| Ac-WEKF-NH₂                    | 649.32                              | 649.4                             | 97               |
| Ac-WERF-NH₂                    | 677.35                              | 677.2                             | 95               |
| Ac-WDKF-NH₂                    | 653.31                              | 653.1                             | 96               |
| Ac-WDRF-NH₂                    | 663.31                              | 663.3                             | 97               |
| Ac-YDHW-NH₂                    | 660.27                              | 660.2                             | 95               |
| Ac-YEHW-NH₂                    | 674.28                              | 674.4                             | 97               |
| Ac-WDHW-NH₂                    | 683.28                              | 683.4                             | > 99             |
| Ac-WEHW-NH₂                    | 697.30                              | 697.2                             | 97               |
| Ac-YDHW-NH₂                    | 679.31                              | 679.4                             | 95               |
| Ac-YDKW-NH₂                    | 651.30                              | 651.3                             | 96               |
| Ac-YEKW-NH₂                    | 655.32                              | 655.3                             | > 99             |
| Ac-YERW-NH₂                    | 693.32                              | 693.4                             | 96               |
| Ac-WEKW-NH₂                    | 688.33                              | 688.2                             | 97               |
| Ac-WERW-NH₂                    | 716.34                              | 716.4                             | 97               |
| Ac-WDKW-NH₂                    | 674.32                              | 674.3                             | 97               |
| Ac-WDRW-NH₂                    | 702.32                              | 702.2                             | 97               |
| z-YERF-NH₂ (z-DTP2)            | 746.35                              | 746.3                             | 97               |
| z-YDHF-NH₂ (z-DTP1)            | 713.29                              | 713.3                             | 97               |
| Compound                                      | Calc, m* | Exp, m* | Prec. (%) |
|-----------------------------------------------|----------|---------|-----------|
| z-YDHQ-NH$_2$ (z-DNC)                         | 694.27   | 694.4   | 96        |
| z-YD(O-methyl)HF-NH$_2$                       | 727.48   | 727.7   | 95        |
| z-YD(O-methyl)HQ-NH$_2$                       | 708.27   | 709.5   | 95        |
| 2Cl-z-YERF-NH$_2$                             | 780.50   | 780.4   | 96        |
| 2Cl-z-YDHF-NH$_2$                             | 747.26   | 747.3   | 96        |
| 2Cl-z-YDHQ-NH$_2$                             | 728.23   | 728.3   | 97        |
| Myr-YERF-NH$_2$                               | 823.68   | 822.9   | 97        |
| Myr-YDHF-NH$_2$                               | 789.60   | 788.8   | 95        |
| Myr-YDHQ-NH$_2$                               | 770.63   | 769.9   | 97        |
| Benzoyl-YERF-NH$_2$                           | 717.90   | 717.0   | 97        |
| Benzoyl-YDHF-NH$_2$                           | 593.90   | 683.6   | 97        |
| Benzoyl-YDHQ-NH$_2$                           | 665.90   | 665.4   | 98        |
| 3-hydroxy-4-methoxy-benzyl-YERF-NH$_2$        | 755.30   | 755.2   | 95        |
| 3-hydroxy-4-methoxy-benzyl-YDHF-NH$_2$        | 722.24   | 722.2   | 95        |
| 3-hydroxy-4-methoxy-benzyl-YDHQ-NH$_2$        | 703.23   | 703.3   | 95        |
| Fmoc-YERF-NH$_2$                              | 834.37   | 834.2   | 99        |
| Fmoc-YDHF-NH$_2$                              | 801.31   | 801.2   | 99        |
| Fmoc-YDFQ-NH$_2$                              | 782.30   | 781.7   | 99        |
| Ac-YERFK(εZ)-NH$_2$                           | 916.32   | 916.2   | 97        |
| Ac-YDHFK(εZ)-NH$_2$                           | 882.60   | 882.8   | 97        |
| Ac-YDHQK(εZ)-NH$_2$                           | 864.42   | 864.5   | 96        |
| Ac-YQRF-NH2                                   | 653.33   | 653.2   | 95        |
| Ac-YNRF-NH2                                   | 639.31   | 639.5   | 95        |
| Ac-YLRF-NH2                                   | 638.35   | 638.5   | 96        |
| Ac-YLFL-NH2                                   | 497.26   | 497.3   | 96        |
| Ac-YNF-NH2                                    | 483.21   | 483.2   | 97        |
| Ac-YPF-NH2                                    | 466.22   | 466.1   | 97        |
| Ac-YHFL-NH2                                   | 506.23   | 506.2   | 96        |
| H-YHFL-NH2                                    | 464.22   | 464.2   | 98        |
| Ac-YRY-NH2                                    | 541.26   | 541.3   | 98        |
| H-YRY-NH2                                     | 499.25   | 499.4   | > 99      |
| Ac-YKF-NH2                                    | 497.26   | 497.1   | 98        |
| Ac-YRF-NH2 (DTP3)                             | 525.27   | 525.3   | > 99      |

calcld, m* = calculated monoisotopic
exp, m* = experimental value
The peptides were lyophilized and dissolved in dimethylsulphoxide (DMSO; Sigma-Aldrich) at a concentration of 10 mg/mL (stock solution) and then stored in aliquots at -20°C until they were used.

High-Resolution Mass Spectrometry (MS) Analysis of z-DTP1, z-DTP2, and DTP3

For a more accurate mass measurement, the lead peptides, z-DTP1, z-DTP2 and DTP3, were analyzed in the positive ion mode using an electrospray hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF) fitted with a Z-spray electrospray ion source. The capillary source voltage and the cone voltage were set at 3,500 V and 35 V, respectively; the source temperature was maintained at 80 °C; nitrogen was used as the drying gas, at the flow rate of approximately 50 L/hr. The time-of-flight analyzer was calibrated using selected fragment ions resulting from the collision-induced decomposition of human (Glu1)-fibrinopeptide B (500 fmol/mL in CH₃CN:H₂O [50:50], 0.1% formic acid) at an infusion rate of 10 μL/min in the TOF MS/MS mode. The mass data were then collected by infusing the samples (3 pmol/μL in 1:1 CH₃CN:H₂O) into the system at a flow rate of 10 μL/min. Data acquisition and processing were carried out using MassLynx 4.1 software. The results of these analyses are reported below:

| compound | calculated Mr [M+H]^+ | experimental Mr [M+H]^+ | Δ (ppm) | Δ (Da) |
|----------|------------------------|-------------------------|---------|--------|
| z-DTP1   | 714.2888               | 714.2897                | 1.4     | 0.0009 |
| z-DTP2   | 747.3466               | 747.3429                | 5.3     | 0.0037 |
| DTP3     | 526.2778               | 526.2784                | 1.9     | 0.0006 |

* calculated considering the monoisotopic masses according to the Pure App Chem. 63(7), 975-90 (1991)

¹H-Nuclear Magnetic Resonance (NMR) Analysis of z-DTP1, z-DTP2, and DTP3

The identity and purity of the lead D-peptides, z-DTP1, z-DTP2 and DTP3, were also determined by ¹H-nuclear magnetic resonance (NMR) analysis. The NMR spectra of the peptides were acquired in D6-DMSO, at a concentration of 2 mg/mL, using a Varian 400 MHz NMR spectrometer. The measured chemical shifts are reported below:

| compound | chemical shift values |
|----------|-----------------------|
| z-DTP1   | δ 5.02 - 4.91 ppm (m, 5H, Z group); δ 7.10, 7.12 ppm (d, 1H, H3 and H5 on Tyr side chain) and δ 6.68, 6.70 ppm (d, 1H, H2 and H6 on Tyr side chain); δ 3.12 – 3.02 ppm (m, 4H, βCH2 Asp, βCH2 His); δ 4.64 – 4.59 ppm (m, 1H, αCH His); δ 9.24 ppm (s, 1H, H2 on imidazole His); δ 8.93 ppm (s, 1H, H4 on imidazole His); δ 7.39 - 722 |
| ppm (m, 5H, H1-5 on the phenyl ring on the Phe side chain) |
|----------------------------------------------------------|
| **z-DTP2**                                               |
| δ 5.03 – 4.94 ppm (m, 5H, Z group); δ 7.12 – 7.14 ppm (d, 1H, H3 and H5 on Tyr side chain); δ 6.68 - 6.70 ppm (d, 1H, H2 and H6 on Tyr side chain); δ 4.30 – 4.21 ppm (m, 1H, αCH Phe); δ 4.51 – 4.45 ppm (m, 1H, αCH Tyr); δ 2.08 – 2.00 ppm (m, 2H, γCH2 Glu); δ 1.67 - 1.58 ppm (m, 2H, βCH2 Glu); δ 4.51 - 4.45 ppm (m, 1H, αCH Glu); δ 4.30 – 4.21 ppm (m, 3H, αCH Ty r, Phe, Arg); δ 7.43 ppm (s, 1H, Nɛ, Arg side chain); δ 6.68 – 6.70 ppm (d, 1H, Nη1, Arg side chain); δ 7.30 – 7.22 ppm (m, hydrogen on Phe side chains) |
| **DTP3**                                                 |
| δ 1.82 ppm (s, 3H, Acetyl); δ 7.10 – 7.08 ppm (d 1H, H3, H5 on Tyr side chain); δ 7.31 – 7.15 ppm (m, 5H, H1-5 on the phenyl ring on the Phe side chain);δ 4.51 – 4.45 ppm (m, 1H, αCH Arg); δ 3.01 – 3.06 ppm (m, 2H, βCH2 Arg); δ 2.90 – 2.84 ppm (m, 2H, γCH2 Arg); δ 2.70 – 2.64 ppm (m, 2H, δCH2 Arg); δ 6.67 – 6.69 ppm (d, 1H, Nη1, Arg side chain); δ 7.43 ppm (s, 1H, Nɛ, Arg side chain) |

**Protein Purification and ELISA**

Recombinant proteins were purified as described previously (Tornatore et al., 2008). Briefly, full-length human (h)GADD45β was expressed as a protein fused to an N-terminal glutathione S-transferase (GST) or a poly-histidine (His$_6$) tag and purified by affinity chromatography using GST-Trap and His-Trap columns (GE Healthcare), respectively. The GST-GADD45β protein preparations were used for kinase assays; The His$_6$-GADD45β protein was used for ELISA, CD and MALDI-TOF-MS analyses.

Full-length human (h)MKK7 was expressed as a protein fused to an N-terminal GST tag (GST-MKK7), purified using GST-Trap as above, and then used for ELISA and spectrofluorimetric analyses. Purified GST was used as a control in all the experiments. The kinase domain (KD) of hMKK7 (amino acids 101-405) was expressed as a protein fused to an N-terminal His$_6$ tag in E. coli (BL21[DE3] strain) using the pETDuet-1 vector (Novagen). The resulting His$_6$-hMKK7-KD protein was purified by affinity chromatography using a His-Trap column, followed by gel filtration, and utilised for CD and MALDI-TOF-MS analyses, following dilution in water or 1 mM ammonium bicarbonate, pH 7.0, as appropriate, from a stock solution prepared at 0.5 mg/mL (13.7 μM) in 25 mM TRIS, 150 mM NaCl buffer at pH 7.0.

The methods used for the ELISA binding and competition assays were described previously (Tornatore et al., 2008). Briefly, His$_6$-GADD45β was biotinylated using the EZ Link® NHS-LC-biotin kit (Pierce), according to the manufacturer’s instructions (Tornatore et al., 2008). Human GST-MKK7 was coated onto wells of 96-well plates by incubation at 42 nM in ELISA buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA) for 16 hr at 4°C, followed by blocking with 2% non-fat dry milk in PBS. Biotin-labelled His$_6$-hGADD45β was then added to the wells at increasing concentrations ranging from 5.2 to 168 nM and incubated in the dark for 1 hr at 37°C, followed by washing with Tween (T)-PBS.
Bound His$_6$-hGADD45$\beta$ was revealed using horseradish peroxidase (HRP)-conjugated streptavidin (1:1,000) and the HRP chromogenic substrate, o-phenylenediamine (OPD). GST-coated wells were used as controls.

The ELISA competition screen used for the isolation of antagonists of the GADD45$\beta$/MKK7 complex was carried out using coated GST-hMKK7 (42 nM), soluble biotin-labelled His$_6$-hGADD45$\beta$ (21 nM), and the competing tetrapeptides or tetrapeptide mixtures at the nominal concentration of 42 nM (Figures S3A-B). The library deconvolution strategy used for this screen was described previously (Sandomenico et al., 2012). General information about this type of screens can be found elsewhere (Houghten et al., 1999; Eichler and Houghten, 1995). Values were expressed as the percentage of inhibition of His$_6$-hGADD45$\beta$ binding to GST-hMKK7 relative to the binding measured in the absence of competitor.

Dose-response, ELISA GADD45$\beta$/MKK7 competition assays were carried out under similar conditions, using coated GST-hMKK7 (42 nM), soluble biotin-labelled His$_6$-hGADD45$\beta$ (21 nM), and the competing peptides at increasing concentrations, ranging from 0.001 to 21 nM (Figures 3A-B, Figure S4A, Figure S5D, Table S3, Tables S4). As part of the SAR analysis and lead optimization process, where appropriate, competitors were tested at a concentration up to 100 nM. In order to determine their stability in biological fluids, peptides were incubated at the concentration of 10 µM in human serum for 48 hr at 37°C and, then, used at increasing concentrations as above in ELISA GADD45$\beta$/MKK7 competition assays (Figure 3B, Figures S4A-B, Figure S5D). IC$_{50}$ values were calculated using at least 7 concentrations of peptide and defined as the mean concentration of peptide inducing 50% inhibition of His$_6$-hGADD45$\beta$ binding to GST-hMKK7 relative to the binding measured in the absence of competitor. Where indicated, values were expressed as the percentage of inhibition of His$_6$-hGADD45$\beta$ binding to GST-hMKK7 relative to the binding measured in the absence of competitor. GST was used as a control in all the experiments. The amino acid sequence of the inactive D-tetrapeptide used as negative control in some of the ELISA and cellular assays was as follows: z-DNC, z-Tyr-Asp-His-Gln-NH$_2$.

Peptide Pull-Down Assays
For the peptide pull-down assays (Figure 5E), DTP3 and SCRB D-peptides were synthesized joined to an N-terminal β-Ala₂ spacer by the solid phase method, as described above (Sandomenico et al., 2012), without N-terminal acetylation in order to preserve a suitable anchoring amino group, and then immobilized onto Sepharose 4 Fast Flow resin (GE Healthcare). Upon synthesis, the peptides were cleaved from the Rink Amide resin used for the solid phase methodology and purified to homogeneity by RP-HPLC. Peptides were then dissolved at a concentration of 1.5 mg/mL in 2.0 mL of 25 mM phosphate buffer, pH 7.0, and incubated for 48 hr at room temperature with 1 mL of Sepharose 4 Fast Flow resin, which had been previously pre-activated with N-hydroxy-succinimide (NHS) and washed twice with the same buffer at 4°C. Coupling reactions were monitored by RP-HPLC analysis of the reaction solutions, and residual NHS groups were finally deactivated by treatment with 2.0 mL of 0.5 M TRIS pH 9.0, following the removal of the reaction solutions by washing. With each of the peptides, the efficiency of immobilization was approximately 90%, yielding peptide preparations containing approximately 2.7 mg of bound peptide per mL of Sepharose 4 Fast Flow resin. The blank resin was prepared under similar conditions, without including the peptide. The resins were stored at 4°C in 20% ethanol until they were used.

For the pull down of endogenous MKK7 from MM cells, 100 µL of resin coated with DTP3, SCRB peptide or no peptide (blank) were washed twice with 3 volumes of a buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, and 0.007% Triton X-100, supplemented with protease inhibitors (Roche), and subsequently incubated for 1.5 hr at room temperature with 300 μg of cell extract prepared from MM cells, as described above for co-immunoprecipitation assays. The resins were then washed three times with the same buffer, containing 0.1% of Triton X-100, and bound proteins were resolved by SDS-PAGE and revealed by western blotting using an anti-MKK7 antibody.

**Circular Dichroism (CD) Analyses**

CD analyses were performed using a JASCO J-710 spectropolarimeter (JASCO Corp) equipped with a Peltier temperature control system and a 110-QS quartz cuvette with 1.0-cm path length, as described previously (Tornatore et al., 2008; Sandomenico et al., 2013). Following optimisation in pilot experiments, the analyses were conducted at a pH of approximately 7.0 and a concentration of His₆-hGADD45β and His₆-hMKK7-KD of 1.25 µM, whether the proteins were used alone or in combination. In order to minimize salt interference from the solution buffers in the far-UV region of the spectrum,
proteins were diluted from their stock aliquots in water. The CD spectra of GADD45β, MKK7-KD and the GADD45β/MKK7-KD complex were acquired in the presence or absence of either DTP3 or SCRB D-peptide at increasing concentrations, ranging from 0.1 nM to 10 μM, as shown, in water, under a constant N₂ flow and using the following settings: wavelength range, 190-280 nm; scanning speed, 20 nm/min; data pitch, 0.2 nm; band width, 1 nm; response time, 4 s. The recorded spectra were then signal-averaged from at least five independent readings and smoothed, and the baseline values were corrected by subtracting the contributions of the peptides, at each of the concentrations ranging from 0.1 nM to 10 μM. Data were analyzed for conformational changes of the proteins in the near-UV region, between 200 and 280 nm.

MALDI-TOF Mass Spectrometry (MS) Analyses

MALDI-TOF-MS analyses were performed in the positive linear reflectron mode using a MALDI-TOF micro MX analyzer (Waters Co., Manchester, UK), equipped with a pulsed nitrogen laser (λ=337 nm). The instrument was calibrated using a mixture (10 pmol/mL) of insulin, cytochrome c, and trypsinogen as standard proteins (Sigma, MO, USA) and the recommended polynomial equation, according to the manufacturer’s instructions. Spectra were processed and analyzed using MassLynx 4.0 software. The instrument source voltage was set at 12 kV. The pulse and detector voltages were set at 1,999 V and 5,200 V, respectively. Measurements were carried out using a suppression mass gate set at 5,000 m/z and an extraction delay of 600 ns. Data were acquired by measuring and averaging at least 10 spectra, chosen randomly over the well surface, and the spectra were subsequently smoothed and overlaid. Spectra acquisition was optimized by performing a scouting procedure prior to the analyses, using saturated sinapinic acid (10 mg/mL in acetonitrile/trifluoroacetic acid 0.1%, 2:3, v:v) and α-cyano-4-hydroxycinnamic acid matrix solution (10 mg/ml in acetonitrile/trifluoroacetic acid 0.1%, 1:1, v:v) as ionizing matrices. To acquire the spectra, 1 μL of the matrix solution, 1 μL of the protein solution in 1 mM of ammonium bicarbonate, pH 7.0, and 1 μL of the matrix were sequentially spotted onto the target plate, letting the solvent evaporate at each of the steps. For the analyses shown in Figure S5J, His₆-hMKK7-KD and His₆-GADD45β were used at the concentration of 5 μM, following incubation for 10 min at room temperature in the presence or absence of a 5-μM concentration of DTP3 or SCRB D-peptide. Peptides were dissolved in water in order to minimize the salt contents of the samples.
Spectrofluorimetric Analyses

The stoichiometry of the interaction of DTP3 with MKK7 was determined by using tryptophan fluorescence quenching analysis. Spectra were acquired with a Cary Eclipse Spectrofluorimeter analyzer (Varian Inc.), equipped with Peltier thermostating system, and a 1-cm quartz cuvette. The analyses were carried out using 1.25 μM of GST-hMKK7 or GST dissolved in 10 mM of phosphate buffer, pH 7.5, and increasing concentrations of DTP3 or control SCRB D-peptide dissolved in the same buffer. The data were acquired at 25.0 °C, using an excitation wavelength of 295.0 nm, in order to avoid interference with tyrosine residues, and a fluorescence emission wavelength ranging from 300 nm to 450 nm. The excitation and emission slits were set at 5 nm; the scan rate was 120 nm/min; the data interval was 1.00 nm; the averaging time was set at 0.500 s; the PMT voltage was set at “high.”

All the spectra were smoothed using the same algorithm. The fluorescence values recorded at 333 nm were extracted, transformed to -ΔFluorescence and, then, plotted against the peptide concentration.

Fluorescence data were fitted using a single-site binding model (equation 1):

\[ PL = P + L \]  

Accordingly, the equilibrium dissociation constant (KD) of the DTP3/MKK7 interaction was given by equation 2:

\[ K_D = \frac{[P][L]}{[PL]} \]  

Where [P], [L] and [PL] are the equilibrium concentrations of the protein, the ligand and the protein-ligand complex, respectively. For each measurement, the observed fluorescence value, \( F_{obs} \), was given by the population-weighted combination of the fluorescence values in the unbound and bound protein states:

\[ F_{obs} = P_b F_b + P_u F_u = P_b (F_b - F_u) + F_u \]  

Where \( P_u \) and \( P_b \) are the populations of the unbound and bound states, respectively, and \( F_u \) and \( F_b \) are the intrinsic fluorescence values for these two states. Note that \( P_u + P_b = 1 \). \( P_b \) can be expressed in terms of [P], [L], [PL] and KD according to:

\[ P_b = \frac{[PL]}{[P]+[PL]} = \frac{[P][L]}{K_D ([P]+[P][L]/K_D)} \]  

The fitting formula employed here is the combination of equations 3 and 4:
The quality of the data fitting suggested that a single site model, i.e. a 1 to 1 stoichiometry of binding, was a highly appropriate model to account for the measured fluorescence trends, resulting in a R square ($R^2$) of 0.985 and a chi square ($X^2$) of 1.436. Based on this assay, the estimated $K_D$ value of the interaction of DTP3 with MKK7 was 64.81 +/- 6.22 nM. Data were fitted using a non-linear regression algorithm (Origin, version 6.0) (Williamson, 2013).

The emission spectra of the D-peptide solutions were acquired under the same conditions, using up to 300 μM of DTP3 or SCRBD-peptide, in order to exclude interference from tyrosine fluorescence.

**GADD45β and MKK7 Modeling**

The structure of human GADD45β was built by homology modeling. The 14 N-terminal amino acids of this protein were predicted by the GeneSilico MetaDisorder server (verified to be the best performing method in preliminary CASP8 and CASP9 blind procedures) (Kozlowski and Bujnicki, 2012) to be disordered and were, therefore, excluded from subsequent analyses. The GADD45β amino acid region spanning residues 15-160 was predicted to fold as a globular domain, with a defined pattern of secondary structure, and was used to search the Protein Data Bank (PDB) with the HHpred server (Söding et al., 2005). The top scoring hit resulting from this search corresponded to the crystal structure of human GADD45γ (PDB ID: 3FFM, chain A), covering the amino acid region from residue 15 to 157, which shares a 58% amino acid sequence identity with GADD45β. The probability of this being a true positive was nearly 100% (E-value = 5.3x10^{-52}). The homology model of GADD45β was subsequently built from the structure of GADD45γ using Modeller (Eswar et al., 2007). We generated 50 models of the GADD45β structure and selected the final model according to the Modeller objective function. Hydrogen atoms were added using HAAD software (Li et al., 2009). The selected model was then refined using the FG-MD molecular dynamics algorithm (Zhang et al., 2011). The quality of this refined model was assessed using the QMEAN (Benkert et al., 2011) and ProQ model quality assessment programs (Wallner and Elofsson, 2003). The QMEAN score of this model was 0.78 (within a range from 0 to 1, with 1 indicating a perfect model); the ProQ scores were as follows:

\[\Delta F = F_{\text{obs}} - F_{\text{cal}} = \frac{[P][I]}{K_D ([P] + [I])} (F_{b} - F_{a})\]
MaxSub = 0.74; LGscore = 5.47 (with MaxSub >0.5 and LGscore >4, corresponding to very good and extremely good model quality, respectively) (Figure S5L).

The deposited crystal structure of the kinase domain of human MKK7 (PDB ID: 2DYL) was re-refined by the use of autoBUSTER-TNT 1.11 (Blanc et al., 2004), because portions of this protein had been incorrectly traced. The phase distribution obtained from this analysis was subjected to density modification as implemented by Parrot (Cowtan, 2010). The resulting map was used for model building by ARP/warp (Cohen et al., 2008). The missing residues were modeled by using the loop tool option available in Modeller. The QMEAN score of the final model of MKK7 was 0.70; the ProQ scores were as follows: MaxSub = 0.6; LGscore = 6.0 (Figure S5M).

**GADD45β/MKK7 Complex Modeling**

The model of the mode of interaction of GADD45β with the kinase domain of MKK7 was built from the models of GADD45β and MKK7, obtained as described above, by applying the protein-protein docking procedure implemented in the ClusPro 2.0 web server (http://cluspro.bu.edu) (Kozakov et al., 2010) with default parameters. Published data from site-directed mutagenesis and in vitro binding studies (Papa et al., 2007) were taken into account by providing the following amino acid residues as restraints: MKK7, residues Val134, Trp135, Lys136, Arg138, Lys149, and Arg162; GADD45β, residues Glu63, Glu64, Asp67, Asp68, His74, Gln109, Thr111, Trp112, Glu113, Arg115, and Leu117. The centroid of the largest cluster of solutions – two solutions were considered within the same cluster when the root mean square deviation of their backbone atoms was below 9 Å – was selected as the model of the complex, based on established criteria (Kozakov et al., 2010). This selected model was finally refined in explicit solvent using the HADDOCK web server (Van Dijk and Bonvin, 2010) (http://haddock.science.uu.nl/services/HADDOCK/haddockserver-refinement.html) (Figure S5N).

**Binding Site Prediction**

The FTSite (Ngan et al., 2012) and FLAP (fingerprints for ligands and proteins) (Carosati et al., 2004) algorithms were used to identify potential ligand-binding sites on the experimental structure of MKK7. The strategy of FTSite consists in exploring the potential interactions of the surface regions of a protein with 16 small organic molecules, which vary in size and shape, and has been shown to be effective in detecting 'hot spots' involved in the binding of proteins to drug-size ligands (Kozakov et al., 2011).
FLAP, which was also used for the building of the pharmacophore model and the docking of this model to the putative ligand-binding region of M KK7 (Figure S5A, Figures S5Q-S; see also Figure S5T), relies on the GRID molecular interaction fields (MIFs) (Baroni et al., 2007) and takes into account shape, hydrophobic interactions, H-bond donor, and H-bond acceptor interactions (Figure S5P).

**Pharmacophore Model Building**

The pharmacophore model was generated using FLAP software (Figure S5A) (Baroni et al., 2007), starting from 12 bioactive peptides exhibiting an IC$_{50}$ value less than or equal to 10 nM (Table S4). The three-dimensional structure of the peptides was built using the MarvinSketch server (http://www.chemaxon.com/marvin/sketch/index.jsp) and subsequently minimized. 100 conformers were generated for each of the peptides used as input for FLAP. The pharmacophore predictive capacity was subsequently tested by using an additional set of 10 active and 16 inactive peptides, defined on the basis of their activity in ELISA GADD45β/M KK7 competition assays as follows: active peptides, exhibiting a percentage of binding inhibition of more than 60%; inactive peptides, exhibiting a percentage of binding inhibition of less than 30% (Table S4; see also Figure S5B).

**Broad-Panel Kinase Profiling**

DTP3 was tested for activity at the concentration of either 1 or 10 µM against a panel of 142 human kinases. The kinases used in the assay and their activities in the presence or absence of DTP3 are listed in Table S5. The profiling study was outsourced to the International Centre for Kinase Profiling, MRC-Protein Phosphorylation Unit, Dundee (http://www.kinase-screen.mrc.ac.uk/), and performed according to in-house protocols therein.

**Pharmacokinetic Analyses**

In vitro and in vivo pharmacokinetic analyses were outsourced to Cyprotex (http://www.cyprotex.com/home/) (Figure 7A, Tables S6). In vitro pharmacokinetic assays were performed according to established, in-house protocols and included: plasma stability (human), thermodynamic solubility (PBS pH 7.4, at 37°C for 14 days), microsomal stability (human), plasma protein binding (PPB; mouse and human), and distribution coefficient (LogD). In vivo pharmacokinetic
analyses were also performed at Cyprotex according to established, in-house protocols. Briefly, the peptides were dissolved in PBS and administered by single intravenous injection to CD1 male mice at the dose of 10 mg/kg (25-30 g of total body weight; n=3 mice per time point per compound). Blood samples were collected in heparinized tubes at up to 7 time points after this injection (i.e. 0.08, 0.25, 0.5, 1, 2, 4 and 8 hr), and the plasma concentrations of the test peptide were determined at each time point by LC-MS/MS, using a five-point standard curve covering a peptide concentration range from 3 to 3,000 ng/mL. Standard curves were prepared using blank plasma matrices and treated in the same manner as the test samples. The main pharmacokinetic parameters determined in these analyses were: terminal half-life (t_{1/2}), plasma clearance (CL), volume of distribution (V_d), and area under the plasma concentration versus time curve (AUC) (Figure 7A, Table S6). Values were calculated from the data of the peptide plasma concentration versus time curves using a non-compartmental model analysis, as described elsewhere (Groulx, 2006; Landaw and Di Stefano 3rd, 1982). The doses of z-DTP2 and DTP3 predicted to achieve the therapeutic plasma concentration of 1 μM at the steady state were calculated from the measured pharmacokinetic values using the following equation: \( k_0 = k_1 \times V_d \times C_p \) (where \( V_d \) is expressed in L/kg; \( C_p \) is the plasma concentration at the steady state expressed in mg/L; \( k_0 \) is the zero order input expressed in mg/kg/hr; \( k_1 \) is the first order elimination constant \([0.693/t_{1/2}]\)). The calculated \( k_0 \) values of DTP3 and z-DTP2 on the basis of this equation were 0.81 mg/kg/hr and 3.61 mg/kg/hr, respectively. The measured plasma concentration of DTP3 after continual administration via osmotic pumps was very similar to the predicted plasma concentration of 1 μM.

**Mouse Xenografts**

Mice were housed in the animal facilities at the Hammersmith Campus, Imperial College London, and experiments were conducted under the Home Office Authority (PPL 70/6874). The PPL was reviewed by the Imperial College Ethical Review Process (ERP) and the Home Office. For experiments in the subcutaneous xenograft models (Figures 7B-E, Figures S7A-B, Figures S7C-D), 6 to 8-week old male NOD/SCID mice (NOD.CB17-Prkdc^{scid}/IcrCrl; Charles River) were injected intraperitoneally with anti-CD122 monoclonal antibody (TM-β1 clone; 200 µg/mouse) (Tanaka et al., 1991) and, 24 hr later, subcutaneously in the left flank with \( 1.0 \times 10^7 \) U266 or KMS-11 human MM cells resuspended in 200 µL of sterile PBS and Matrigel (1:1) (BD Biosciences), using a 1-mL Luer-Lok™ Tip syringe (BD Biosciences) with a 25-gauge needle (Bhutani et al., 2007). U266 and KMS-11 cells were harvested
from exponentially growing cultures, washed once with serum-free medium, and resuspended in PBS immediately before injection (Bhutani et al., 2007). The anti-CD122 antibody was affinity purified using HiTrap Protein G HP columns (GE Healthcare), according to the manufacturer’s instructions. One week after injection of the cells, when tumor volumes had reached approximately 100 mm³ (U266 cells) or tumors had yet to be palpable (KMS-11 cells), mice were randomized into treatment groups (n=16 or n=4 per group, respectively; day 0) (Bhutani et al., 2007) and, then, treated by continual infusion of DTP3 at a dose of 14.5 mg/kg/day, yielding a plasma concentration of 0.70 ± 0.09 μM (SEM; n=16), or PBS. Treatments were administered using Alzet osmotic pumps (model 1004; 28-day delivery at the rate of 0.11 µL/hr; Charles River), implanted surgically subcutaneously into the right flank of each mouse, according to the manufacturer’s instructions (Gullapalli et al., 2012). For systemic administration, DTP3 was dissolved at the concentration of 147 mg/mL in PBS, and mice were euthanized on day 28 of the start of treatment with CO₂. Tumor diameters were measured at the indicated times using a vernier caliper, and volumes were estimated from caliper measurements using the following equation: volume = AxB²/2 (where A is the larger diameter, and B is the smaller diameter of the tumor) (Mauro et al., 2011). Results were reproduced at least twice. The plasma concentration of DTP3 was measured at the steady state by LC-MS/MS at Cyprotex, as described above.

For the in vivo analyses of apoptosis and JNK activation (Figures 7D-E, Figure S7B), mice were randomized into treatment groups when tumor volumes were approximately 200 mm³ and, then, treated with DTP3 or PBS as above. At the times indicated, mice were sacrificed with CO₂, and tumors were isolated for analysis. For PI nuclear staining analyses, tumors were minced with a scalpel and incubated for 40 min at 37°C with collagenase D (5 mg/mL; Roche) in 5 mL of complete RPMI-1640 medium, as described previously (Robinson et al., 2003). Single cell suspensions were prepared by passing the digested tumor tissue through a 70-µm cell strainer. Cells were then washed with PBS, stained with PI (5.0x10⁵ cells/sample), and analyzed by flow cytometry with a FACSCalibur automated system (Becton Dickinson) and FlowJo software, as described above for cell lines (Riccardi and Nicoletti, 2006). TUNEL assays were performed on paraffin-embedded sections of the tumor tissue as reported previously (Papa et al., 2008), using the In Situ Cell Death Detection kit (Roche), according to the manufacturer’s instructions, and images were acquired using a Leica SP5 confocal microscope (Leica). Western blots were performed using tumor homogenates prepared in lysis buffer as described previously (Papa et al., 2008).
For experiments in the medullary xenograft model (Figure 7F), 6 to 8-week old male NOD/SCID mice (NOD.CB17-Prkdc<sup>s Tod</sup>/IcrCrl; Charles River) were sub-lethally irradiated (3.50 Gy) and, then, injected intraperitoneally with purified anti-CD122 monoclonal antibody (200 µg/mouse), as above. 24 hr later, mice were injected intravenously with KMS-12-BM human MM cells (1.0x10<sup>7</sup> cells/mouse) resuspended in 100 µL of PBS, as described previously (Rabin et al., 2007). As for subcutaneous xenografts, KMS-12-BM cells were harvested from exponentially growing cultures, washed once with serum-free medium, and resuspended in PBS immediately before the injection into mice. Three days after injection of the cells, mice were randomized into two treatment groups (n=8 per group; day 0), as shown in Figure 7F. Treatments were maintained for a period of 8 weeks, and the experiment was stopped on day 161 of treatment start. DTP3 was administered intermittently by infusion using Alzet osmotic pumps (model 1002; 14-day delivery at the rate of 0.25 µL/hr; Charles River) implanted subcutaneously, as described above, two-week on/two-week off, at the dose of 29.0 mg/kg/day. PBS in the control group was also administered for 8 weeks. Animals were monitored daily for weight loss and other clinical signs of medullary disease, such as hind limb paralysis and behavioural changes, and euthanized with CO<sub>2</sub> on day 161 of treatment start or when the disease severity reached any of the end-points stated in the PPL. Results were reproduced at least twice.

Patients

Patients were recruited at several Italian Institutions under the umbrella of the “Gruppo Italiano Malattie Ematologiche dell’Adulto” (GIMEMA) and at the Haematology Clinic at Hammersmith Hospital (Imperial College Healthcare NHS Trust), in accordance with ethics committee approval reference numbers VMP-VMPT trial 163 and 11/LO/1628, respectively. The studies were approved by the Ethics Committee of the “Ospedale San Giovanni Battista” (Turin, Italy) or the London Harrow Research Ethics Committee. Informed written consent was obtained from all the subjects involved in the studies. For the analyses shown in Figure 1A, the relative GADD45B mRNA expression was determined by qRT-PCR in purified monoclonal CD138<sup>+</sup> cells from 101 MM patients, monoclonal CD138<sup>+</sup> cells from 14 patients with monoclonal gammapathy of undetermined significance (MGUS), and polyclonal CD138<sup>+</sup> cells from 10 patients with unrelated diseases and without PC dyscrasia. The monoclonality or polyclonality of the isolated PC populations was verified by flow cytometry, as described previously (Rawstro et al., 2008). The analyses shown in Figures 1B-C were conducted on the 69 of the 101 MM
patients in Figure 1A who were enrolled in the VMP arm of the VMP-VMPT trial (clinicaltrial.gov NCT01063179) (Palumbo et al., 2010). The inclusion and exclusion criteria for this trial are described elsewhere (Palumbo et al., 2010). All the patients included in the analysis shown in Figures 1B-C were transplant-ineligible and uniformly treated with nine cycles of velcade-melphalan-prednisone (VMP). The median follow up was of 49 months. PCs were purified at diagnosis from bone marrow aspirates of these patients and stored in the tissue bank of the Hematology Department at the University of Turin. The median age of the patients at diagnosis was 71 years (range 65-86 years), with a female/male ratio of 33/36. The median albumin level was 3.85 g/dL (range 2.2-5.0 g/dL); the median β2-microglobulin level was 4.7 mg/L (range 1.9-12.1 mg/L); and the disease stage according to the International Staging System (Greipp et al., 2005) was as follows: 15 patients (21%), stage 1; 26 patients (37%), stage 2; 15 patients (22%), stage 3; 13 patients (19%), undetermined stage. Based on their FISH profile, 45 patients (66%) were standard risk, and 23 patients (34%) were high risk.

The difference in GADD45B mRNA expression between PCs from MGUS and MM patients was calculated using unpaired, two-tailed t-test (Figure 1A). The treatment response of the 69 patients shown in Figures 1B-C was assessed using the standard International Myeloma Working Group (IMWG) Uniform Response Criteria (Durie et al., 2006). Patients were stratified into two groups on the basis of the GADD45B mRNA levels in CD138+ cells at diagnosis, as determined by qRT-PCR, using as cut-off the median GADD45B expression value as follows: low GADD45B, bottom 50th percentile; high GADD45B, top 50th percentile. The progression free survival (PFS) and overall survival (OS) of these patients (Figures 1B-C) were defined according to the standard IMWG Uniform Response Criteria (Durie et al., 2006) and calculated using the Kaplan-Meier method. The comparisons between the two groups were analyzed using the log-rank test. At the measured endpoint (i.e. 70.7 months), the PFS was 52% for the patients with low GADD45B mRNA expression as compared with the PFS of 17% for the patients with high GADD45B mRNA expression. The OS at the same endpoint was 72% for the patients with low GADD45B mRNA expression, and it was 42% for those with high GADD45B mRNA expression. Results were considered significant with a p value <0.05.

All the other subjects, including seventeen additional MM patients, three patients with Waldenström's macroglobulinemia, and one healthy volunteer, were recruited at the Haematology Clinic at the Hammersmith Hospital, adopting the same diagnostic criteria used above (Durie et al., 2006).
For external validation of the data shown in Figures 1B-C, we examined two independent gene expression datasets of newly diagnosed patients with MM (Figures S1A-B). The first dataset was obtained from the MRC Myeloma IX study performed on 274 patients using the Affymetrix Gene-Chip Human Genome U133 Plus 2.0 Array platform and deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database, under the accession number, GSE15695 (Dickens et al., 2010; Wu et al., 2011). The trial consisted of two treatment pathways: an intensive treatment pathway, comprising high dose melphalan (HDM) and autologous stem cell transplantation after induction with CTD (cyclophosphamide, thalidomide, dexamethasone) or CVAD (cyclophosphamide, vincristine, doxorubicin, dexamethasone), for younger and fitter patients, and a non-intensive treatment pathway, comprising either attenuated CTD (CTDa) or MP (melphalan, prednisolone), for older and less fit patients (Dickens et al., 2010; Wu et al., 2011). The second dataset was obtained from the Dutch-Belgian/German HOVON-65/GMMG-HD4 study performed on 320 MM patients using the same Affymetrix platform and deposited in the GEO database under the accession number, GSE19784 (Broyl et al., 2010). This trial consisted of two treatment arms: a bortezomib based treatment arm (bortezomib, adriamycin, dexamethasone) (PAD), and a vincristine based treatment arm (vincristine, adriamycin, dexamethasone) (VAD), as induction therapy, followed by HDM treatment with autologous stem cell rescue and maintenance treatment with thalidomide (VAD arm) or bortezomib (PAD arm) (Broyl et al., 2010, Sonneveld et al., 2008).

Gene expression values in the two datasets were both normalized using robust multi-array average (RMA) (Irizarry et al., 2003). In order to obtain homogeneous datasets, patients were separated according to therapeutic arm. Analyses were performed on the 155 patients present in the intensive arm of the MRC Myeloma IX study (GSE15695), and the 149 patients present in the VAD arm of the HOVON-65/GMMG-HD4 study (GSE19784). Sample purity was as reported previously (Broyl et al., 2010, Dickens et al., 2010). For each study, patients were stratified using k-mean clustering based on the GADD45B expression values (Hartigan and Wong, 1979). Survival analysis was performed in R using package survival (http://www.R-project.org/). Differences between curves were tested for statistical significance using the log-rank test (Harrington and Fleming, 1982), with p value < 0.05 taken as the level of significance.

**Statistical Analyses**
Results were expressed as the mean of the indicated number of samples. The statistical error was calculated as either standard deviation (SD) or standard error of the mean (SEM), as indicated, according to the typology of the experiments. Unless otherwise stated, the statistical significance of the values was determined using the Student's t-test. A value of $p<0.05$ was considered statistically significant. In Figure 1A, the difference in GADD45B mRNA expression between PCs from MGUS and MM patients was calculated using unpaired, two-tailed t-test. The PFS and OS of MM patients in Figures 1B-C and Figures S1A-B were calculated using the Kaplan-Meier method, and comparisons between the cohorts of patients were analyzed using the log-rank test. The correlations between GADD45B mRNA expression and D-peptide cellular activity were analyzed by calculating the Spearman correlation coefficient ($r_s$) and the corresponding $p$ value (two-tailed) (Figure 5C, Figure S5U). The median OS for the cohorts of tumor-bearing mice shown in Figure 7F was calculated using GraphPad software, and comparisons between the cohorts were analyzed using the log-rank test. The sample size for the analyses shown was not pre-determined. No samples or animals were excluded from the analyses. No specific method of randomization was used for the animal studies. The animal experiments were not blinded.
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