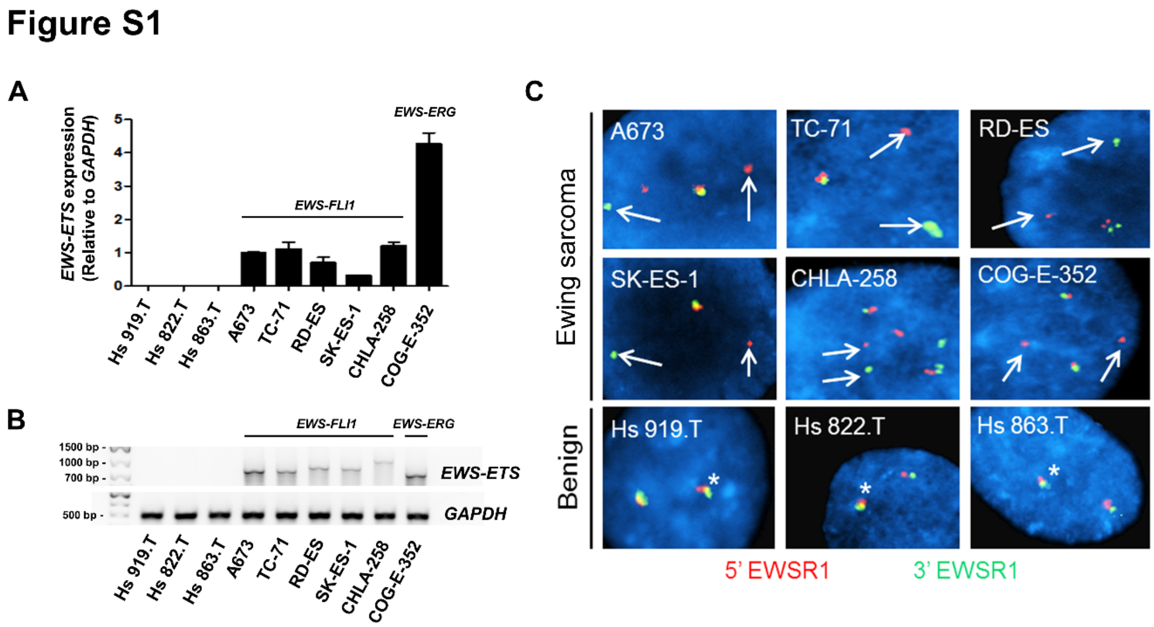
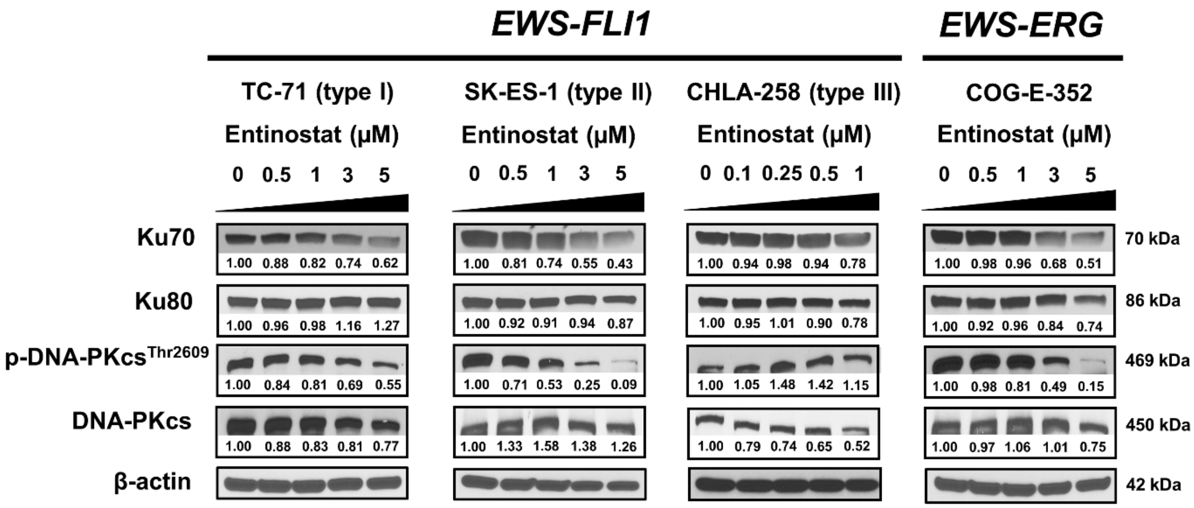
Supplementary Figures



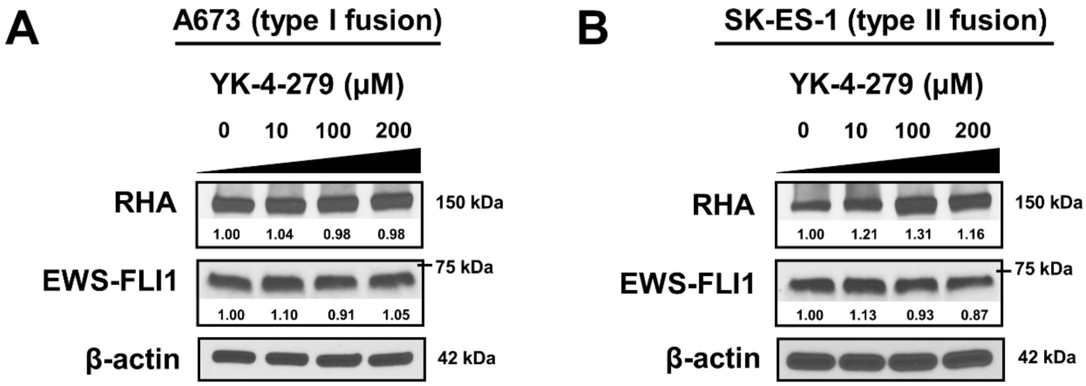
Supplementary Figure S1. qRT-PCR and FISH analysis of *EWS-ETS* fusion genes in ES cells used in the study. (**A**) A qRT-PCR gene expression panel of *EWS-FLI1* and *EWS-ERG* in ES cells (A673, TC-71, RD-ES, SK-ES-1, CHLA-258 and COG-E-352) and benign control cells (Hs 919.T, Hs 822.T and Hs 863.T). Graph shows relative fold expression calculated using ΔΔCt value changes from A673 (set at 1.0). (**B**) Electrophoresis image of amplified cDNA fragments from all tested cell lines. (**C**) FISH analysis showing the red and green probes separated in the nuclei of ES cells, which mark the 5' and 3' loci in the *EWSR1* gene, respectively, and are indicated by the arrows. However, Hs 919.T, Hs 822.T and Hs 863.T cells are negative for the *EWSR1* rearrangement as shown by the red and green signals staying together (asterix).

Figure S2



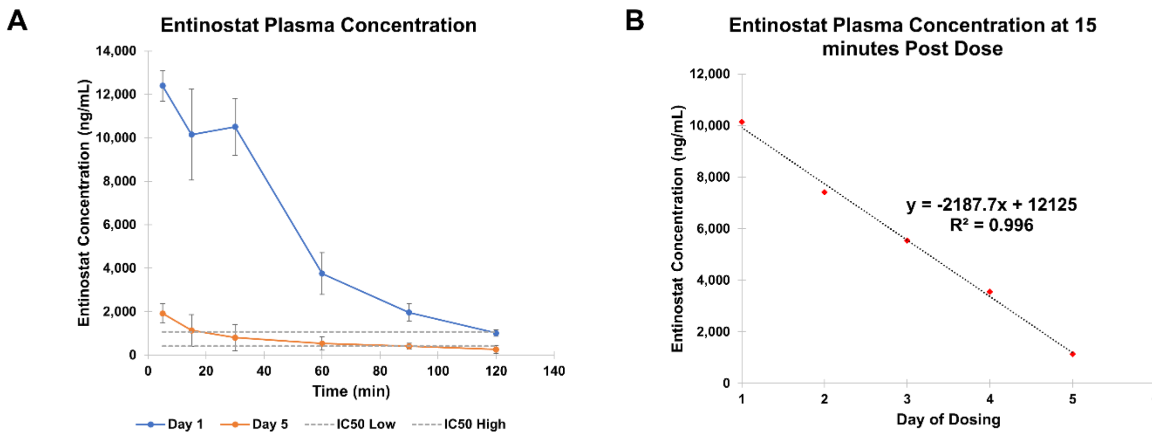
Supplementary Figure S2. Entinostat regulates the expression of DNA repair proteins on the non-homologus end joining (NHEJ) pathway in a cell line-dependent manner. Representative western blots showing changes in protein expression in TC-71, SK-ES-1, CHLA-258 and COG-E-352 cells after 48 h incubation with entinostat. Cell lysates were probed with the indicated antibodies. β-actin served as a protein loading control. The relative intensities of protein bands are shown under the immunoblots after normalization for the levels of β-actin.

Figure S3



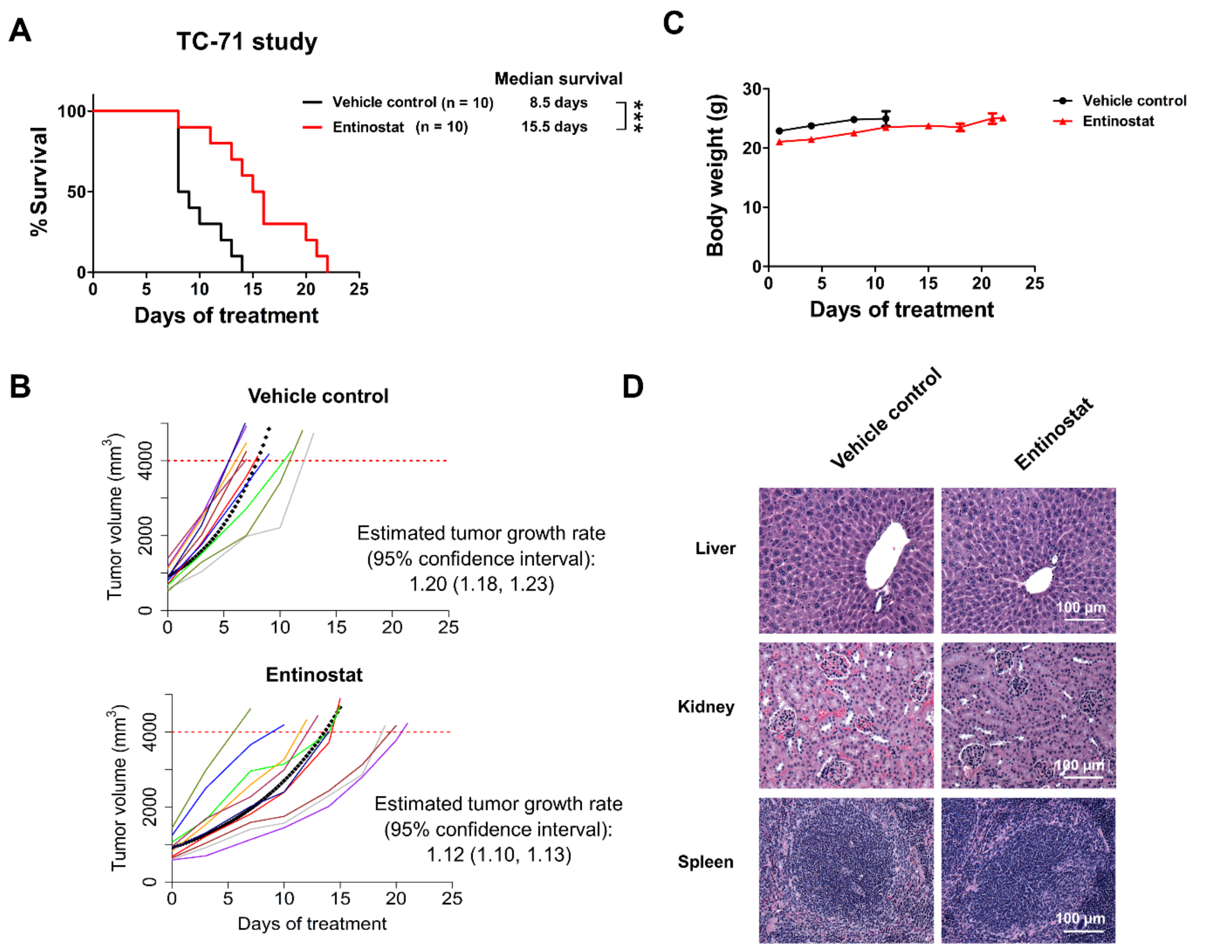
Supplementary Figure S3. YK-4-279 barely affects the expression of RHA and EWS-FLI1 in A673 (A) and SK-ES-1 (B) cells 12 hours post treatment. β-actin served as a loading control. The relative intensities of protein bands are shown under the immunoblots after normalization for the levels of β-actin.

Figure S4



Supplementary Figure S4. The plasma concentrations of entinostat in BALB/c mice after administration. (A) Plasma drug concentration-time profiles for days 1 and 5 for entinostat administered orally at 24.5 mg/kg following daily consecutive doses are shown. **(B)** Entinostat plasma concentrations at 15 minutes post dose from day 1 to day 5 is shown.

Figure S5



Supplementary Figure S5. Entinostat significantly prolonged the median survival of TC-71 xenograft-bearing mice. **(A)** Kaplan-Meier survival curves for the TC-71 tumor-bearing nude mice treated with vehicle or entinostat (25 mg/kg, p.o., qd) for 26 days. N = 10 mice per treatment group. *** $p < 0.001$ by log-rank test. **(B)** Tumor growth curves of individual mouse from each treatment group with the model-predicted values (dotted lines) overlaid in black. Estimated tumor growth rate for each treatment group was indicated. **(C)** Mice body weights throughout the treatment period in the TC-71 xenograft mouse study. Data, mean ± SEM. **(D)** Representative H&E staining photographs of livers, kidneys and spleens from each treatment group in the TC-71 xenograft mouse study. Bar = 100 µm.

Supplementary Tables

Supplementary Table S1. The targeting sequences for siRNAs

siRNA	***Company***	***Catalog number***	***Targeting sequences***
siFLI1-#1	Dharmacon	D-003892-01	5'-GCACAAACGAUCAGUAAGA-3'
siFLI1-#3	Dharmacon	D-003892-03	5'-AGUCGUCCAUGUACAAGUA-3'
siERG-#6	Dharmacon	J-003886-06	5'-GUUAGAUCCUUAUCAGAUU-3'
siERG-#8	Dharmacon	J-003886-08	5'-CCUCAGACCUCCCGUACAU-3'
siGENOME non-targeting siRNA #2 (siControl)	Dharmacon	D-001210-02-05	5'-UAAGGCUAUGAAGAGAUAC-3'
siHDAC1-#3	Qiagen	SI00070623	5'-CCACAGCGATGACTACATTAA-3'
siHDAC1-#6	Qiagen	SI02663472	5'-CACCCGGAGGAAAGTCTGTTA-3'
siHDAC3-#1	Qiagen	SI00057316	5'-CAGCCGGTTATCAACCAGGTA-3'
siHDAC3-#6	Qiagen	SI03057901	5'-CACCCGCATCGAGAATCAGAA-3'
AllStars Negative Control siRNA (siNC)	Qiagen	1027280	Proprietary
siGENOME human PLK1 SMARTpool (siPLK1)	Dharmacon	M-003290-01-0010	5'-CAACCAAAGUCGAAUAUGA-3' 5'-CAAGAAGAAUGAAUACAGU-3' 5'-GAAGAUGUCCAUGGAAAUA-3' 5'-CAACACGCCUCAUCCUCUA-3'

Supplementary Table S2. The antibodies used for Western blot assays

Antibody	*Company*	*Catalog number*	*WB*
anti-pH$_2$AX^{Ser139}	Cell signaling	#9718	1:1000
anti-BRCA1	Cell signaling	#14823	1:1000
anti-BRCA2	R&D Systems	MAB2476	1:1000
anti-RAD51	Cell signaling	#8875	1:1000
anti-Ku70	Cell signaling	#4588	1:1000
anti-Ku80	Cell signaling	#2180	1:1000
anti-pDNA-PKcs^{Thr2609}	Thermo Fisher	PA5-12913	1:350
anti-DNA-PKcs	Santa Cruz	sc-9051	1:4000
anti-p21$^{Waf1/Cip1}$	Cell signaling	#2947	1:1000
anti-cyclin D1	Cell signaling	#2922	1:1000
anti-FLI1	Santa Cruz	sc-356	1:200
anti-ERG	Cell signaling	#97249	1:1000
anti-RHA	Abcam	ab26271	1:1000
anti-HDAC1	Proteintech	10197-1-AP	1:500
anti-HDAC3	Cell signaling	#3949	1:500
anti-acetyl-histone H3	Millipore	#06-599	1:1000
anti-histone H3	Cell signaling	#4499	1:1000
anti-HSP90	Cell signaling	#4877	1:1000
anti-acetylated lysine	Cell signaling	#9441	1:500
anti-β-actin	Thermo Fisher	MA5-15739	1:5000
HRP-goat-anti-rabbit IgG	Cell signaling	#7074	1:5000
HRP-horse-anti-mouse IgG	Cell signaling	#7076	1:5000

Supplementary Table S3. Primer sequences and PCR fragments' sizes in ChIP-PCR assay

Primer Pair	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	PCR fragment size (bp)
1	GGTGTTGTCCACTGGTCTATCC	CTTCTCTAGGAAGCTCCGAAGATC	161
2	TACCGGTGATTTGGTTCTGGTTC	TCCTTCTCTGTTCTTCCCTCCC	200
3	CACAAGCAGGAAGAGCATATATAGC	GTCTTAGACGTGCTTGTTTCAGATC	183
4	TCTCTACTGTGTCCTCATTCATGTC	GCATGATAGCAAGTAGGTAAAAGGG	155

Supplementary Table S4. Summary of the *EWS-FLI1* or *EWS-ERG* fusion characteristics of the Ewing sarcoma cell lines used in the studies

Cell line	Disease	Fusion gene	Exons in *EWS* gene	Exons in *FLI1* or *ERG* gene	Fusion type
A673	ES	*EWS-FLI1*	1-7	6-9	type I
TC-71	ES	*EWS-FLI1*			
RD-ES	ES	*EWS-FLI1*	1-7	5-9	type II
SK-ES-1	ES	*EWS-FLI1*			
CHLA-258	PNET	*EWS-FLI1*	1-10	6-9	type III
COG-E-352	PNET	*EWS-ERG*	1-7	8-12	N/A

ES, Ewing sarcoma; PNET, primitive neuroectodermal tumor; N/A, not applicable.

Supplementary Table S5. The IC_{50} values of entinostat, growth rate and the doubling time of non-*EWS*-rearranged benign cells and *EWS*-rearranged Ewing sarcoma cells

Cell line	Entinostat IC_{50} (μM) [a]	Growth rate (hours^{-1})	Doubling time (hours)
Hs 919.T	70.41 ± 14.72	0.003481	199
Hs 822.T	43.18 ± 7.05	0.005228	133
Hs 863.T	83.86 ± 6.51	0.005522	126
A673	2.86 ± 0.15	0.028487	24
TC-71	2.51 ± 0.12	0.027435	25
RD-ES	1.98 ± 0.08	0.013527	51
SK-ES-1	1.64 ± 0.13	0.01511	46
CHLA-258	1.11 ± 0.06	0.007467	93
COG-E-352	2.58 ± 0.08	0.013299	52

[a]Data, mean ± SEM.

Supplementary Table S6. Pharmacokinetic parameters of entinostat in BALB/c mice following oral administration of entinostat (24.5 mg/kg) for 5 consecutive days*

Pharmacokinetic parameter	**Day 1**	**Day 5**
Elesclomol C_{max} (ng/mL)	12,400	1,920
T_{max} (min)	5	5
$AUC_{0-\infty}$ (min*ng/mL)	690,044	101,058
K_{el} (min^{-1})	0.0217	0.0120
$T_{1/2}$ (min)	32	58
CL_{oral} (mL/min/kg)	36	247

*Data, mean values. C_{max}, maximum concentration; T_{max}, time of maximum concentration; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; K_{el}, elimination rate constant; $T_{1/2}$, half-life; CL_{oral}, oral clearance.

Supplementary Materials and Methods

Real-time qRT-PCR assay

RNA was isolated from cells at ~80% confluency using RNeasy (Qiagen) according to the manufacturer's instructions. cDNA was made using SuperScript™ III Reverse Transcriptase (Invitrogen). Real-time PCR was done using EvaGreen 2X mastermix (BullsEye). Primers are listed as follows. For *EWS-FLI1* detection, Forward Primer EWS1 – 5' GTCAACCTCAATCTAGCACAGGG 3' and Reverse primer FLI1 – 5' CTGTCGGAGAGCAGCTCCAG 3'. For *EWS-ERG* detection, the reverse primer was substituted with ERG1- 5' GAGGGGTACTTGTACAGAGATGA 3'. GAPDH primers were forward 5' TGAACGGGAAGCTCACTGG 3' and reverse 5' TCCACCACCCTGTTGCTGTA 3'. PCR was run on a BioRad CFX 96 under the following conditions: 95°C for 30 seconds, then 40 cycles of 95°C for 15 seconds followed by 64°C for 45 seconds. Expression of the fusion transcript was evaluated after normalization to GAPDH.

Fluorescence in situ hybridization (FISH)

FISH was performed using the EWSR1 (22q12.2) break-apart probe set (Cytocell, Cambridge, UK). Cultured tumor cells were spread on microscope slides. FISH probes were added, the sample with probes were codenatured and hybridized overnight, and washed in stringency buffer. DAPI counterstain was applied and analysis of interphase nuclei was performed using a fluorescent microscope.

Pharmacokinetic *in vivo* studies

The pharmacokinetic studies were approved by the University of Kansas (KU) - Lawrence IACUC. Female BALB/c mice were used for the pharmacokinetic studies of entinostat. The entinostat was dosed by oral gavage at 24.5 mg/kg once daily for 5 consecutive days. The entinostat oral dosing solution was 10 mg/ml and contained 2% DMSO and 30% polyethylene glycol in sterile water for injection. Blood samples for the analysis of entinostat were taken at 5, 15, 30, 60, 90 and 120 minutes on days one and five and at 15, 30 and 60 minutes on days two, three and four. Samples from 3 individual mice were taken at each time point by cardiac puncture. Prior to cardiac puncture, the mice were given inhaled isoflurane to achieve deep anesthesia. Blood samples were held on ice immediately after collection and centrifuged to collect the plasma. Plasma samples were stored at -80 ˚C until they were thawed for assay.

LC-MS/MS analysis for quantitation of entinostat

The LC-MS/MS analytical method for entinostat was modified from a method published by Zhao et al [1]. Plasma samples for entinostat were prepared by the addition of 0.02 ml of plasma to a 1.5 ml Eppendorf centrifuge tube. Acetonitrile (0.07 ml) and 0.03 ml of the internal standard solution (d3-paclitaxel in acetonitrile) were added and the solution was vortexed for 10 seconds. Deionized water (0.1 ml) and n-butyl chloride (0.5 ml) were added and the solution was vortexed for 5 minutes. The solution was frozen in a dry ice IPA bath and the frozen layer was removed and placed in a clean 1.5 ml Eppendorf centrifuge tube. This layer was evaporated to dryness and the resulting solid was reconstituted in 0.1 ml of 1:1 acetonitrile:water and vortexed for 30 seconds followed by ultra-sonification for 5 minutes. The solution (250 µl) was transferred to a polyethylene insert in an HPLC autosampler vial. The HPLC injection volume was 20 µl. QC samples were prepared in an identical manner with a solution of entinostat of a known concentration added to blank mouse plasma.

A Shimadzu HPLC with a Waters X-Terra C18 column and a RP18 guard column was linked with a Sciex 3200QTrap LC-MS/MS system. The mobile phase was a 70/30 v/v acetonitrile / 2 mM ammonium acetate solution containing 0.1% formic acid. An isocratic flow at 0.2 ml/min was used. All QC samples were within FDA guidelines over the range of linearity from 10 to 5,000 ng/ml. The limit of quantitation was 10 ng/ml. The concentration-time profiles were evaluated by using Phoenix WinNonlin software version 6.2 (Certara, St. Louis, MO).

Histology staining

At the time of sacrifice, tumor tissues were collected and weighed. Tumors were either snap frozen in liquid nitrogen or fixed in 10% neutral buffered formalin and paraffin embedded. Tumors were then subjected to hematoxylin and eosin (H&E) staining and immunohistochemical staining. Three major organs (livers, kidneys, and spleens, 6 mice per group) were also collected, fixed and then subjected to H&E staining for histopathological analysis. Photos were captured using Nikon Eclipse 80i microscope equipped with QImaging QIClick™ digital CCD camera. Images were analyzed by the MetaMorph Imaging System (Molecular Devices, CA).

Supplementary References

1. Zhao M, Rudek MA, Mnasakanyan A, Hartke C, Pili R, Baker SD (2007) A liquid chromatography/tandem mass spectrometry assay to quantitate MS-275 in human plasma. J Pharm Biomed Anal 43: 784-787. DOI 10.1016/j.jpba.2006.08.006