Supplemental information

Supplemental data

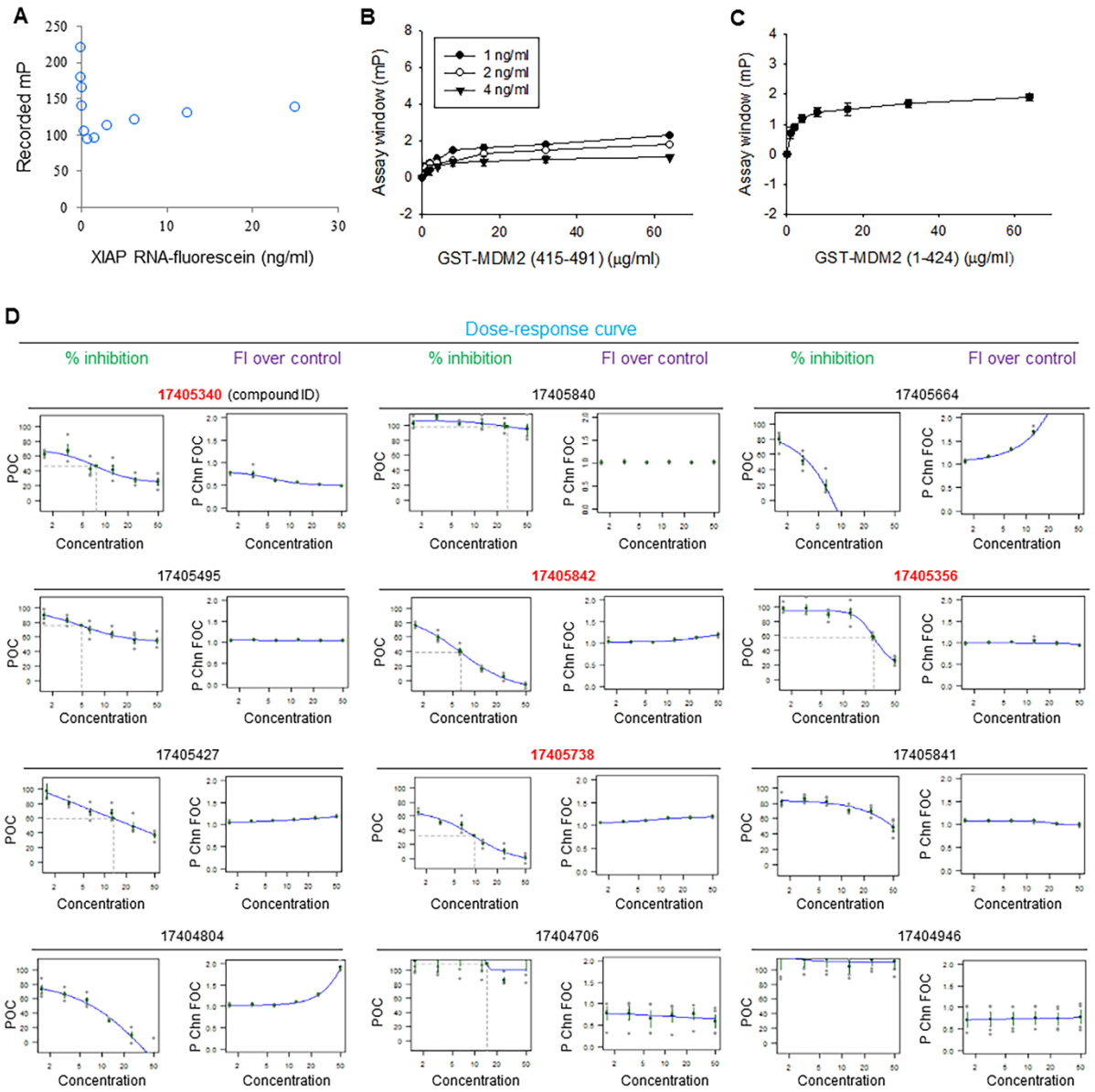

Figure S1, related to Figure 1. (A) Polarization signals, expressed as mP, for different concentrations of XIAP IRES-fluorescein. (B and C) FP assays for interaction between MDM2 RING protein and control XIAP RNA (non-IRES upstream 5'-UTR of XIAP mRNA) (B) and binding between MDM2 protein with a deleted RING domain and XIAP IRES (C); data represent mean ± SD of three independent experiments. (D) Representative graphs of FP dose-response assays to validate potential positive hits identified by HTS in LOPAC library. Hits were selected from the library, and a dose-response test was carried out in 1536-well format with 5 doses (1.6, 3.1, 6.3, 12.5 25 and 50 µM) at 4 replicates. Both % control (left side) and FI over control (right side) are plotted against compound concentration for each compound. The IC50 values were obtained using GraphPad Prism software; data represent mean ± SD

of three independent experiments. The 4 compounds (red) with IC50<25 μM and FI over vehicle control <2 were further screened by secondary cell-based assay.

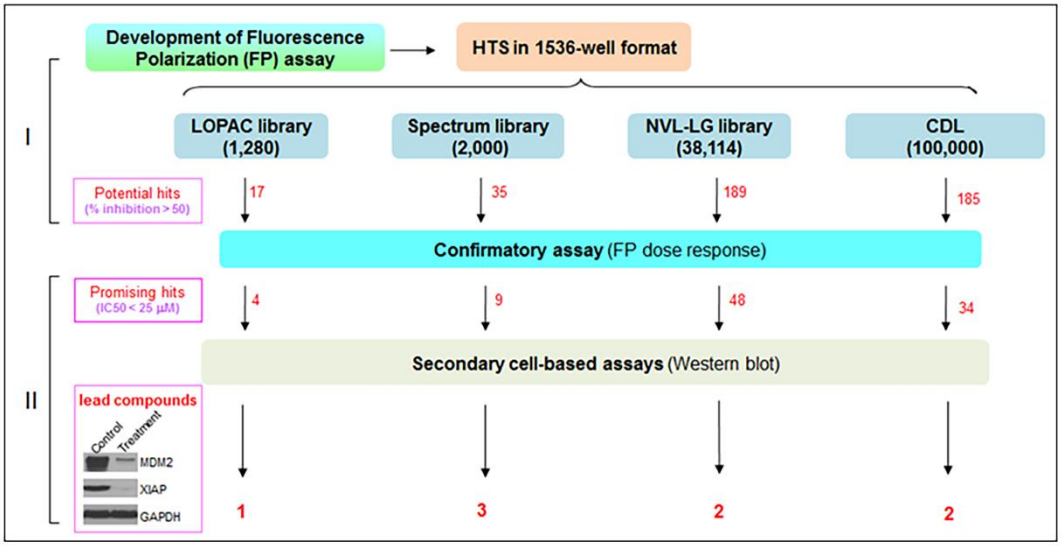

Figure S2, related to Figure 2. Summary of hits and leads selected by HTS, FP dose-response and Western blot assays, respectively, from four libraries: LOPAC, Spectrum, NVL-LG (Novelty and LeadGene) from Asinex and CDL from ChemDiv.

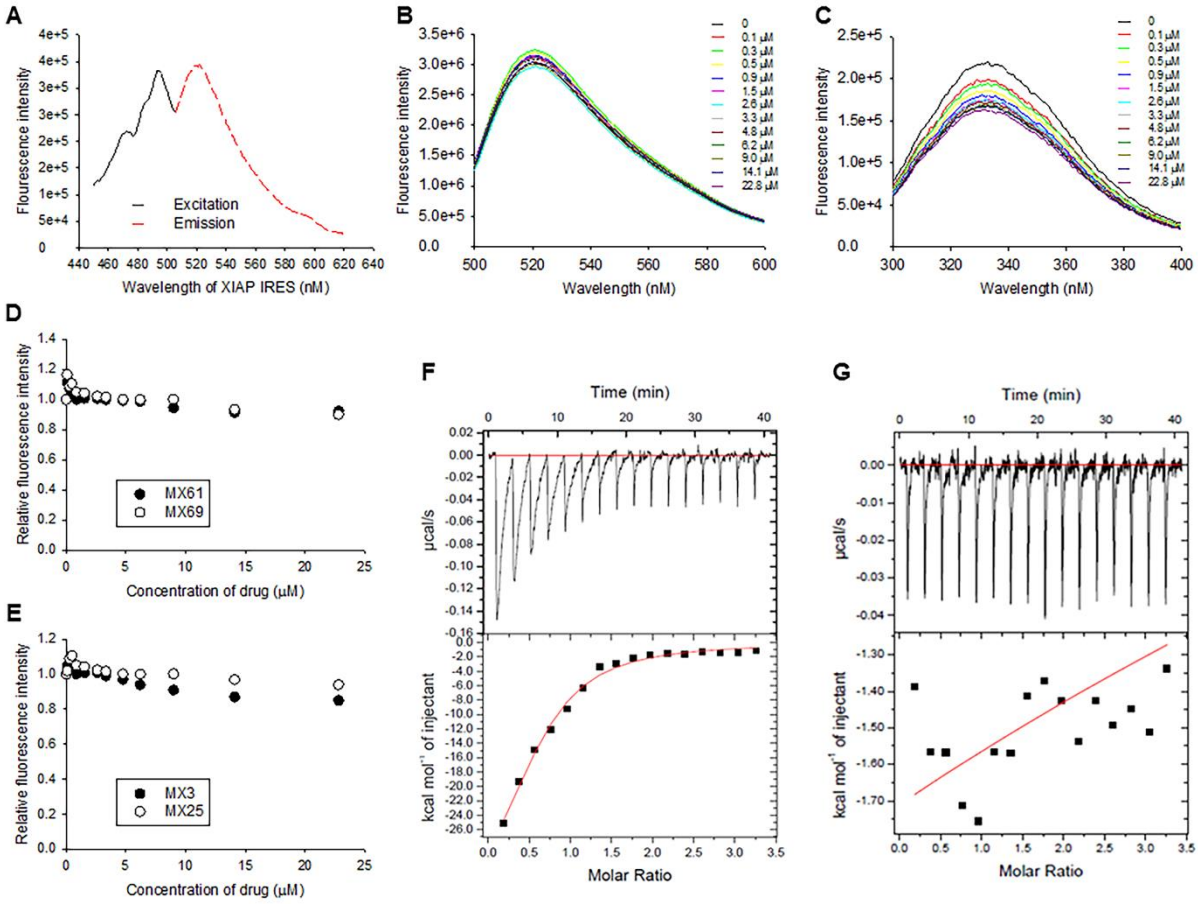

Figure S3, related to Figure 3. (A) Excitation and emission spectra of fluorescence-labeled XIAP IRES. (B and C) Representative graphs showing little or no fluorescence changes when XIAP IRES RNA was titrated with MX69 (B) and MDM2 RING protein titrated with MX3 (C). (D and E) The control GST protein was titrated with MX61 or MX69 (D) and the XIAP non-IRES titrated with MX3 or MX25 (E), respectively: no binding activity was detected. (F and G) Confirmation of the binding between XIAP IRES and MX3 (F) and non-binding between XIAP IRES and MX69 (G) as detected by ITC. The upper box is raw heating power over time and the lower box is a fit of integrated energy values, normalized for each injection. Each fluorescent titration or ITC assay was performed at least three times.

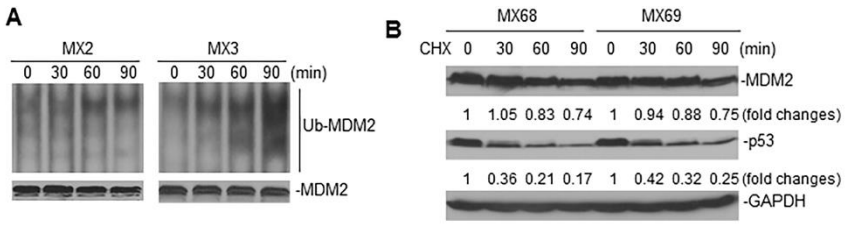

Figure S4, related to Figure 4. (A) IP and Western blot assay using anti-MDM2 and anti-ubiquitin antibodies, respectively, to detect effects of MX3 (1 μM) on ubiquitination of endogenous MDM2 in NB-1691 cells. MX2 served as controls. (B) CHX pulse-chase assay for MDM2 and p53 protein turnover in SK-N-SH cells stably transfected with mutant MDM2 (MDM2-C464A) following treatment with MX69.

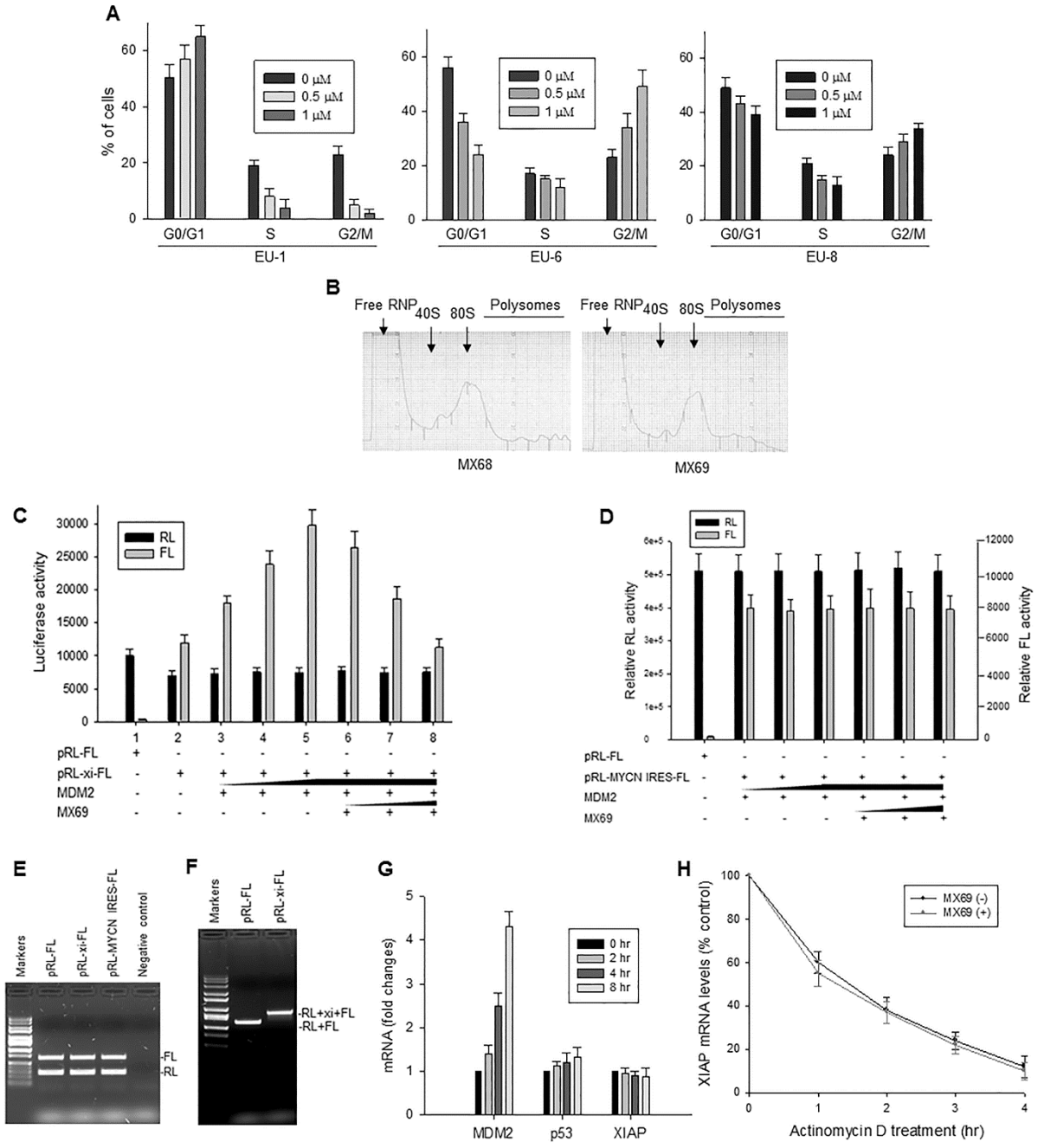

Figure S5, related to Figure 5. (A) Cell-cycle distribution analysis of three ALL cell lines EU-1, EU-6 and EU-8 treated with MX3 for 8 hr. (B) Representative polyribosomal profile for EU-1 cells treated with either 10 μM MX69 or MX68 (control) for 4 hr. The 254 nm traces obtained during fraction collection are shown. (C) Effect of MX69 on MDM2-mediated XIAP IRES activity. 293T cells were co-transfected with 5 μg pRL-xi-FL, and increasing amounts of MDM2 plasmid (2.5, 5, and 10 μg) or a constant amount of MDM2 (10 μg), in the presence or absence of increasing amounts of MX69 (2.5, 5 and 10 μM). Controls included transfection of pRL-FL empty vector alone. Quantitative RL and FL activities were detected using the Dual-Luciferase Reporter System. (D) Similar gene transfection and reporter assay for effect of MDM2 and MX69 on MYCN IRES activity. (E) RL and FL mRNA levels analyses following transfection of

293T cells with various plasmids as described in (C and D). Total RNA was extracted, and RT-PCR was performed with specific primers targeted to RL and FL, respectively. (F) 293T cells were transfected with pRL-FL or pRL-xi-FL, and mRNAs as indicated were detected by RT-PCR using primers targeted to both RL and FL. (G) EU-1 cells were treated with 10 μM MX69 for different times, as indicated. The mRNA levels of MDM2, p53 and XIAP relative to GAPDH were determined by quantitative RT-PCR. (H) EU-1 cells were treated with or without 10 μM MX69 for 4 hr, followed by addition of 5 μg/ml actinomycin D and then harvested at the indicated time points. XIAP mRNA was determined by quantitative RT-PCR. Data in (A, C, D, G and H) represent mean ± SD of three independent experiments.

Table S1, related to Figure 6. MDM2 and XIAP expression levels and p53 status of 12 cancer cell lines (6 ALL and 6 NB), and cytotoxicity of eight selected leads against the 12 cancer cell lines.

Cell lines		p53 status	MDM2 expression	XIAP expression	Compounds IC50 (μM)*							
					MX3	MX5	MX6	MX11	MX25	MX61	MX69	MX92
ALL	EU-1	WT	++++**	++	0.28	15.3	32.4	15.8	11.9	43.4	7.5	15.7
	EU-3	WT	+++	++	0.19	13.7	31.4	14.3	10.7	42.1	6.7	14.6
	SUP-B13	WT	++++	++	0.32	14.6	43.5	12.4	12.9	37.9	7.4	14.9
	UOC-B1	WT	+++	++	0.35	11.2	38.6	13.5	11.4	41.3	8.9	16.8
	EU-6	M	++	++	0.76	28.8	45.6	34.6	23.3	50.6	12.3	18.6
	EU-8	N	-	++	0.98	48.9	>100	>100	>100	>100	35.5	>100
NB	NB-1691	WT	+++++	+++	0.92	19.4	>100	34.9	18.5	49.6	15.4	21.5
	NB-1643	WT	++	++	0.84	17.3	>100	34.2	20.1	54.8	11.2	19.3
	SH-EP1	WT	+++	+++	1.15	20.1	>100	36.9	19.6	65.7	17.9	18.9
	IMR-32	WT	+++	++	1.23	17.9	>100	43.8	17.5	47.7	16.2	17.6
	SK-N-SH	WT	+	++	0.96	21.3	>100	35.6	19.8	43.8	17.4	17.4
	LA1-55N	N	++	++	1.76	15.6	>100	32.4	28.9	>100	28.9	32.6

Abbreviations: WT, wild-type**;** M, mutation**;** N, null

*IC50, concentration of compounds required to produce 50% decrease in cell viability during 24 hr WST assay.

**+-++++, expression levels detected by Western blot assay; -, negative

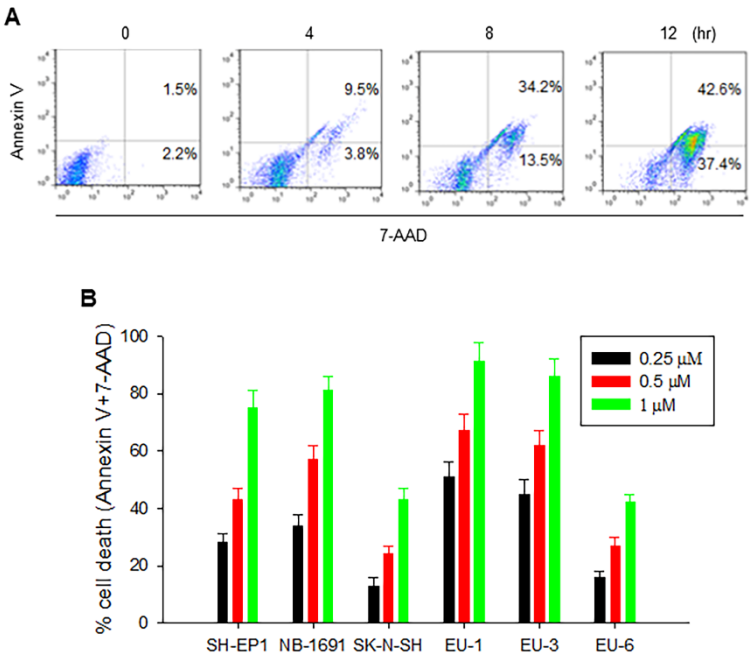

Figure S6, related to Figure 6. (A) Representative flow cytometric graphs for detection of apoptosis (annexin V+) and cell death (7-AAD+) in EU-1 ALL cells treated with MX3. (B) MX3 apoptosis dose-response profiles for 6 tumor cell lines treated as indicated for 24 hr and quantitatively detected by flow cytometry; data represent mean of three independent experiments, bars ± SD.

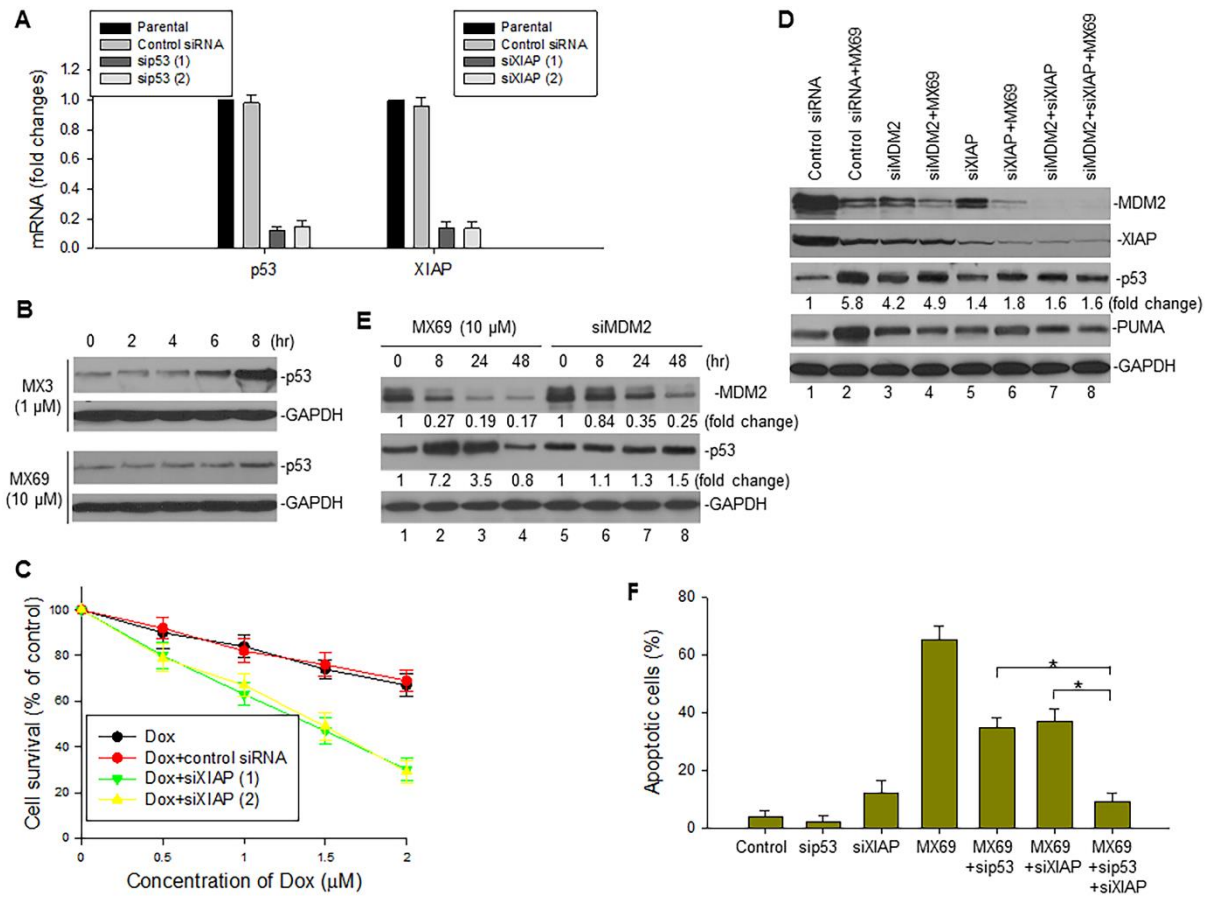

Figure S7, related to Figure 7. (A) The expression levels of p53 and XIAP mRNA relative to GAPDH in SH-EP1 and LA1-55N cells transfected with sip53 and siXIAP, respectively, were determined by quantitative RT-PCR. (B) Western blot showing p53 induction in NBMM cells after treatment with either MX3 or MX69. (C) LA1-55N cells stably transfected with MDM2 in presence or absence of siXIAP were treated with doxorubicin (Dox) and cell survival detected by WST assays. (D) Western blot showing inhibition of MDM2 and XIAP and induction of p53 and PUMA in NB1691 cells treated with MX69 (10 μM for 24 hr) in presence or absence of siRNA as indicated. (E) Kinetic reduction of MDM2 and induction of p53 in MX69 (10 μM) treated and siMDM2 transfected NB-1691 cells. (F) NB-1691 cells were treated with sip53 (1), siXIAP (1), MX69 (10 μM for 24 hr) alone or their combinations as indicated and apoptotic cells quantitatively detected by flow cytometry, *p<0.01. Data in (A, C and F) represent mean of three independent experiments.

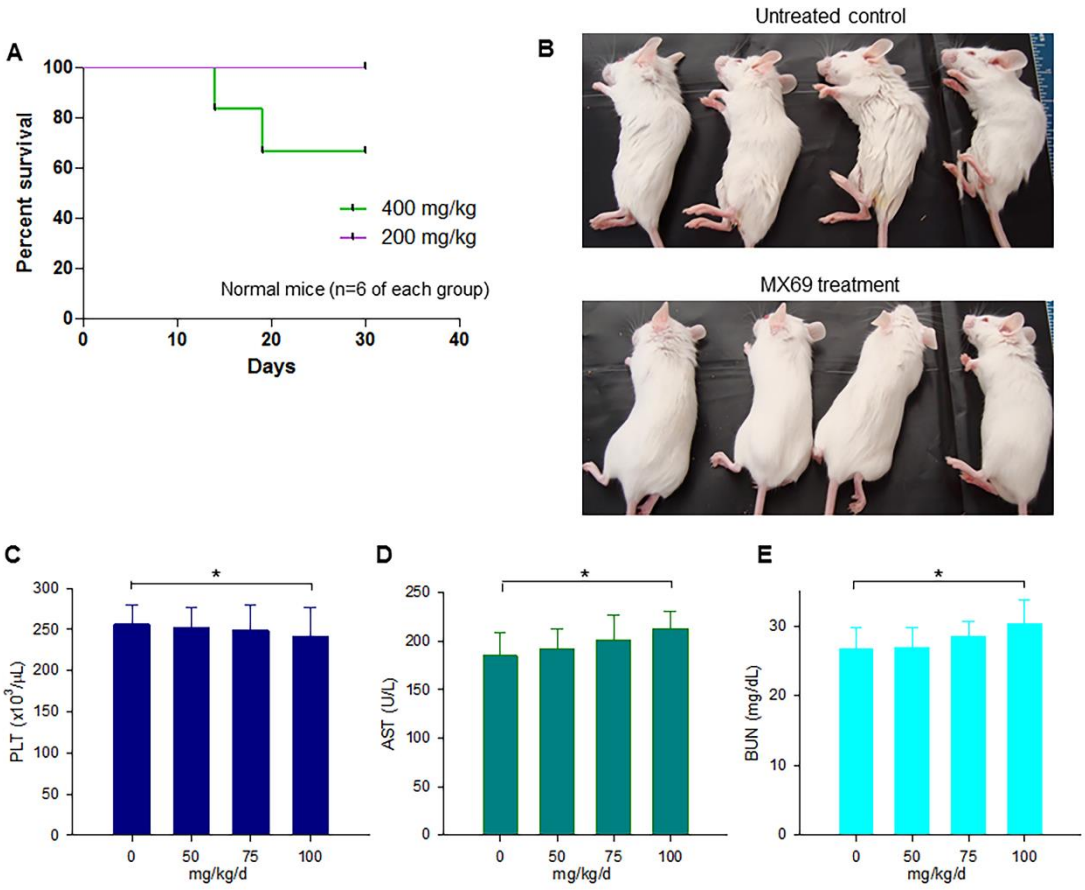

Figure S8, related to Figure 8. (A) Event-Free Survival (EFS) curves for apparent Maximum Tolerated Dose (MTD, 200 mg/kg) and toxic dose (400 mg/kg) of MX69 in normal Hsd:ICR (CD-1) mice. (B) comparison of SCID mice xenografted with EU-1 human leukemia cells, showing difference between the no- treatment control group and mice treated with 100 mg/kg of MX69 (40 days post-inoculation and treatment). (C-E) Normal Hsd:ICR (CD-1) mice were treated with various doses of MX69, and then blood was collected and analyzed for the levels of PLT (C), AST (D) and BUN (E); data represent mean of three independent experiments, bars ± SD, (*p>0.5).

Supplemental Experimental Procedures

Plasmid and siRNA

To generate the XIAP IRES RNA expression plasmid, we first annealed the primers 5'-GATCCTTTCACATTTTGGATTTCCTAATATAATGTTCTCTTTTTAGAAAAGGTGGA-3' and 5'-GAAAGTGTAAAACCTAAAGGATTATATTACAAGAGAAAAATCTTTTCCACCTTC GA-3' that contain the fragment from -34 to -62 of the XIAP 5'-UTR (underlined), which is the RNP core-binding-site and is bound by MDM2 (Gu et al., 2009; Holcik et al., 1999); the primers were then inserted immediately downstream of the CMV promoter of the pRNA-CMV3.1-puro vector (Genscript, Piscataway, NJ). A control RNA plasmid was generated similarly by annealing primers containing 28-nt of non-IRES upstream 5'-UTR of XIAP mRNA (sequence not shown) and inserting into the pRNA-CMV3.1-puro vector. The MDM2 CRISPR/Cas9 HS0000146336 by cloning sequence CCAGCTTCGGAACAAGAGACCC (targeting exon 2) into pLV-U6g-EPCG vector and control CRISPR/Cas9 were purchased from Sigma. MDM2 KO was carried out using CRISPR/Cas9 and stable clones were selected after about 1 month of culture. The clone with significant inhibition of MDM2 was selected for experiments.

The sip53-1 (sense GAAAUUUGCGUGUGGAGUAdTdT, antisense UACUCCACACGCAAAUUUCdCdT), sip53-2 (sense GGUGAACCUUAGUACCUAAdTdT, antisense UUAGGUACUAAGGUUCACCdAdA), siXIAP-1 (sense GGAUAUACUCAGUUAACAAdTdT, antisense UUGUUAACUGAGUAUAUCCdAdT), siXIAP-2 (sense GAAUCUUAAUAUUCGAAGUdTdT, antisense ACUUCGAAUAUUAAGAUUCdCdG), siMDM2 (sense CUAUGAAAGAGGUUCUUUUdTdT, antisense AAAAGAACCUCUUUCAUAGdTdA) and control siRNA were purchased from Santa Cruz, and transient transfection of siRNA was carried out using the HiPerFect transfection reagent (Qiagen), following the manufacturer's manual.

Protein expression and purification

The expression and purification of GST-fused MDM2 proteins were performed as described previously (Gu et al., 2009). Briefly, after transfection of GST-fused MDM2 plasmids into BL21 *Escherichia coli*, the cells were incubated in LB medium at 30^0 C for protein expression. The cells were harvested after incubation with 0.1 mM IPTG for 2 hr. Purification of the GST-fused MDM2 proteins was performed by lysing the induced cells with sonication, followed by isolation with glutathione-agarose beads (Pharmacia). The purity and correct expression of each of the GST-fused MDM2 proteins were analyzed by gel electrophoresis and Coomassie G250 staining as well as western blot assay using anti-GST antibody.

Synthesis and labeling of the XIAP IRES probe

A DNA fragment 5'-TAATACGAGTCACTATAGGGATTTCACATTTTGGATTTCCTAATAT AATGTTCTCTTTTTAGAAAAGGTGG-3', containing the 28-nt XIAP IRES sequence and the T7 promoter sequence (underlined) was used as the template for an in vitro transcription reaction using the MAXIscript kit (Ambion, Austin, TX, USA) to generate a single-stranded RNA. This RNA was labeled with fluorescein at its 5' end using the 5' EndTag Nucleic Acid Labeling System (Vector Laboratories, Burlingame, CA, USA). A DNA fragment containing 28-nt of non-IRES sequence upstream 5'-UTR of XIAP was similarly transcripted and labeled

served as control.

Compound-protein and compound-RNA binding assays

The binding activity of selected leads to either MDM2 protein or XIAP IRES was examined by fluorescence titration and isothermal titration calorimetry (ITC) assays. The GST-MDM2 RING protein has spontaneous fluorescence; XIAP IRES was labeled with fluorescein as described above. Titration was performed using PTI Quanta-Master spectrometer (Photon Technology International, Birmingham, NJ). The steady-state fluorescence of the protein-compound or RNA-compound mixtures was acquired using a 3 ml cuvette. The slit widths for excitation and emission were adjusted to minimize photobleaching of the sample, while achieving sufficient fluorescent signal intensity. The fluorescence measurements, as a function of reagent concentration, were fitted with the hyperbolic function $F = F_f + (F_b - F_f)[ligand_f]/(K_d + [ligand_f])$, where F is the observed fluorescence, F_f is the fluorescence of unbound protein or RNA, F_b is the fluorescence from the protein-compound or RNA-compound complex, $ligand_f$ is the concentration of the compound, and K_d is its dissociation constant.

ITC assay was performed using the auto-iTC200 instrument (MicroCal, GE). MDM2 protein or XIAP IRES was loaded into a 96 DeepWell PP plate, and then compound was titrated stepwise into the protein or RNA sample cell using a syringe, for a total of 16 injections (except for the first injection, which was 0.4 μl). The equilibrium time between two adjacent injections was 210 s. The binding stoichiometry (n), binding constant (K_d), and thermodynamic parameters (ΔH and ΔS) were determined by fitting the titration curve to a one-site binding mode, using the Origin software provided by the manufacturer.

Gene transfection and reporter assay

The dicistronic reporter plasmid pRL-xi-FL was generated by inserting the XIAP IRES into the dicistronic vector pRL-FL, kindly provided by Dr. Steve Haines (University of Nottingham, UK), which contains an upstream *Renilla* luciferase cistron and a downstream *Firefly* luciferase (FL) cistron. The pRL-MYCN IRES-FL plasmid was generated as described previously (Gu et al., 2012). Transient transfections were performed in 293T cells using Lipofectamine™ 2000 reagents (Invitrogen). Transfected cells treated with or without MX69 were resuspended in 10 ml of RPMI containing 10% FBS, and then incubated 24–36 hr. Cell extracts were prepared with 1x lysis buffer, then 20 μl aliquots of the supernatant were mixed first with 100 μl of Luciferase Assay Reagent II (Promega) to measure the FL activity and next, the RL activity was determined by adding Stop & Glo® Reagent to the same sample. These luciferase activities were analyzed via Microplate Instrumentation (BioTek).

Antibodies used for immunoprecipitation and Western blot

Antibodies used for immunoprecipitation and Western blot were as follows: MDM2 antibody (SMP14, Sigma); p53 (DO-1), Bcl-2 (N-19) and caspase 3 antibodies (Santa Cruz); XIAP (2F1), cIAP1 (ab108361), cIAP2 (ab137393), PUMA (ab9346) and PARP (E-102) antibodies (Abcam); p21 (12D1) antibodies (Cell Signaling). All antibodies were used according to the manufacturers' instruction.

UV cross-linking and RNA-protein binding assays

The DNA templates for synthesis of the XIAP IRES RNA probe (XIAP IRES) and a control RNA probe from non-IRES upstream 5'-UTR of XIAP mRNA (XIAP non-IRES) were generated by PCR using specific primer pairs (XIAP IRES, forward: 5′-TAATACGACTCACTATAGGGCGAAATTAGAATGTTTCTTAGCGGTC-3', reverse: 5′-CTTCTCTTGAAAATAGGAC-3'; XIAP non-IRES, forward: 5'-TAATACGACTCACTATAGGGCGATATATTCTGCATCACAGTTTAC-3', reverse: 5′-CTAAATACTAGAGTTCGACATTAC-3'. The forward primers incorporated the T7 promoter sequence (underlined). Internally labeled RNA probes were synthesized by in vitro transcription with T7 polymerase (MAXIScript T7 RNA polymerase kit, Ambion) in the presence of [α-^{32}P] UTP (Amersham). The GST-MDM2 protein was mixed with ^{32}P-labeled probes in the presence or absence of selected leads. UV cross-linking of the RNA-protein complexes was performed using a 254-nm UV light source set at 400,000μJ/cm^2. The UV-irradiated RNA-protein complexes were then treated with RNase T1 and resolved by 10% SDS-PAGE gel and visualized by autoradiography.

Polysome preparation and analysis

Cells were incubated with 100 μg/mL cycloheximide (CHX) for 15 min to arrest polyribosome migration, and then lysed (in order to isolate cytoplasmic extracts) in a buffer containing 20 mM Tris-HCl at pH 8.0, 100 mM NaCl, 5mM $MgCl_2$, 0.5% Triton X-100, 500 U/mL RNAsin, and a cocktail of protease inhibitors. Fractionation was performed on a 15-45% (w/v) sucrose gradient at 39,000 rpm for 1 hr (SW41Ti rotor). Fractions were collected by upward replacement in a fractionator (Isco, Lincoln, NE). The RNA from each fraction was subjected to quantitative RT-PCR.

Quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed with a mixture of random monomers and oligo-dT as primers. Amplification was performed with a 7500 Real-Time PCR System (Applied Biosystems), using the QuantiFast SYBR Green RT-PCR kit (Qiagen), according to the manufacturer's instructions. All gene-specific primers were purchased from Qiagen.

Pulse-chase assay

Protein turnover was assessed by a standard protein-synthesis-inhibitor CHX assay. Briefly, cells were treated with 50 μg/mL CHX for different times before lysis, in the presence or absence of reagents; these were then tested by Western blot analysis to reveal concurrent expression levels of tested proteins.

The mRNA degradation rate was examined using a standard actinomycin D analysis: At different times after addition of 5 μg/mL of actinomycin D, in the presence or absence of MX69, the cells were harvested and their total RNA isolated. The MDM2 mRNA was detected by quantitative RT-PCR, as described above.

Clonogenic assay

The effect of leads on normal human hematopoiesis in vitro was determined with a soft-agarose colony assay. Briefly, a bottom layer of a low-melting-point agarose solution containing 0.5% agarose, in a final concentration of 1X RPMI 1640 medium supplemented with 10% FBS, was poured into gridded 35 mm dishes and allowed to gel. A top layer of 0.35% agarose and the 1 X medium as the diluent contained the prepared cells, reagents or compounds. Cells were cultured at 37˚ C in a humidified atmosphere containing 5% CO_2. After 2 weeks, the cultures were fixed with formalin and colonies were scored.

Animal studies

For the in vivo toxicity assay, normal Hsd:ICR(CD-1) mice were injected i.p. with MX69 at dose levels and in a schedule that were the same as for treatment of the xenografts. After 14 days of MX69 treatment, the mice were euthanized and their whole blood (250 μL) collected in EDTA-coated tubes via cardiac puncture for hematology studies on white blood cell (WBC) and platelet (PLT) components. Serum (250ul) was also collected for examination of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN). The organs (specifically heart, liver and kidney) of treated mice were collected for histopathological evaluation.