

Genome-wide identification of genetic determinants for the cytotoxicity of perifosine

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Abstract

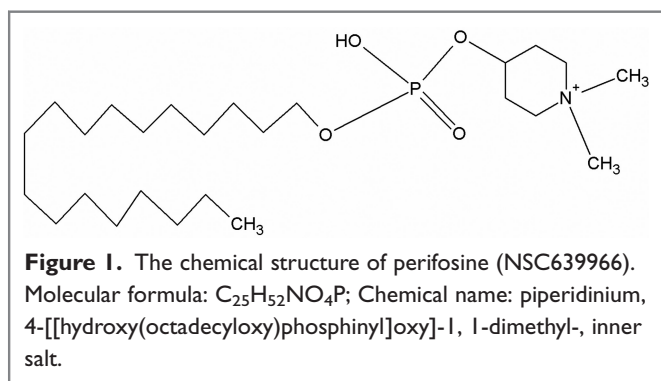
Perifosine belongs to the class of alkylphospholipid analogues, which act primarily at the cell membrane, thereby targeting signal transduction pathways. In phase I/II clinical trials, perifosine has induced tumour regression and caused disease stabilisation in a variety of tumour types. The genetic determinants responsible for its cytotoxicity have not been comprehensively studied, however. We performed a genome-wide analysis to identify genes whose expression levels or genotypic variation were correlated with the cytotoxicity of perifosine, using public databases on the US National Cancer Institute (NCI)-60 human cancer cell lines. For demonstrating drug specificity, the NCI Standard Agent Database (including 171 drugs acting through a variety of mechanisms) was used as a control. We identified agents with similar cytotoxicity profiles to that of perifosine in compounds used in the NCI drug screen. Furthermore, Gene Ontology and pathway analyses were carried out on genes more likely to be perifosine specific. The results suggested that genes correlated with perifosine cytotoxicity are connected by certain known pathways that lead to the mitogen-activated protein kinase signalling pathway and apoptosis. Biological processes such as 'response to stress', 'inflammatory response' and 'ubiquitin cycle' were enriched among these genes. Three single nucleotide polymorphisms (SNPs) located in *CACNA2D1* and *EXOC4* were found to be correlated with perifosine cytotoxicity. Our results provided a manageable list of genes whose expression levels or genotypic variation were strongly correlated with the cytotoxicity of perifosine. These genes could be targets for further studies using candidate-gene approaches. The results also provided insights into the pharmacodynamics of perifosine.

Keywords: perifosine, cytotoxicity, NCI-60, gene expression, genotype

Introduction

Perifosine (NSC639966; Figure 1) belongs to the class of phospholipid analogues or alkylphospholipids, which have anticancer activity in both *in vitro* (cell culture studies) and *in vivo* (animal model-based studies) model systems.^{1,2} Functionally, perifosine resembles natural phospholipids and acts primarily at the cell membrane, thereby targeting

signal transduction pathways. Perifosine has been shown to inhibit, or otherwise modify, signal transduction through a number of different pathways, including mitogen-activated protein kinase (MAPK) and Akt.²⁻⁵ Preclinical studies suggest that perifosine inhibits protein kinase B/Akt phosphorylation and induces *in vitro* and *in vivo* cytotoxicity in cancer cell lines such as multiple myeloma



cells,⁴ HeLa cells³ and prostate carcinoma cells.² Clinical studies have focused on daily oral dosing (after a loading dose), with two partial responses noted in soft tissue sarcoma (STS) patients, including one patient each with chondrosarcoma and leiomyosarcoma,^{6,7} as well as patients with renal cell carcinomas.⁷ Furthermore, the phase II studies in STS patients were not designed to look for disease stabilisation, a potentially important endpoint for drugs targeting signal transduction pathways.⁸

The genetic determinants that are responsible for perifosine's activity have not been comprehensively studied, however. Traditional candidate-gene approaches require *a priori* knowledge and the selection of a small number of candidate genes for hypothesis testing, while an *in silico* genome-wide approach could be used to identify any associated genes as potential candidates in an unsupervised way. The US National Cancer Institute (NCI)-60 resources have allowed genome-wide studies using a panel of 60 human cancer cell lines.⁹ In addition to the genetic determinants, the NCI-60 resources also provide tools such as COMPARE¹⁰ to identify compounds that show correlated cytotoxic patterns with a particular agent. These compounds, for example, could be potential agents for enhancing the response to a candidate drug or as a substitute for that drug.

The NCI-60 human cancer cell lines have been used in anti-cancer drug screens conducted by the NCI since the late 1980s.⁹ The cell lines represent nine distinct tumour types: leukaemia, colon, lung, brain, renal, melanoma, ovarian, breast and prostate. The Developmental Therapeutics Program (DTP) at NCI¹¹ has maintained a database for the cytotoxicity data, as represented by the GI_{50} (the

concentration required to inhibit cell growth by 50 per cent) on >40,000 cytotoxic agents, including perifosine.¹² A handful of gene expression datasets using high-throughput platforms such as the Affymetrix oligonucleotide microarrays and cDNA arrays of the untreated NCI-60 cell lines are now publicly available at the DTP/NCI website (Table 1). Recently, the NCI-60 cell lines were genotyped for ~120,000 single nucleotide polymorphism (SNP) markers using the Affymetrix Human 125K Mapping Array manufacturers details.¹³ By associating gene expression or SNP genotypes in untreated NCI-60 cell lines, investigators have been able to predict the chemosensitivity of various cytotoxic compounds.^{14–16} Here, we report a list of candidate genes whose expression levels or genotypic variation were found to be strongly correlated with the cytotoxicity of perifosine using these publicly available NCI-60 databases. The genes identified could be studied further using a candidate-gene approach. They also could provide new insights into the pharmacodynamics of perifosine.

Materials and methods

Cytotoxicity data

The 60 NCI-60 human cancer cell lines were originally exposed to >40,000 compounds at NCI/NIH and outside laboratories. The growth inhibitory effects of each compound were measured for each cell line and reported as the GI_{50} (for details, see the DTP/NCI website¹⁷) and maintained in the DTP/NCI online databases. Cytotoxicity data on perifosine (NSC639966) and other agents were obtained as the normalised $-\log_{10}[GI_{50}]$ values (released in September 2005). The NSC numbers and common names for the standard agents were retrieved from the DTP/NCI website.

COMPARE analysis

The COMPARE software^{10,18,19} maintained at the DTP/NCI, was used to screen >40,000 synthetic or natural compounds for agents that showed correlated cell growth (GI_{50}) patterns with that of perifosine. COMPARE generates rank-ordered lists of compounds based on the similarities of cytotoxicity

Table 1. NCI-60 microarray expression datasets

Dataset	Institution	Platform	No. of genes on chip	No. of genes analysed ^a
MP-6800	Millenium Pharmaceuticals, Inc.	Affymetrix Human 6800	7,451	2,955
GL-U95	Gene Logic, Inc.	Affymetrix U95	63,175	23,223
NP-U95	Novartis Pharmaceuticals, Inc.	Affymetrix U95A	12,626	10,063
NS-cDNA ^{41,42}	NCI and Stanford University	cDNA array	9,703	5,291

^aGenes or probe sets that had missing data in more than six cell lines (10 per cent) were not included in the analysis datasets. NCI; National Cancer Institute.

patterns. Every compound from one of several specially prepared databases is ranked for similarity of its *in vitro* cell growth pattern to the *in vitro* cell growth pattern of a selected seed or probe compound (ie perifosine). Top-ranking agents based on Pearson correlation coefficient r , whose GI₅₀ patterns correlated with that of perifosine, were reported by the software. To control false correlations due to small sample size, the minimum number of cell lines in common for two compounds to be included in the calculation was 50. We further set the cut-off for COMPARE analysis at $|r| = 0.6$ (equivalent to nominal $p < 0.000001$, assuming 40,000 compounds and $n = 60$, Bonferroni corrected $p < 0.05$).

NCI-60 microarray expression datasets

The NCI-60 microarray expression datasets (released in August 2005) were downloaded from the DTP/NCI Molecular Target Databases.²⁰ These datasets comprise gene expression data on untreated NCI-60 cell lines using different microarray platforms (Table 1). Genes or probe sets that had missing data in more than six cell lines (10 per cent) were not included in the final analysis dataset.

NCI-60 SNP genotypes

The genotype calls for 125,937 SNPs in 58 NCI-60 cell lines were to be downloaded from the DTP/NCI website using the Affymetrix Human 125K Mapping array.¹³ We removed uninformative SNPs, such as those with identical genotypes across all cell lines or those with missing data in more than six cell lines (10 per cent). Only SNP markers with at least two data points per genotype were included in the association studies. This left 34,040 highly informative SNPs in the final analysis dataset. Three exploratory genetic models (additive, dominant, recessive) were used to evaluate the association between genotype and cytotoxicity. Given the genotypes of a SNP marker (AA, AB, BB), the genotypes were encoded as (AA = 0, AB = 1, BB = 2) in the additive model, (AA/AB = 1, BB = 0) in the dominant model and (AA/AB = 0, BB = 1) in the recessive model.

Identifying associated copy number alterations

Data on copy number alterations in the NCI-60 cell lines as reported by Garraway and colleagues¹³ were downloaded from the DTP/NCI website.

Table 2. Agents correlated with the GI₅₀ values of perifosine, as reported by COMPARE ($r > 0.6$)

NSC#	r	Chemical name
605583	0.81	Miltefosin C; choline, hexadecyl hydrogen phosphate, inner salt
643826	0.75	Choline, hydroxide, 3-methoxy-2-[methyl(octadecyl)amino]propyl hydrogen phosphate, inner salt
643828	0.68	Choline, hydroxide, 2-methoxy-3-[methyl(octadecyl)amino]propyl hydrogen phosphate, inner salt
324368	0.68	Edelfosine; 1-octadecyl-2-methylphosphorylcholine
643827	0.68	Choline, hydroxide, 3-methoxy-1-[methyl(octadecyl)amino]-2-propyl hydrogen phosphate, inner salt
18268	0.65	Actinomycin D
678144	0.62	4H-1,3,6,2-dioxazaphosphocinium, 4-hexadecyltetrahydro- 2,6,6-trimethyl-, bromide, 2-oxide
337591	0.62	ES 12H; choline, hydroxide, 3-(dodecyloxy)propyl hydrogen phosphate, inner salt
87222	0.62	Actinomycin C3
266763	0.61	2-Propenamide, N-[2-(decylsulfinyl)-1-(hydroxymethyl)ethyl]-3-(1,2,3,4-tetrahydro-6-methyl-2,4-dioxo-5-pyrimidinyl)-
207895	0.60	Benzofurazan, 4-(4-methyl-1-piperazinyl)-7-nitro-, 3-oxide

Linear regression model

We performed genome-wide associations between the gene expression (or genotype) and cytotoxicity data. Pearson correlation coefficients and the associated p -values were computed using a linear regression model, which was implemented as the `lm` function in the R Statistical Package.²¹ Specifically, the cytotoxicity, as represented by $-\log_{10}[\text{GI}_{50}]$, was modelled as dependent on either gene expression or genotype. To adjust for multiple tests, the false discovery rate (FDR) was controlled using the Benjamini and Hochberg step-up FDR procedure²² (FDR_{BH}). An FDR cut-off of 10 per cent was used to identify candidates for further analyses.

Associations with standard agents

Associations between the identified genes and the cytotoxicity data on the 171 anti-cancer agents in the NCI Standard Agent Database²³ were performed to evaluate perifosine specificity for our gene list. The standard agents cover a variety of mechanisms, besides being phospholipid analogues, and were originally determined by Boyd.²⁴ The same cut-off ($\text{FDR}_{\text{BH}} < 0.10$) was used to determine if an identified gene was associated significantly with any standard agents. The genes that

showed no significant associations with any of the 171 standard agents using any dataset were denoted 'perifosine specific'. Genes that showed associations with any of the 171 standard agents using any dataset were denoted 'non-specific'.

Gene ontology and pathway analyses

We used Onto-Express and Pathway-Express^{25–27} to search enriched biological processes and known physiological pathways among the perifosine-specific genes from the Gene Ontology (GO)²⁸ and Kyoto Encyclopaedia of Genes and Genomes (KEGG) databases.^{29,30} GO terms or KEGG pathways that were over-represented relative to the corresponding analysis sets (two hits or more, binomial test at $\text{FDR}_{\text{BH}} < 0.05$) were called 'enriched' in our gene list.

STS expression database

The identified perifosine-specific genes were queried against a STS expression database, which characterised eight gastrointestinal stromal tumours, eight monophasic synovial sarcomas, four liposarcomas, one myxoid, 11 leiomyosarcomas, eight malignant fibrous histiocytomas and two benign peripheral nerve sheath tumours (Schwannoma).³¹

Table 3a. Genes with expression levels specifically associated with the cytotoxicity of perifosine ($FDR_{BH} < 0.10$)

Gene symbol	Gene title	r^a	p	Response
GL-U95				
REG4	Regenerating islet-derived family, member 4	-0.58	1.3E-06	Sensitivity
SLCO4A1	Solute carrier organic anion transporter family, member 4A1	-0.52	2.3E-05	Sensitivity
RPL18A	Ribosomal protein L18a	-0.50	4.7E-05	Sensitivity
OAZ2	Ornithine decarboxylase antizyme 2	0.53	1.6E-05	Resistance
DZIP3	Zinc finger DAZ-interacting protein 3	0.58	1.5E-06	Resistance
NP-U95				
STK39	Serine threonine kinase 39 (STE20/SPS1 homologue, yeast)	-0.48	1.2E-04	Sensitivity
FAM32A	Family with sequence similarity 32, member A	-0.46	2.5E-04	Sensitivity
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3	-0.45	3.0E-04	Sensitivity
RAB8A	RAB8A, member Ras oncogene family	-0.44	4.7E-04	Sensitivity
STK17B	Serine/threonine kinase 17b (apoptosis-inducing)	-0.44	5.8E-04	Sensitivity
TCF3	Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	-0.44	5.8E-04	Sensitivity
PARP4	Poly (ADP-ribose) polymerase family, member 4	-0.43	6.1E-04	Sensitivity
PSMA2	Proteasome (prosome, macropain) subunit, alpha type, 2	-0.43	6.3E-04	Sensitivity
DGKE	Diacylglycerol kinase, epsilon 64 kDa	-0.43	6.9E-04	Sensitivity
PVT1	Pvt1 oncogene homologue, MYC activator (mouse)	0.42	1.0E-03	Resistance
ELOVL2	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 2	0.42	9.5E-04	Resistance
SMARCA3	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 3	0.42	9.3E-04	Resistance
USP6	TL132 protein	0.43	7.1E-04	Resistance
IGF1R	Insulin-like growth factor 1 receptor	0.44	5.3E-04	Resistance

Continued

Table 3a. Continued

Gene symbol	Gene title	r^a	p	Response
NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	0.44	5.4E-04	Resistance
POU4F1	POU domain, class 4, transcription factor 1	0.44	5.1E-04	Resistance
PDLIM3	PDZ and LIM domain 3	0.44	5.0E-04	Resistance
CBS	Cystathionine beta-synthase	0.44	4.4E-04	Resistance
ARMCX2	Armadillo repeat containing, X-linked 2	0.45	3.9E-04	Resistance
OPHN1	Oligophrenin 1	0.46	2.5E-04	Resistance
ZNF609	Zinc finger protein 609	0.46	2.3E-04	Resistance
ATN1	Atrophin 1	0.47	1.5E-04	Resistance
DZIP3	Zinc finger DAZ-interacting protein 3	0.48	1.3E-04	Resistance
PPBPL2	Pro-platelet basic protein-like 2	0.49	9.3E-05	Resistance
MPDZ	Multiple PDZ domain protein	0.53	1.3E-05	Resistance
SKIV2L	Superkiller viralicidic activity 2-like (<i>Saccharomyces cerevisiae</i>)	0.56	4.6E-06	Resistance
GABRG3	Gamma-aminobutyric acid (GABA) A receptor, gamma 3	0.60	4.9E-07	Resistance
NS-cDNA				
ATF2	Activating transcription factor 2	-0.56	4.8E-06	Sensitivity
TRA2A	Transformer-2 alpha	-0.44	8.3E-04	Sensitivity
ETS2	V-ets erythroblastosis virus E26 oncogene homologue 2 (avian)	-0.44	5.3E-04	Sensitivity
UBE2D3	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homologue, yeast)	-0.42	8.5E-04	Sensitivity
ANP32A	Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	0.49	1.6E-04	Resistance

^aPearson correlation coefficients were calculated by linear regression in which cytotoxicity ($-\log_{10}[GI_{50}]$) was dependent on gene expression. A positive r -value indicates that a gene is correlated with resistance, while a negative r -value indicates that a gene is correlated with sensitivity.

Genes differentially expressed among different sarcomas were provided by the database using significance analysis of microarrays (SAM).³²

Results

COMPARE analysis

At $p < 0.05$ after Bonferroni correction, the COMPARE software^{10,18} identified 24 agents with

positive correlation with the cytotoxicity pattern of perifosine. By contrast, no agents with significant negative correlation were identified. Table 2 shows some top-ranking agents ($r > 0.6$) with well-characterised chemical names. Among them, some clearly belong to the same drug class as perifosine: miltefosine (NSC605583, $r = 0.81$) and edelfosine (NSC324368, $r = 0.68$). Edelfosine was further used as a representative of phospholipid analogues

Table 3b. SNPs associated with the cytotoxicity of perifosine ($FDR_{BH} < 0.10$)

dbSNP ^a	Gene locus	Location	r	p	Model
rs4236669	CACNA2D1	Intron	0.64	2.8E-07	Recessive
rs1468400	CACNA2D1	Intron	0.62	8.8E-07	Recessive
rs1345938	EXOC4	Intron	0.58	2.6E-06	Recessive

^adbSNP Build 126 (May, 2006).

to verify the associations detected from perifosine (Supplementary Table 1).

Genes with expression associated with perifosine cytotoxicity and GO and pathway analyses

Table 3a lists the perifosine-specific genes identified from the microarray expression datasets. The non-specific genes are listed in Supplementary Table 2. The GO and pathway analyses were then carried out to find any enriched biological processes and known KEGG pathways among the perifosine-specific genes (Table 4).

Table 4. Enriched Gene Ontology biological processes among the perifosine-specific genes

GO ID	Process	p	Gene symbol
<i>NP-U95</i>			
GO:0006950	Response to stress	3.8E-04	STK39 MAPKAPK3
GO:0006511	Ubiquitin-dependent protein catabolism	7.9E-04	PSMA2 USP6
GO:0006954	Inflammatory response	4.3E-03	NFATC4 PARP4
GO:0006512	Ubiquitin cycle	6.5E-03	DZIP3 USP6
GO:0006366	Transcription from RNA polymerase II promoter	7.8E-03	PPBPL2 NFATC4
<i>NS-cDNA</i>			
GO:0006355	Regulation of transcription, DNA-dependent	5.6E-03	EST2 ATF2

At $FDR_{BH} < 0.10$, no genes were associated with perifosine cytotoxicity using the MP-6800 dataset, although at a more lenient cutoff ($FDR_{BH} < 0.25$), one gene, *FABP5* (encoding fatty acid binding protein 5), could be described as being significantly correlated with the sensitivity response to perifosine. The expression of *FABP5* was denoted as non-specific, as it was also associated with one standard agent. For the two Affymetrix U95 series of microarray datasets (GL-U95 and NP-U95), one gene, *DZIP3* (encoding zinc finger DAZ-interacting protein 3), was correlated with the resistance response to perifosine using both datasets ($FDR_{BH} < 0.10$). *DZIP3* was denoted as perifosine specific, as it showed no associations with any standard agents. In total, ten genes were found to be correlated with perifosine cytotoxicity ($FDR_{BH} < 0.10$) using the GL-U95 dataset: five each with sensitivity and resistance. Of these, five did not show associations with any standard agents. The GO biological process 'ubiquitin cycle' was enriched among all ten genes (two hits or more, binomial test at $FDR_{BH} < 0.05$); however, it was not significant among the five perifosine-specific genes. No KEGG pathways were enriched among the identified genes. By contrast, 79 genes were found to be correlated with perifosine cytotoxicity ($FDR_{BH} < 0.10$) in the NP-U95 dataset. Among them, 30 genes were correlated with sensitivity, while 49 genes were correlated with resistance. Five GO biological processes were enriched among the 27 perifosine-specific genes (two hits or more, binomial test, $FDR_{BH} < 0.05$). No KEGG pathways were enriched among the identified genes. Using the NS-cDNA dataset, 23 genes were identified, with significant associations with perifosine cytotoxicity ($FDR_{BH} < 0.10$).

Among them, 12 genes were correlated with sensitivity and 11 genes were correlated with resistance. One GO biological process, 'DNA-dependent regulation of transcription', was enriched among the five perifosine-specific genes. No KEGG pathways were enriched among the identified genes.

SNPs associated with perifosine cytotoxicity

Three SNPs under the recessive model were found to be significantly correlated with the resistance response to perifosine ($FDR_{BH} < 0.10$; Table 3b, Figure 2). These included two SNPs located in the introns of *CACNA2D1* (calcium channel, voltage-dependent, alpha 2/delta subunit 1). The third SNP is located in an intron of *EXOC4* (exocyst complex component 4). Using both additive and dominant models, these three SNPs did not show significant associations with any standard agents. By contrast, rs1468400 in *CACNA2D1* was correlated with one standard agent under the recessive model.

Copy number alterations and perifosine cytotoxicity

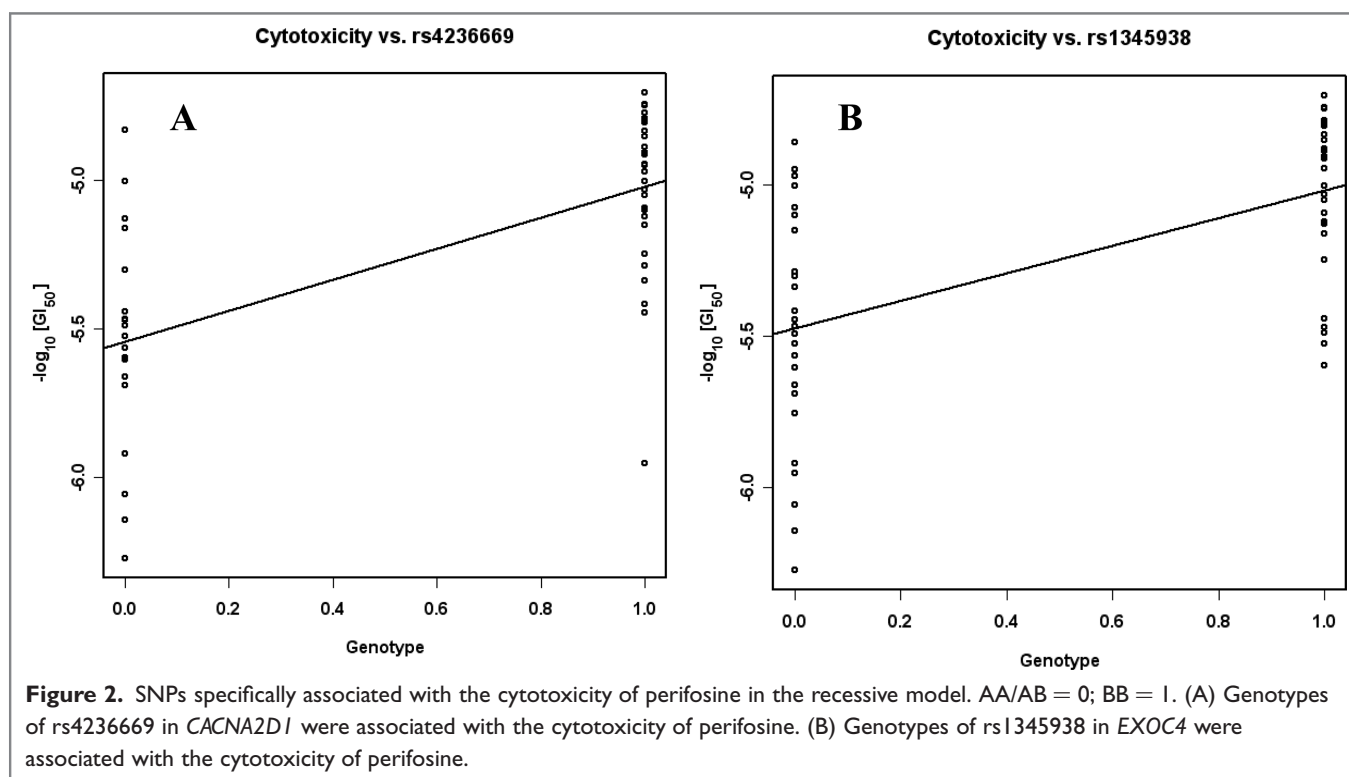
At $FDR_{BH} < 0.10$, no copy number alterations or gene amplifications were found to be correlated with perifosine cytotoxicity.

Querying gene expression patterns in STS cells

Perifosine-specific genes in Table 3a were queried against the STS expression database.³¹ Genes that are either up- or downregulated in each type of tumour are listed in Supplementary Table 3. Six genes (*STK17B*, *IGF1R*, *POU4F1*, *CBS*, *MPDZ*, *EST2*) were included in the database. With the exception of *EST2*, the other five genes were found to be up- or downregulated in certain STS cells.

Discussion

We performed a genome-wide analysis to identify genes whose expression levels were significantly

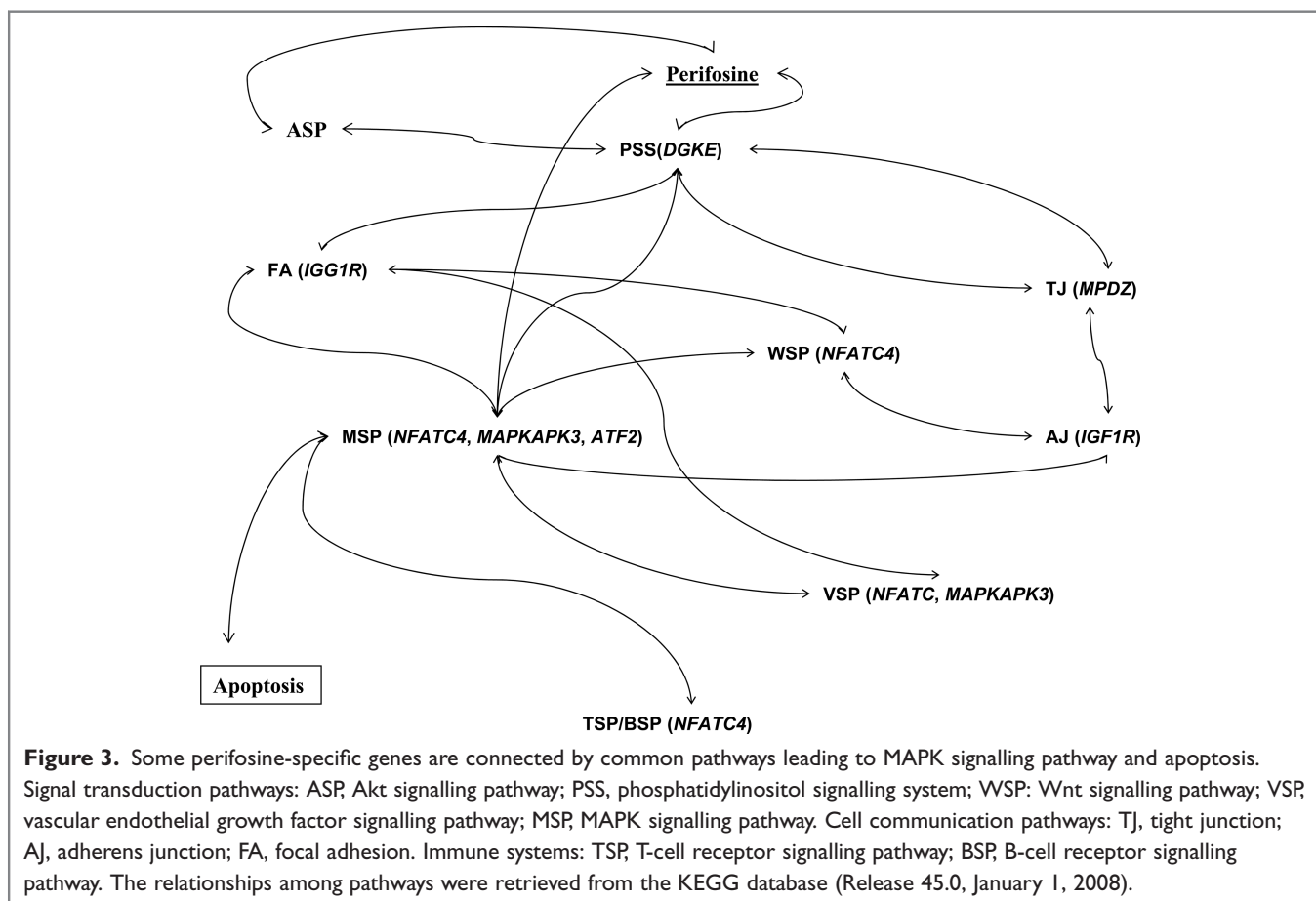


associated with perifosine's activity, as represented by its cytotoxicity (GI_{50}). Four independent gene expression datasets of untreated NCI-60 cancer cell lines (Table 1), using different microarray platforms, were used to evaluate the association between cytotoxicity and gene expression. We further focused on the identified genes that are more likely to be perifosine specific (Table 3). Previous studies, using traditional candidate-gene approaches, have suggested that perifosine inhibits, or otherwise modifies, signal transduction through a number of different pathways, including MAPK and Akt.²⁻⁴ An *in silico* genome-wide scan without *a priori* knowledge in this work provided more candidate genes in an unsupervised way.

The use of COMPARE^{10,18} allowed us to identify compounds that have similar cell growth patterns with perifosine (Table 2). To limit the effects due to factors such as small sample size and multiple comparisons, we took measures to control potential false positives. Compounds including those belonging to the same drug class as perifosine (such as miltefosine and edelfosine) were among the top-ranking agents with strong positive correlation coefficients ($r > 0.6$, $p < 0.05$ after Bonferroni correction). Not surprisingly, a majority of the perifosine-specific genes were also significantly associated (nominal $p < 0.05$) with edelfosine, which was used to represent phospholipid analogues (Supplementary Table 1). The remaining few genes showed at least marginal associations (nominal $p < 0.10$) with edelfosine. This suggests that our list of perifosine-specific genes also contains a set of common genes that determines the pharmacodynamics of this drug class. To our knowledge, this is the most comprehensive list of associated genes for phospholipid analogues. The COMPARE program also retrieved drugs acting through different mechanisms (Table 2). The shared cytotoxicity profiles could be explained by the common pathways between these drugs and perifosine. For example, the correlation with actinomycin, which inhibits transcription by binding DNA at the transcription initiation complex and preventing elongation by

RNA polymerase,³³ could be explained via general transcriptional modulation (Table 4).

We wanted to know the interactions among the perifosine-specific genes with known biological processes or pathways. Searches against the GO and KEGG databases identified six biological processes that were enriched among the perifosine-specific genes (Table 4). Among them, the biological process of the ubiquitin cycle was identified with *DZIP3* and *USP6*. Notably, *DZIP3* was significantly associated with resistance to perifosine, using two of the Affymetrix U95 series of arrays (Table 3a). The function of *DZIP3*, a ubiquitin ligase,³⁴ in the pharmacodynamics of perifosine has not been investigated, although, given the potential of ubiquitin ligases as anti-cancer targets,^{35,36} *DZIP3* and the role of ubiquitin-dependent protein degradation could be an interesting candidate for further studies. The perifosine-specific genes also over-represented such biological processes as 'response to stress' and 'inflammatory response', which are more evidently related to drug response. Although no particular known KEGG pathways were found to be enriched among the perifosine-specific genes, many of these genes could be connected by a network of known physiological pathways (Figure 3) which have interactions with perifosine through known mechanisms that lead to the MAPK signalling pathway and apoptosis. For example, perifosine can affect the phosphatidylinositol signalling pathway, Akt signalling pathway and MAPK signalling pathway.^{37,38} Some of our identified perifosine-specific genes are known to be involved in these pathways; for example, *DGKE* (the phosphatidylinositol signalling pathway) and *MAPKAPK3* (the MAPK signalling pathway). Furthermore, the gene product of *DGKE* is involved in the phosphatidylinositol signalling system pathway and interacts with the phosphatidylinositol 3-kinase/phosphatase and tensin homologue deleted on chromosome 10 (PTEN)/Akt pathways,³⁰ suggesting its potential role in the perifosine response. The connected pathways can be divided into three categories:²⁹ cell communication (tight junction, adherens junction and focal adhesion); immune systems (T/B cell receptor



signalling pathways); and signal transduction (MAPK, Wnt, vascular endothelial growth factor and phosphatidylinositol signalling pathways). Given the fact that perifosine, as well as edelfosine, significantly affects the pathway of extrinsic apoptosis,^{38–40} our findings showed that while perifosine was involved in such pathways as the MAPK and phosphatidylinositol signalling pathways that can lead to apoptosis,^{2–4} it could also influence other interconnected pathways, such as those in cell communication.

Variation in DNA sequence is partially responsible for gene expression;^{41,42} therefore, we performed an association test between SNP genotypes¹³ and the cytotoxicity of perifosine. Different models (additive, dominant and recessive) were used to explore the genetic relationships between genotypes and cytotoxicity. Two SNPs (rs4236669 in *CACNA2D1* and rs1345938 in

EXOC4) showed strong perifosine-specific associations under the recessive model (Figure 2). Since the expression of *CACNA2D1* was not found to be significantly correlated with perifosine cytotoxicity, the relationship between gene expression and its genotypes is not straightforward. Given that *CACNA2D1* is involved in the MAPK signalling pathway,²⁹ however, these SNPs could be interesting candidates for further studies.

Studies have shown that alkylphospholipids are a class of anti-cancer agents that perturb signal transduction pathways through inhibition of MAPK and Akt phosphorylation.^{2–4} These drugs have shown consistent clinical anti-cancer activity, but their systemic application has been limited by toxicity. Therefore, one impact of our list of genes could be to help to identify better targeted cancer types for perifosine. One potential candidate, for example, could be multiple myeloma, given the

fact that the *PSMA2* gene (associated with the sensitivity response to perifosine; Table 3a) was found to be highly upregulated in multiple myeloma cells.⁴³ In fact, perifosine activity has been reported in myeloma preclinically.^{4,39} A recent multicentre phase II study of perifosine alone and in combination with dexamethasone for patients with relapsed or relapsed/refractory multiple myeloma suggested promising activity (eg stabilisation of disease) as combination therapy, with manageable toxicity.⁴⁴ Our results thus warrant further clinical trials for this tumour type. There is some evidence of perifosine having activity in STS, with responses reported in chondrosarcoma and leiomyosarcoma.^{6,7} Based on these studies, continued assessment of perifosine in STS also appears to be warranted. Given the heterogeneity of STS, it is a plausible hypothesis that there is an identifiable subset of tumours that will respond to this agent.⁴⁵ A search against a STS expression database³¹ further indicated that a type of leiomyosarcomas that does not express calponin showed the best correlated pattern of gene expression with our perifosine-specific genes (Supplementary Table 4). For example, *STK17B* (associated with the sensitivity response to perifosine; Table 3a) is significantly upregulated in this tumour type, while *POU4F1* and *MPDZ* (associated with the resistance response to perifosine; Table 3a) are significantly downregulated in this tumour type, suggesting that this type of leiomyosarcoma could be a better target for perifosine. As the available STS expression dataset contains only ~5,000 genes,³¹ a more comprehensive dataset could provide more insights.

In summary, we used the public NCI-60 resources to identify a list of genes potentially relevant to the cytotoxicity of perifosine. Although there were some limitations; such as the gene coverage of the current microarray platforms, relatively small sample size of 60 cell lines and severity of multiple comparisons, our results not only confirmed that perifosine is involved in some known pathways (eg MAPK signalling) that can lead to apoptosis, but also suggested that it could influence some new candidate genes and pathways. Our

unsupervised *in silico* analyses, therefore, could provide targeted candidates that are globally associated with the perifosine response for further studies.

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Supplementary Table 1a. A majority of perifosine-specific gene expression levels are associated with the cytotoxicity of edelfosine

Symbol	Perifosine (p-value)	Edelfosine (p-value)	Perifosine (r-value)	Edelfosine (r-value)	Notes
NS-cDNA					
ATF2	0.0000	0.0075	-0.560	-0.348	Significant
TRA2A	0.0008	0.0005	-0.438	-0.456	Significant
ETS2	0.0005	0.0004	-0.438	-0.449	Significant
UBE2D3	0.0009	0.0026	-0.419	-0.382	Significant
VEGFB	0.0006	0.0001	0.443	0.502	Significant
ANP32A	0.0002	0.0003	0.492	0.469	Significant
GL-U95					
REG4	0.0000	0.0004	-0.579	-0.443	Significant
SLCO4A1	0.0000	0.0060	-0.518	-0.351	Significant
RPL18A	0.0000	0.0093	-0.500	-0.333	Significant
OAZ2	0.0000	0.0018	0.527	0.394	Significant
DZIP3	0.0000	0.0001	0.580	0.478	Significant
NP-U95					
STK39	0.0001	0.0004	-0.479	-0.449	Significant
FAM32A	0.0002	0.0529	-0.460	-0.253	Marginal
MAPKAPK3	0.0003	0.0920	-0.454	-0.221	Marginal
RAB8A	0.0005	0.0018	-0.441	-0.399	Significant
STK17B	0.0006	0.0243	-0.435	-0.293	Significant
TCF3	0.0006	0.0054	-0.435	-0.358	Significant
PARP4	0.0006	0.0000	-0.433	-0.519	Significant
PSMA2	0.0006	0.0060	-0.432	-0.354	Significant
DGKE	0.0007	0.0019	-0.429	-0.396	Significant
PVT1	0.0010	0.0018	0.418	0.398	Significant
ELOVL2	0.0009	0.0005	0.419	0.438	Significant
SMARCA3	0.0009	0.0038	0.420	0.371	Significant
USP6	0.0007	0.0094	0.428	0.335	Significant
NFATC4	0.0005	0.0305	0.437	0.282	Significant
IGF1R	0.0005	0.0570	0.437	0.249	Marginal
POU4F1	0.0005	0.0023	0.439	0.390	Significant

Continued

Supplementary Table 1a. Continued

Symbol	Perifosine (<i>p</i> -value)	Edelfosine (<i>p</i> -value)	Perifosine (<i>r</i> -value)	Edelfosine (<i>r</i> -value)	Notes
<i>PDLIM3</i>	0.0005	0.0058	0.440	0.355	Significant
<i>CBS</i>	0.0004	0.0094	0.443	0.336	Significant
<i>ARMCX2</i>	0.0004	0.0008	0.447	0.425	Significant
<i>OPHN1</i>	0.0003	0.0194	0.459	0.304	Significant
<i>ZNF609</i>	0.0002	0.0091	0.462	0.337	Significant
<i>ATN1</i>	0.0001	0.0754	0.474	0.233	Marginal
<i>DZIP3</i>	0.0001	0.0093	0.479	0.336	Significant
<i>PPBPL2</i>	0.0001	0.0081	0.487	0.342	Significant
<i>MPDZ</i>	0.0000	0.0001	0.534	0.498	Significant
<i>SKIV2L</i>	0.0000	0.0007	0.557	0.427	Significant
<i>GABRG3</i>	0.0000	0.0000	0.601	0.533	Significant

Supplementary Table 1b. The perifosine-specific SNPs are associated with the cytotoxicity of edelfosine

dbSNP	Perifosine (<i>p</i> -value)	Perifosine (<i>r</i> -value)	Edelfosine (<i>p</i> -value)	Edelfosine (<i>r</i> -value)	Notes
rs4236669	2.80E-07	0.64	1.75E-03	0.42	Significant
rs1468400	8.80E-07	0.62	6.27E-04	0.46	Significant
rs1345938	2.60E-06	0.58	3.66E-05	0.52	Significant

Supplementary Table 2. Genes whose expression levels were associated with the cytotoxicity of perifosine ($FDR_{BH} < 0.10$) but were not perifosine specific

Symbol	Gene title	<i>r</i>	<i>p</i>	Control total ^a
GL-U95				
<i>FNBP3</i>	Formin-binding protein 3	-0.53	3.5E-05	75
<i>MOBK2A</i>	MOB1, Mps One Binder kinase activator-like 2A (yeast)	-0.50	5.5E-05	43
<i>TP53INP2</i>	Tumour protein p53 inducible nuclear protein 2	0.51	3.9E-05	24
<i>FBXO44</i>	F-box protein 44	0.52	2.4E-05	53
<i>TMF1</i>	TATA element modulatory factor 1	0.53	1.7E-05	57
NP-U95				
<i>HNRPDL</i>	Heterogeneous nuclear ribonucleoprotein D-like	-0.49	6.9E-05	3
<i>DDX39</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	-0.49	7.3E-05	22
<i>MRPL23</i>	Mitochondrial ribosomal protein L23	-0.49	8.7E-05	2
<i>RPS24</i>	Ribosomal protein S24	-0.47	1.9E-04	21
<i>LBR</i>	Lamin B receptor	-0.47	2.0E-04	73
<i>ERCC5</i>	Excision repair cross-complementing rodent repair deficiency, complementation group 5	-0.47	2.0E-04	11
<i>HDAC1</i>	Histone deacetylase 1	-0.46	2.2E-04	2
<i>GTF3A</i>	General transcription factor IIIA	-0.45	3.1E-04	11
<i>EEF1B2</i>	Eukaryotic translation elongation factor 1 beta 2	-0.44	4.3E-04	68
<i>ICAM3</i>	Intercellular adhesion molecule 3	-0.44	5.1E-04	43
<i>SNRPF</i>	Small nuclear ribonucleoprotein polypeptide F	-0.44	5.2E-04	83
<i>SH2D1A</i>	SH2 domain protein 1A, Duncan's disease (lymphoproliferative syndrome)	-0.44	5.8E-04	61
<i>KIR3DL1</i>	Killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	-0.43	7.3E-04	76
<i>RPL35</i>	Ribosomal protein L35	-0.42	8.1E-04	47
<i>NUPL2</i>	Nucleoporin-like 2	-0.42	8.8E-04	1
<i>PTMA</i>	Prothymosin, alpha (gene sequence 28)	-0.42	9.0E-04	33
<i>CORO1A</i>	Coronin, actin-binding protein, 1A	-0.42	9.3E-04	104

Continued

Supplementary Table 2. Continued

Symbol	Gene title	r	p	Control total ^a
<i>LCN1</i>	Lipocalin 1 (tear prealbumin)	-0.42	9.3E-04	21
<i>POLE3</i>	Polymerase (DNA directed), epsilon 3 (p17 subunit)	-0.42	9.2E-04	31
<i>RPS27A</i>	Ribosomal protein S27a	-0.42	9.4E-04	79
<i>TRIM14</i>	Tripartite motif-containing 14	-0.42	9.3E-04	60
<i>LHFPL2</i>	Lipoma HMGIC fusion partner-like 2	0.42	1.0E-03	99
<i>DOK5</i>	Docking protein 5	0.42	9.5E-04	1
<i>EIF4G1</i>	Eukaryotic translation initiation factor 4 gamma, 1	0.42	9.6E-04	28
<i>RGS19</i>	Regulator of G-protein signalling 19 interacting protein 1	0.42	9.2E-04	116
<i>COPB2</i>	Coatomer protein complex, subunit beta 2 (beta prime)	0.42	8.9E-04	1
<i>TLE2</i>	Transducin-like enhancer of split 2 (E(sp1) homologue, <i>Drosophila</i>)	0.42	9.0E-04	4
<i>ITGA7</i>	Integrin, alpha 7	0.42	8.6E-04	13
<i>SEMA3C</i>	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	0.42	8.2E-04	22
<i>S100A13</i>	S100 calcium binding protein A13	0.43	7.8E-04	88
<i>FLOT1</i>	Flotillin 1	0.43	6.5E-04	2
<i>MLF1</i>	Myeloid leukemia factor 1	0.43	6.0E-04	11
<i>ARHGEF11</i>	Rho guanine nucleotide exchange factor (GEF) 11	0.44	5.5E-04	9
<i>COL15A1</i>	Collagen, type XV, alpha 1	0.44	4.9E-04	1
<i>DAG1</i>	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	0.44	4.9E-04	118
<i>IFNA14</i>	Interferon alpha 14	0.44	4.8E-04	2
<i>PHLDB1</i>	Pleckstrin homology-like domain, family B, member 1	0.44	4.5E-04	12
<i>PTPRS</i>	Protein tyrosine phosphatase, receptor type S	0.45	4.0E-04	2
<i>SASH1</i>	SAM and SH3 domain containing 1	0.45	4.1E-04	52
<i>ACVR1B</i>	Activin A receptor, type 1B	0.45	3.2E-04	18
<i>CTNNA1</i>	Catenin (cadherin-associated protein), alpha 1, 102 kDa	0.46	3.0E-04	30

Continued

Supplementary Table 2. Continued

Symbol	Gene title	<i>r</i>	<i>p</i>	Control total ^a
<i>IL6ST</i>	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	0.46	2.9E-04	68
<i>ATP6VID</i>	ATPase, H ⁺ transporting, lysosomal 34kDa, VI subunit D	0.46	2.5E-04	2
<i>SUOX</i>	Sulphite oxidase	0.46	2.1E-04	58
<i>TFAP2A</i>	Transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	0.48	1.3E-04	51
<i>GRINA</i>	Glutamate receptor, ionotropic, N-methyl D-aspartate-associated protein 1 (glutamate binding)	0.49	9.3E-05	1
<i>ABCB6</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 6	0.49	8.2E-05	8
<i>CTSF</i>	Cathepsin F	0.49	7.4E-05	9
<i>VEGFB</i>	Vascular endothelial growth factor B	0.50	4.6E-05	6
<i>GGCX</i>	Gamma-glutamyl carboxylase	0.52	2.1E-05	23
<i>LAPTM4B</i>	Lysosomal-associated protein transmembrane 4 beta	0.53	1.6E-05	84
<i>FYN</i>	Sialidase 1 (lysosomal sialidase)	0.55	6.3E-06	59
NS-cDNA				
<i>LBR</i>	Lamin B receptor	-0.47	1.3E-04	1
<i>TRA2A</i>	Transformer-2 alpha	-0.44	8.3E-04	13
<i>ETS2</i>	V-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	-0.44	5.3E-04	30
<i>UBE2D3</i>	Ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homologue, yeast)	-0.42	8.5E-04	4
<i>RRM1</i>	Ribonucleotide reductase M1 polypeptide	-0.41	9.8E-04	1
<i>ATP6VIC1</i>	ATPase, H ⁺ transporting, lysosomal 42kDa, VI subunit C, isoform 1	0.42	7.7E-04	51
<i>VEGFB</i>	Vascular endothelial growth factor B	0.44	6.3E-04	57
<i>RDX</i>	Radixin	0.45	4.2E-04	29
<i>APOD</i>	Apolipoprotein D	0.45	3.1E-04	3
<i>PTMS</i>	Parathymsin	0.47	1.8E-04	2

^aThe total number of associated standard agents (see Methods).

Supplementary Table 3. Perifosine-specific genes whose expression levels are up- or downregulated in STS

Gene	r^a	Calponin-positive leiomyosarcoma ^b	Calponin-negative leiomyosarcoma ^b	GIST ^b	Synovial sarcoma ^b	Liposarcoma ^b	MFH ^b	Schwannoma ^b
STK17B	-0.44	Downregulated	Upregulated					
IGF1R	0.44	Upregulated						
POU4F1	0.44	Downregulated	Downregulated		Upregulated			Downregulated
CBS	0.44	Upregulated				Downregulated		
MPDZ	0.53	Upregulated	Downregulated					
ESTZ	-0.44							

^aCorrelation coefficients (see Table 3 in the text).^bSTS type (see Nielsen et al. 2002).³¹