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Oligoamine analogues in combination with 2-difluoromethylornithine synergistically induce re-expression of aberrantly silenced tumour-suppressor genes

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Epigenetic gene silencing is an important mechanism in the initiation and progression of cancer. Abnormal DNA CpG island hypermethylation and histone modifications are involved in aberrant silencing of tumour-suppressor genes. LSD1 (lysine-specific demethylase 1) was the first enzyme identified to specifically demethylate H3K4 (Lys[4] of histone H3). Methylated H3K4 is an important mark associated with transcriptional activation. The flavin adenine dinucleotide-binding amine oxidase domain of LSD1 is homologous with two polyamine oxidases, SMO (spermine oxidase) and APAO (N^1-acetylpolyamine oxidase). We have demonstrated previously that long-chain polyamine analogues, the oligoamines, are inhibitors of LSD1. In the present paper we report the synergistic effects of specific oligoamines in combination with DFMO (2-difluoromethylornithine), an inhibitor of ornithine decarboxylase, in human colorectal cancer cells. DFMO treatment depletes natural polyamines and increases the uptake of exogenous polyamines. The combination of oligoamines and DFMO results in a synergistic re-expression of aberrantly silenced tumour-suppressor genes, including *SFRP2* (secreted frizzled-related protein 2), which encodes a Wnt signalling pathway antagonist and plays an anti-tumorigenic role in colorectal cancer. The treatment-induced re-expression of *SFRP2* is associated with increased H3K4me2 (di-methyl H3K4) in the gene promoter. The combination of LSD1-inhibiting oligoamines and DFMO represents a novel approach to epigenetic therapy of cancer.

Key words: epigenetic, histone, lysine-specific demethylase 1 (LSD1), ornithine decarboxylase, polyamine.

INTRODUCTION

Aberrant epigenetic silencing of gene expression is an important mechanism in the genesis and progression of cancer [1]. Epigenetic changes in chromatin, including DNA methylation of gene promoter region CpG islands, collaborate with histone modifications, including methylation, acetylation and phosphorylation, to regulate gene expression. The acetylation of histones is generally associated with active gene transcription [2]. However, the role of histone methylation is context dependent. Typically, methylation at H3K4 (Lys[4] of histone 3), H3K36 and H3K79 is involved in active transcription, whereas methylation at H3K9 (Lys[9] of histone H3) and H3K27 (Lys[27] of histone H3) is associated with transcriptional silencing [3]. Histone methylation was considered an irreversible process until the discovery of LSD1 (lysine-specific demethylase 1) [4]. LSD1 (also named AOF2, BHC110, KDM1 and KIAA0601) interacts with the co-repressor complex CoREST (repressor element 1-silencing transcription factor co-repressor) and HDAC1/2 (histone deacetylase 1/2) and is able to demethylate H3K4me1 (mono-methyl H3K4) and H3K4me2 (di-methyl H3K4) on nucleosomes [5]. LSD1 is a FAD-dependent amine oxidase and its catalytic domain has over 60 % amino acid sequence similarity with two important enzymes in polyamine catabolism, SMO (spermine oxidase) and APAO (N^1-acetylpolyamine oxidase) [4,6]. This homology suggests the strategy of pursuing polyamine analogues as LSD1 inhibitors to regulate chromatin remodelling. Our laboratory has demonstrated previously that specific polyamine analogues function as potent inhibitors of LSD1 [6–9]. In specific instances, inhibition of LSD1 by polyamine analogues results in the re-expression of genes that are aberrantly silenced in cancer. These results have been confirmed both *in vitro* and *in vivo* [7,10]. *In vivo* treatment of established human tumours in nude mice demonstrated that long-chain polyamine analogues (oligoamines) effectively inhibit LSD1 *in situ*, resulting in a dramatic decrease in tumour size. *In vitro* results with the oligoamines demonstrated that treatment of cancer cells results in increased methylated H3K4, the target of LSD1, and increased expression of various previously silenced genes. Each of these results is consistent with the hypothesis that inhibition of LSD1 by the oligoamines is responsible for the re-expression of the silenced genes.

The natural polyamines are cationic alkylamines that are positively charged at physiological pH (Figure 1) [11–14]. They are closely associated with chromatin and are thought to have a role in the regulation of multiple cellular functions, including gene expression. DFMO (2-difluoromethylornithine), an inhibitor of the first rate-limiting enzyme in polyamine biosynthesis, ODC (ornithine decarboxylase) [15], can be used to reduce intracellular polyamine concentrations, both *in vitro* and *in vivo* [16]. We hypothesized that the reduction of the natural polyamines

Abbreviations used: CDKN2A, cyclin-dependent kinase inhibitor 2A; ChIP, chromatin immunoprecipitation; dcSAM, decarboxylated *S*-adenosylmethionine; DFMO, 2-difluoromethylornithine; DNMT, DNA methyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA, GATA-binding protein; HDAC, histone deacetylase; H3K4, Lys[4] of histone H3; H3K4me1, mono-methyl H3K4; H3K4me2, di-methyl H3K4; H3K9, Lys[9] of histone H3; H3K9me2, di-methyl H3K9; LSD1, lysine-specific demethylase 1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; ODC, ornithine decarboxylase; PARP, poly(ADP-ribose) polymerase; qPCR, quantitative PCR; SFRP, secreted frizzled-related protein; TSS, transcriptional start site.

[1] Robert Casero and Laurence Marton have applied for a patent to cover the combination of agents described in the present paper.

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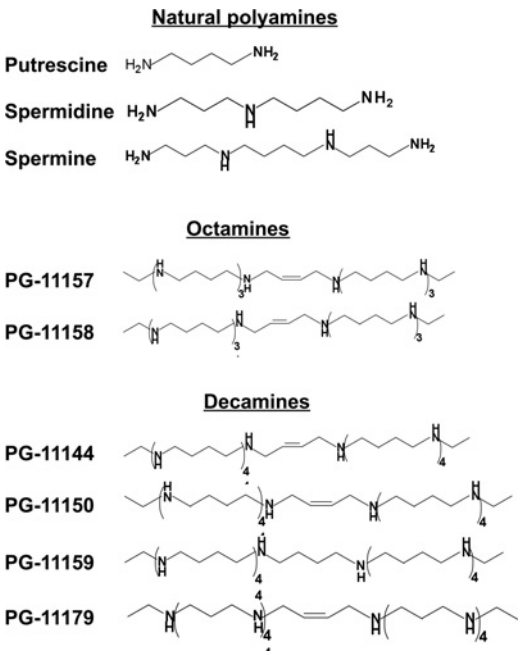

Figure 1 Structures of the natural polyamines and oligoamines

in cancer cells by pre-treatment with DFMO would enhance the epigenetic effects of oligoamine treatment through two mechanisms: (i) the reduction of the natural polyamines would allow the analogues to have greater access to their targets; and (ii) the reduction of natural polyamines would result in increased uptake of the oligoamines, thus rapidly increasing the effective intracellular analogue concentration. Therefore we examined the effects of treating human tumour cells with the combination of DFMO and specific oligoamine analogues.

The results of the present study indicate that this combination of an ODC inhibitor with a polyamine analogue-based LSD1 inhibitor can produce synergistic effects with regard to increased expression of aberrantly silenced tumour-suppressor genes and enrichment of H3K4me2 active marks at the gene promoter. This combination therefore represents a completely novel anti-proliferative therapeutic approach to the treatment of cancer.

EXPERIMENTAL

Compounds, culture conditions and treatment

DFMO was from the DFMO repository at the Medical University of South Carolina, Charleston, SC, U.S.A. (Professor Patrick Woster). Tranylcypromine was purchased from Sigma. Polyamine analogues (Figure 1) were provided by Progen Pharmaceuticals. Stock solutions of each compound were diluted with medium to the desired concentrations for specific experiments. HCT116 human colorectal carcinoma cells were maintained in McCoy's 5A medium and HT-29 colorectal adenocarcinoma cells were maintained in DMEM (Dulbecco's modified Eagle's medium), each supplemented with 9 % FBS (fetal bovine serum; Atlanta Biologicals) and 1 % penicillin/streptomycin (Mediatech), and grown at 37 °C in a 5 % CO_2 atmosphere. Where indicated, the cells were first treated for 24 h with 5 mM DFMO, followed by another 24 h treatment of replenished 5 mM DFMO, either alone or simultaneously with a polyamine analogue or tranylcypromine, at the indicated concentrations.

Determination of cell viability and mechanism of cell death

HCT116 cells were seeded at 6000 cells/well in 100 μl of medium in 96-well plates. Cells were first treated for 24 h with 5 mM DFMO, followed by another 24 h treatment with replenished 5 mM DFMO and PG-11144, alone or simultaneously, at the indicated concentrations. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] reagent (20 μl; Promega) was added to the medium. The cells were incubated for another 2 h and absorbance was measured at 490 nm on a microplate reader (Molecular Devices) to determine the cell viability. To determine whether cell death induced by the combination treatment occurred through apoptosis, PARP [poly(ADP-ribose) polymerase] cleavage analysis was performed as reported previously [7].

Western blotting

Whole-cell lysates were extracted using RIPA buffer [10 mM sodium phosphate (pH 7.5), 150 mM NaCl, 1 % Nonidet P40, 0.5 % sodium deoxycholate and 0.1 % SDS]. Primary antibodies against PARP and actin were from Invitrogen and Santa Cruz Biotechnology respectively. Dye-conjugated secondary antibodies were used for quantification of the Western blot results using the Odyssey infrared detection system and software (LI-COR Biosciences).

HPLC

HCT116 cell extract was prepared using SSAT (spermidine/spermine acetyltransferase) breaking buffer [5 mM Hepes (pH 7.2) and 1 mM dithiothreitol)]. Concentrations of individual polyamine pools and PG-11144 were quantified using dansyl chloride labelling followed by HPLC, as described previously [17].

RNA isolation and qPCR (quantitative PCR)

RNA was extracted using TRIzol® reagent (Invitrogen). First-strand cDNA of HCT116 cells was synthesized using M-MLV (Moloney murine leukaemia virus) reverse transcriptase with an oligo$(dT)_{12–18}$ primer. For HT-29 cells, Superscript III reverse transcriptase with oligo$(dT)_{20}$ primers (Invitrogen) was used to synthesize first-strand cDNA. qPCR was performed in a MyiQ single-colour real-time PCR detection system (Bio-Rad Laboratories) with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal control. The *CDKN2A* (cyclin-dependent kinase inhibitor 2A) primers used were: sense, 5′-CAATCGGGGATGTCTGAGGGAC-3′ and antisense, 5′-CGGAGGCCGATCCAGGTCATG-3′ (annealing at 67.3 °C). The *GATA4* (GATA-binding protein 4) primers used were: sense, 5′-GGCCGCCCGACACCCCAATCT-3′ and antisense, 5′-ATAGTGACCCGTCCCATCTCG-3′ (annealing at 65 °C for HCT116 cells and 58 °C for HT-29 cells). The *GATA5* primers used were: sense, 5′-CCTGCGGCCTCTACCACAA-3′ and antisense, 5′-GGCGCGGCGGGACGAGGAC-3′ (annealing at 64.3 °C for HCT116 cells and 65 °C for HT-29 cells). The *SFRP1* (secreted frizzled-related protein 1) primers used were: sense, 5′-GGCCCATCTACCCGTGTCG-3′ and antisense, 5′-GATGGCCTCAGATTTCAACTCGT-3′ (annealing at 60 °C). The *SFRP2* primers used were: sense, 5′-AAGCCTGCAAAAATAAAAATGATG-3′ and antisense, 5′-TGTAAATGGTCTTGCTCTTGGTCT-3′ (annealing at 63 °C for HCT116 cells and 59.3 °C for HT-29 cells). For HCT116 cells, the *SFRP4* primers used were: sense, 5′-TCTTCTTGCCAGTGTCCAC-3′ and antisense, 5′-GGCTGTTTTCTTCTTGTCCTG-3′ (annealing at 61.2 °C). For HT-29 cells, the *SFRP4* primers used

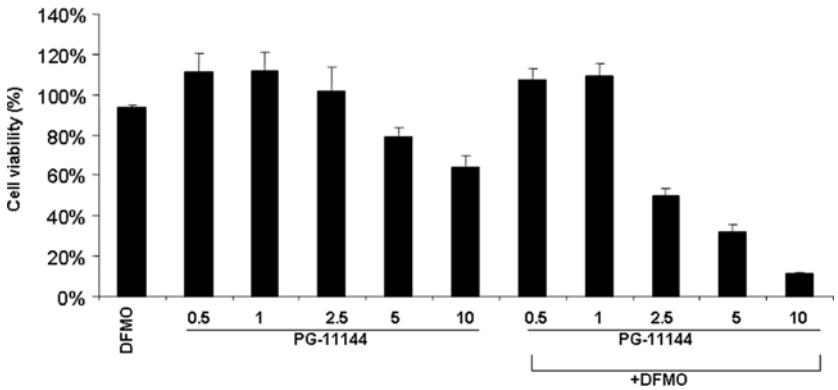

Figure 2 Viability of HCT116 cells following treatment with DFMO in combination with PG-11144

Cells were treated for 24 h with 5 mM DFMO followed by a 24 h treatment with replenished 5 mM DFMO and PG-11144 at the indicated concentrations (μM), alone or simultaneously, and cell viability was measured by an MTS assay. Shown is the percentage of viable cells in treatment groups relative to untreated control cells. Results are means $\pm$ S.D. of treatments performed in quadruplicate from a representative experiment performed three times.

were: sense, 5′-TCTATGACCGTGGCGTGTGC-3′ and antisense, 5′-ACCGATCGGGGCTTAGGCGTTTAC-3′ (annealing at 65 °C). The *SFRP5* primers used were: sense, 5′-GCGAGGAGTACGACTACTATG-3′ and antisense, 5′-AAAGAGCGAGCACAGGAAG-3′ (annealing at 61.2 °C for HCT116 cells and 65 °C for HT-29 cells). Amplification conditions consisted of a 15 min denaturation step followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at the designated temperature for 30 s and extension at 72 °C for 30 s.

ChIP (chromatin immunoprecipitation)

ChIP analysis was performed using Protein A and Protein G Dynabeads® (Invitrogen). Cells were exposed to 1 % formaldehyde to cross-link proteins, and 2×10^6 cells were used for each ChIP assay. The antibody against pan H3 was from Abcam and antibodies against H3K4me1, H3K4me2, H3K9me2 (di-methyl H3K9), acetyl-H3K9 and LSD1 were from Millipore. Quantitative ChIP was performed using qPCR on the MyiQ single-colour real-time PCR detection system. PCR primers spanning the proximal *SFRP2* promoter region from approximately -800 bp to -600 bp relative to the TSS (transcriptional start site) were used for amplification. The sequences of the primers were: sense, 5′-CCTGTGTGACTGGTGAGACTC-3′ and antisense, 5′CGGCGAACTTCGTTTTCCCTC-3′ (annealing at 63.3 °C). Sheared genomic DNA was used as a positive control (input) and for normalization of DNA immunoprecipitated by LSD1. DNA immunoprecipitated by the anti-(pan H3) antibody was used for normalization for the histone H3 modifications.

Bisulfite sequencing

Genomic DNA from HCT116 cells was bisulfite modified using the EZ DNA Methylation kit (Zymo Research) and bisulfite sequencing of the *SFRP2* gene promoter was performed using the following primers: sense, 5′-GGTAATTTAGTAGAAATTTCGGATTG-3′ and antisense, 5′-ACTAATCACTACTTTCTAAATCTAATT-3′. A total of 15 clones were analysed for each treatment condition.

RESULTS

The combination of DFMO and PG-11144 leads to decreased cell viability

To determine the effects of the combination of DFMO and an oligoamine on cell proliferation, HCT116 human colorectal carcinoma cells were first treated for 24 h with 5 mM DFMO, followed by another 24 h treatment with DFMO and PG-11144 at the indicated concentrations. Treatment with PG-11144 alone was also evaluated. An MTS assay was used to determine cell viability (Figure 2). The results demonstrated that PG-11144 alone leads to a moderate reduction in viable cells at high concentrations (79 % of cells are viable at 5 μM and 64 % at 10 μM, relative to control), whereas, as expected, DFMO alone had no significant effects on cell viability. The anti-proliferative effect of PG-11144 was augmented when DFMO was added to the combination, with 2.5 μM and 5 μM PG-11144 reducing viable cells to 50 % and 32 % respectively. PG-11144 (10 μM) plus DFMO produced the most prominent cytotoxicity, decreasing viable cells to 11 % of that of untreated cells.

To determine whether the increased cell death following the combined treatment of PG-11144 and DFMO was a result of increased apoptosis, we examined the changes in PARP cleavage as an indicator of apoptosis as we have published previously [7]. Western blot analysis indicates that PG-11144 alone at 5 and 10 μM induces robust PARP cleavage. When combined with DFMO, PG-11144 at a lower concentration (2.5 μM) is capable of inducing significant PARP cleavage (Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420693add.htm). The PARP cleavage data are entirely consistent with the observed synergy of the combination and indicate that apoptosis is the underlying mechanism of the observed cell death.

Effects of treatment with the combination of DFMO and the oligoamine PG-11144 on polyamine pools

Natural polyamines are known to associate with DNA and result in conformational changes and aggregation of DNA [18,19]. Specific polyamine analogues utilize the polyamine transporter for entry into the cell, where they exert effects on natural polyamine concentrations via regulation of polyamine biosynthesis and catabolism, potentially resulting in changes in chromatin structure [20]. We hypothesized that the addition of DFMO, an inhibitor of a rate-limiting enzyme in polyamine synthesis, ODC [15], would increase uptake of PG-11144 and decrease levels of natural polyamines, allowing greater access of the analogue to chromatin. Therefore the effects of the combination of DFMO and PG-11144 treatment on polyamine pools in HCT116 cells were determined by HPLC (Table 1). As expected, the cellular levels of putrescine and spermidine were depleted by DFMO treatment. Treatment

Table 1 Effects of combining treatment with DFMO and the oligoamine PG-11144 on polyamine pools in HCT116 cells

HCT116 cells were treated for 24 h with or without 5 mM DFMO, followed by another 24 h treatment with refreshed medium containing 5 mM DFMO and the indicated doses of PG-11144, alone or in combination. Intracellular concentrations of putrescine, spermidine, spermine and PG-11144 were measured by HPLC. Each of the values represents the mean of two determinations of one treatment from a representative experiment performed three times.

	Polyamine concentration (nmol/mg of protein)			
Treatment	Putrescine	Spermidin	Spermine	PG-11144
Untreated	2.5	9.5	15.2	0.0
DFMO 5 nM	0.0	0.2	6.7	0.0
PG-11144 0.5 μM	2.7	5.0	7.6	0.0
PG-11144 1 μM	2.5	4.3	6.4	0.5
PG-11144 2.5 μM	2.2	3.2	4.3	1.0
PG-11144 5 μM	0.0	3.2	4.6	1.7
PG-11144 10 μM	0.0	3.2	6.2	2.3
DFMO 5 mM + PG-11144 0.5 μM	0.0	1.1	6.7	2.3
DFMO 5 mM + PG-11144 1 μM	0.0	1.2	4.1	1.9
DFMO 5 mM + PG-11144 2.5 μM	0.0	1.2	4.6	2.8
DFMO 5 mM + PG-11144 5 μM	0.0	1.1	5.7	6.0
DFMO 5 mM + PG-11144 10 μM	0.0	0.9	7.8	11.5

with PG-11144 alone led to a decrease in all three polyamines in a dose-dependent manner. This decrease is probably due to the down-regulation of ODC caused by PG-11144 as we have published previously [21,22]. The combination of PG-11144 with DFMO did not lead to a greater decrease in natural polyamine pools than that achieved with DFMO alone. However, intracellular levels of PG-11144 were significantly increased when DFMO was used in combination with PG-11144, as compared with treatment with PG-11144 alone. In the presence of DFMO, the addition of PG-11144 at a dose as low as 0.5 μM resulted in a level of intracellular PG-11144 comparable with that seen following treatment with 10 μM PG-11144 alone. Furthermore, treatment with 5 or 10 μM PG-11144 in combination with DFMO led to a significant increase in the intracellular concentration of PG-11144. These data validate our hypothesis that DFMO, in combination with an oligoamine, not only reduces natural polyamines, but also enhances intracellular levels of the analogue, which may result in greater effectiveness of oligoamines on their targets.

The combination of DFMO plus oligoamines results in synergistic re-expression of multiple aberrantly silenced genes

To determine whether the combination of the oligoamines and DFMO results in synergistic re-expression of previously silenced genes, we examined the expression level of the Wnt-signalling antagonist SFRP2. *SFRP2* is a gene frequently silenced in colon cancers, as represented by the HCT116 colorectal cancer cell line [23]. HCT116 cells were pre-treated for 24 h with 5 mM DFMO, followed by a 24 h co-treatment with replenished 5 mM DFMO, either alone or with a polyamine analogue or tranylcypromine at 5 μM. The results indicate that each of the oligoamines tested in combination with DFMO induced synergistic re-expression of the previously silenced *SFRP2* gene. We also observed that PG-11144 and PG-11150 were effective as single agents in increasing *SFRP2* expression level, as reported previously [7] (Figure 3A). The synergistic gene re-activation effects were not limited to HCT116 cells. Another human colorectal cancer cell line, HT-29, was also examined, and the combination of DFMO and oligoamine treatment showed increases not only in re-expression of *SFRP2*, but also in the expression of the aberrantly silenced *CDKN2A* (p16) gene (Figure 3B). In addition, genes of the GATA family of transcription factors, *GATA4* and *GATA5*, and of three other members of the SFRP family, *SFRP1*, *SFRP4* and *SFRP5*, were examined in HCT116 cells (Supplementary Table S1a at http://www.BiochemJ.org/bj/442/bj4420693add.htm); these genes were also examined in HT-29 cells except *GATA5* and *SFRP1* (Supplementary Table S1b). In both of the colorectal cancer cell lines, the combination of DFMO with the various oligoamines resulted in a cell type-specific response with regard to which specific genes were re-expressed, with the *SFRP2* gene demonstrating the greatest re-expression. Tranylcypromine, a non-selective MAO (monoamine oxidase) inhibitor commonly used as antidepressant medication, has been demonstrated to inhibit LSD1-mediated demethylation of H3K4 [24–26]. However, treatment with tranylcypromine, either alone or in combination with DFMO, did not induce re-expression of the aberrantly silenced *SFRP2* gene in HCT116 or HT-29 cells, in clear contrast with the results seen with the oligoamines. A modest increase in expression of genes other than *SFRP2* was observed in HCT116 cells (*SFRP1*) and in HT-29 cells (*CDKN2A* and *SFRP5*) when these cells were treated with tranylcypromine alone or in combination with DFMO. It should be noted that tranylcypromine lacks the cationic nature of the oligoamines, thus, unlike the oligoamines, it is not targeted to chromatin. These data suggest that inhibition of LSD1 by tranylcypromine and the oligoamines differs in functional outcome.

Synergistic re-expression of *SFRP2* in response to combined DFMO and PG-11144 is dose-dependent on PG-11144

To further evaluate that DFMO pre-treatment increased the effectiveness of PG-11144, we examined the dose dependency of *SFRP2* gene re-expression with increasing concentrations of PG-11144 in combination with 5 mM DFMO. A dose as low as 0.5 μM PG-11144 demonstrated synergy with DFMO. The synergy reached its maximum when 5 μM PG-11144 was used in combination with DFMO (Figure 4). Higher concentrations actually resulted in less *SFRP2* expression, probably as a result of increased cytotoxicity.

The combination of DFMO with PG-11144 alters histone modifications in the promoter region of the *SFRP2* gene

To investigate whether the synergistic re-expression of aberrantly silenced genes was linked to LSD1 inhibition and changes in chromatin that favour transcription, the levels of various histone

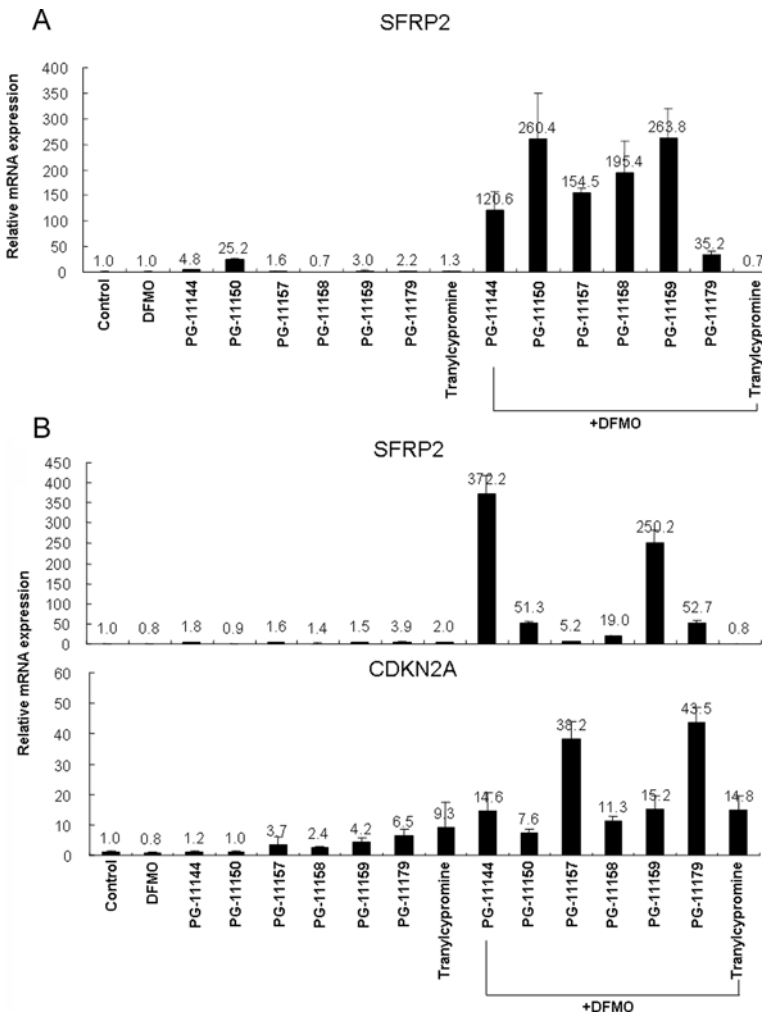

Figure 3 Synergy of oligoamines and DFMO in the re-expression of aberrantly silenced genes

HCT116 cells (**A**) or HT-29 cells (**B**) were first treated for 24 h with 5 mM DFMO, followed by another 24 h treatment with replenished 5 mM DFMO, either alone or simultaneously with a polyamine analogue or tranylcypromine at 5 μM. (**A**) Transcript expression of *SFRP2* in HCT116 cells was analysed by qPCR. (**B**) For HT-29 cells, qPCR was performed to analyse the transcript expression levels of *SFRP2* and *CDKN2A*. For both cell lines, GAPDH was used as an internal control. Results are the means $\pm$ S.D. of three independent treatments with the real-time PCR performed in triplicate. The transcript level for control untreated samples was set to a value of 1.

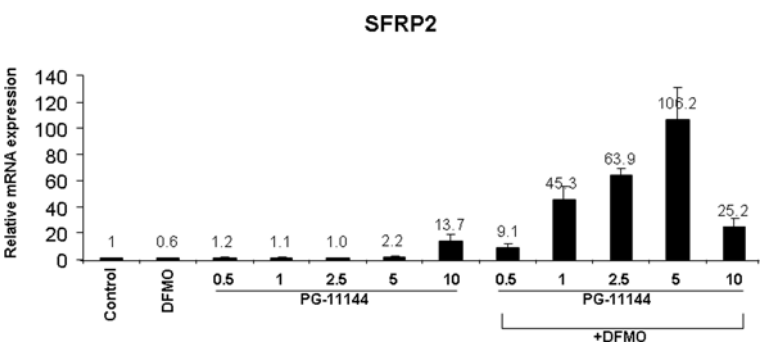

Figure 4 Dose response to PG-11144, in co-treatment with DFMO, for the synergistic re-expression of *SFRP2*

HCT116 cells were treated for 24 h with 5 mM DFMO, followed by another 24 h treatment with replenished 5 mM DFMO and PG-11144 in the indicated doses (μM), alone or simultaneously. Results are means $\pm$ S.D. of real-time PCR analysis of three independent treatments with the PCR performed in triplicate. GAPDH was used as an internal control. The transcript level for control samples was set to a value of 1.

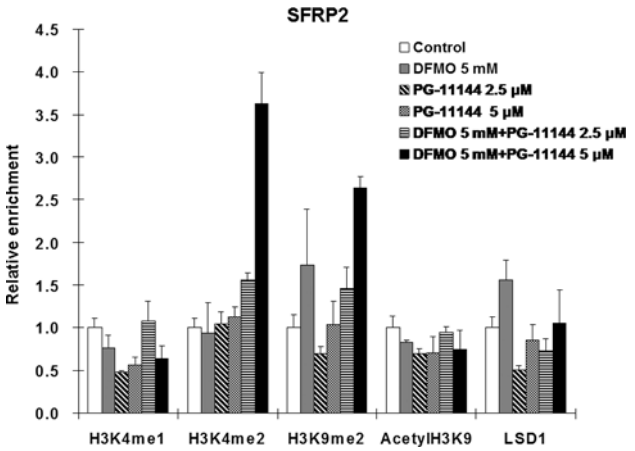

Figure 5 Effects of DFMO in combination with PG-11144 on various histone marks and LSD1 at the promoter of *SFRP2*

HCT116 cells were first treated for 24 h with 5 mM DFMO followed by another 24 h treatment with replenished 5 mM DFMO and PG-11144 in the indicated doses, alone or simultaneously. Quantitative ChIP analysis was performed with H3 as control for immunoprecipitation of protein–DNA complexes. Results are means ± S.D. of three independent real-time ChIP experiments with PCR performed in triplicate. The relative enrichment of histone marks or LSD1 levels for untreated control samples were set to a value of 1.

modifications in the promoter of *SFRP2* were examined in HCT116 cells. ChIP analysis of the promoter region of *SFRP2* clearly demonstrated a significant increase in H3K4me2, the target of LSD1 and a transcriptional activating mark, when 5 μM PG-11144 was combined with DFMO (Figure 5). However, no induction was detected for the relative enrichment of H3K4me1, which is another substrate of LSD1. Interestingly, the level of the repressive mark H3K9me2 in the *SFRP2* promoter region exhibited a modest increase when treated with DFMO, either alone or in combination with PG-11144. In contrast, a slight decrease in the H3K9me2 mark was observed when cells were treated with 2.5 μM PG-11144 alone. The level of the activating mark, acetyl-H3K9, demonstrated a marginal decrease, probably because acetylation and methylation at the same histone site are mutually exclusive [27]. Occupancy of LSD1 at the promoter of *SFRP2* was reduced by PG-11144 treatment alone. The LSD1 level in the adjacent *SFRP2* promoter region (spanning approximately -1000 bp to -800 bp relative to the TSS) was also examined, and a consistent reduction of LSD1 was observed after exposure to PG-11144, alone or in combination with DFMO (results not shown).

DNA methylation status of the *SFRP2* promoter is not affected by DFMO in combination with PG-11144

DFMO inhibits ODC and consequently depletes putrescine. Reducing the level of putrescine leads to accumulation of dcSAM (decarboxylated *S*-adenosylmethionine), which is the aminopropyl group donor in spermidine and spermine synthesis [28]. dcSAM acts as a competitive inhibitor of *S*-adenosylmethionine, the methyl group donor for methylation reactions, and could subsequently result in interference with DNMT (DNA methyltransferase) activity and changes in DNA methylation [29]. Bisulfite sequencing was performed to determine the CpG island methylation status in the *SFRP2* promoter with and without treatment (Figure 6). No significant changes in *SFRP2* promoter methylation were observed with either DFMO or PG-11144, alone or in combination (frequencies of unmethylated CpG sites for control, 5 mM DFMO alone, 5 μM PG-11144 alone or DFMO in combination with PG-11144 were 4.37 %, 5.29 %, 4.37 % and 4.60 % respectively). These results indicate that the increase in transcription induced by the combination of DFMO and PG-11144 is not dependent upon changes in promoter DNA CpG island methylation.

DISCUSSION

Epigenetic silencing of gene expression plays a key role in the aetiology and progression of cancer [1,30,31]. Strategies to reverse aberrant gene silencing have been demonstrated to be efficacious in specific cancers, and clinical trials are ongoing to evaluate drugs targeting epigenetic regulation of gene expression [32,33]. Targeting epigenetic changes is an attractive strategy, as these changes, unlike gene loss or mutation, are reversible. To date, most of the drugs studied that alter epigenetic gene regulation have targeted either the DNMTs or the HDACs. However, other significant targets exist. With the discovery of the transcriptionally repressive lysine-specific demethylase LSD1 [4] it was hypothesized that inhibition of this enzyme could lead to the re-expression of some aberrantly silenced genes. As an FAD-dependent amine oxidase, LSD1 is structurally similar to, and functions in a mechanistically similar manner as, the polyamine oxidases [4,7,34,35]. We therefore postulated, and have since demonstrated, that specific polyamine analogues can effectively inhibit LSD1 activity, increase promoter-bound levels of H3K4me2 and lead to re-expression of previously silenced genes [7,10].

The studies presented in the present paper demonstrate that pre-treatment of human colon cancer cells with the ODC inhibitor DFMO, followed by co-treatment with DFMO and the polyamine analogue LSD1 inhibitor, results in intracellular polyamine depletion, increased accumulation of analogue, synergistic re-expression of an aberrantly silenced gene and increased cell death through apoptotic mechanisms. However, the determination of the precise gene expression changes leading to increased apoptosis will require additional study.

The natural polyamines are known to play a role in chromatin structure and their cationic nature at physiological pH makes them important counter ions to the phosphate backbone of DNA [11]. There are no data to indicate that the natural polyamines are inhibitors of, or substrates for, LSD1 [4]; however, it is possible that the reduction of intracellular polyamine concentrations alters chromatin conformation and/or makes the target, LSD1, more accessible to the oligoamine inhibitors.

The ChIP analysis of the present study demonstrates that PG-11144 alone down-regulates LSD1 occupancy in specific regions of the *SFRP2* promoter (spanning approximately -800 bp to -600 bp relative to the TSS) (Figure 4). We also observed that in the adjacent region, approximately -1000 bp to -800 bp relative to the TSS, the LSD1 level was reduced by PG-11144, both alone and in combination with DFMO (results not shown). PG-11144 is a long-chain polyamine analogue that possesses a multivalent ($+10$) cationic structure, and thus has higher affinity for chromatin than natural polyamines [36]. PG-11144 could bind to, and alter, chromatin structure, thus decreasing the ability of LSD1 to target the nucleosome at specific chromatin sites, thereby leading to a decrease of LSD1 occupancy at the *SFRP2* promoter. In the presence of DFMO, this effect could be augmented in certain promoter regions due to the lower level of natural polyamines and higher accessibility of the oligoamine to

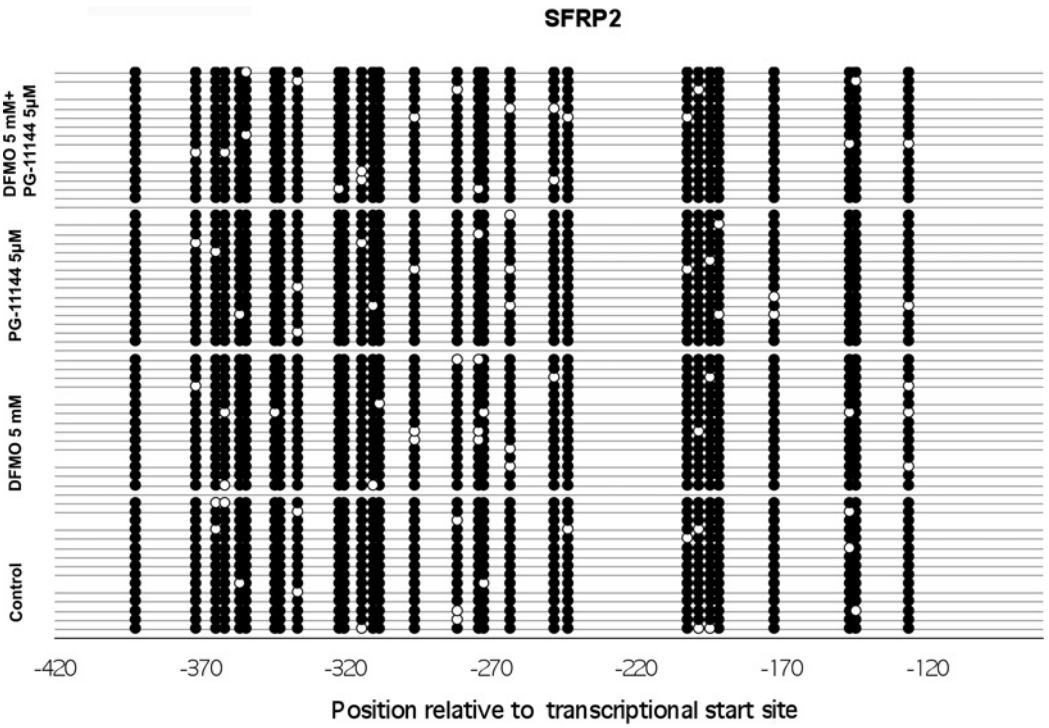

Figure 6 Effects of oligoamine and DFMO treatment on promoter CpG island methylation of *SFRP2*

HCT116 cells were first treated for 24 h with 5 mM DFMO followed by another 24 h treatment with replenished 5 mM DFMO and 5 μM PG-11144, alone or simultaneously. Genomic DNA from HCT116 cells was bisulfite modified and sequencing of the promoter of the *SFRP2* gene was performed. The methylation status of 29 CpG sites in the promoter region of *SFRP2* was assessed. Each circle represents one CpG site that is methylated (closed circle) or unmethylated (open circle) and each horizontal line shows the methylation status of CpG sites for a single cloned allele. A total of 15 clones were analysed for each treatment condition.

chromatin. Further work will be necessary to specifically test this hypothesis at this and other gene promoters.

It is notable that the results of the present study demonstrate that not only the transcriptional activating mark H3K4me2, but also the repressive mark H3K9me2, were increased by the combination treatment. LSD1 was reported to demethylate H3K9me2 when interacting with androgen receptor or oestrogen receptor [37–41]; however, more studies will be necessary to determine whether this increased H3K9me2 is correlated with changes in LSD1 activity or is potentially due to broader effects on repressor complex formation. The simultaneous increase in both transcriptional activating and repressive marks indicates the complexity of the gene regulation mechanisms underlying the response to co-treatment with DFMO and the oligoamines.

DFMO-induced reduction of the levels of putrescine and spermidine results in a subsequent accumulation of dcSAM, which could contribute to DNMT inhibition [29]. In addition, OAZ (ODC antizyme-1), a molecule that promotes ODC degradation and thereby induces polyamine depletion, was reported to induce hypomethylation of genomic DNA and decrease H3K9me2 in a human oral cancer cell line [42]. However, the results of the present study demonstrate that treatment with DFMO, alone or in combination with PG-11144, had no effects on the methylation level of CpG islands in the *SFRP2* promoter. Furthermore, the addition of the DNMT inhibitor, 5-azacytidine, to the DFMO and PG-11144 combination treatment regimen in HCT116 cells showed no advantage with respect to *SFRP2* re-expression (results not shown). These results imply that the chromatin-modifying effects induced by DFMO in combination with the oligoamines might be sufficient to result in the high level of *SFRP2* re-expression detected, independent of changes in DNA CpG island methylation.

The combination of DFMO and the oligoamines may have particular significance in colon cancer. Chemoprevention studies by Gerner and colleagues [43] demonstrated that DFMO is a well-tolerated and promising drug for the prevention of sporadic colorectal adenomas. These studies, combined with our own finding that PG-11144 alone is effective in shrinking established HCT116 human colon tumours in nude mice [7], suggest the potential for added benefit in the colon tumour setting. However, further *in vivo* studies will be necessary to determine the efficacy and any potential toxic side effects of this combination in both therapeutic and chemopreventive settings.

Increased intracellular polyamines have been implicated in alterations in histone acetyltransferases and HDACs, potentially resulting in epigenetic changes that lead to the initiation and progression of tumours in a transgenic mouse model where ODC, the target of DFMO, is overexpressed [44–46]. Another previous report indicates that polyamine depletion by DFMO can induce differentiation in cardiac myocytes through epigenetic mechanisms [47]. Taken together, these findings are relevant in that they suggest that inhibition of polyamine synthesis by DFMO may reduce or block some epigenetic changes necessary for cancer. Thus the effective combination of the oligoamine LSD1 inhibitors with DFMO may be a result of multiple beneficial mechanisms.

In summary, we have demonstrated that the LSD1-inhibiting oligoamines, in combination with the ODC inhibitor DFMO,

results in increased expression of important tumour-suppressor genes, including *SFRP2* and *CDKN2A*, which are often aberrantly silenced in colon cancer, as well as other solid tumour and haematological malignancies. Importantly, this combination results in a synergistic response in treated cells that alters local chromatin and results in the re-expression of these important tumour-suppressor genes. This combination represents a promising and entirely unique strategy for the targeting of epigenetically silenced genes in the treatment of cancer.

AUTHORS CONTRIBUTION

Yu Wu, Tracy Murray-Stewart, Laurence Marton and Robert Casero designed the experiments; Yu Wu and Nora Steinbergs performed the experiments; Yu Wu, Nora Steinbergs, Tracy Murray-Stewart, Laurence Marton and Robert Casero analysed the data; Yu Wu, Tracy Murray-Stewart, Laurence Marton and Robert Casero wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Oligoamine analogues in combination with 2-difluoromethylornithine synergistically induce re-expression of aberrantly silenced tumour-suppressor genes

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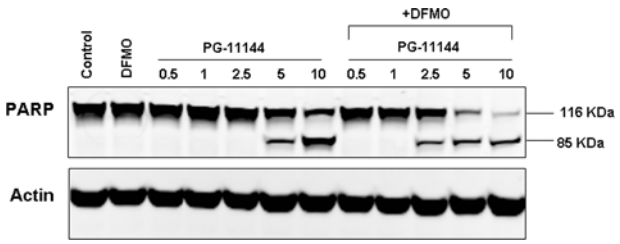

Figure S1 Induction of PARP cleavage by PG-11144 alone or simultaneously with DFMO

HCT116 cells were first treated for 24 h with 5 mM DFMO followed by another 24 h treatment of replenished 5 mM DFMO and PG-11144 in the indicated doses (μM) alone or simultaneously. Total protein was extracted and analysed by Western blotting using antibodies that recognize full-length and cleaved PARP. The positions of the 116 kDa full length and the 85 kDa cleaved PARP are indicated on the right. Actin was used as a loading control. Molecular mass is given in kDa on the right-hand side.

[1] Robert Casero and Laurence Marton have applied for a patent to cover the combination of agents described in the present paper.
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Table S1 Effects of treatment with the combination of DFMO and oligoamine on transcript expression levels of aberrantly silenced genes

HCT116 (a) and HT-29 (b) cells were first treated for 24 h with 5 mM DFMO, followed by another 24 h treatment with replenished 5 mM DFMO, either alone or simultaneously with a polyamine analogue or tranylcypromine at 5 μM. (a) Transcript expression of *GATA4*, *GATA5*, *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* in HCT116 cells was analysed by qPCR. (b) For HT-29 cells, qPCR was performed to analyse the transcript expression levels of *CDKN2A*, *GATA4*, *SFRP2*, *SFRP4* and *SFRP5*. *GAPDH* was used as internal control for all of the qPCR analyses. The results are the means of three independent treatments with the real-time PCR performed in triplicate. The transcript level for untreated control samples was set to a value of 1.

(a)

Treatment	GATA4	GATA5	SFRP1	SFRP2	SFRP4	SFRP5
DFMO 5 mM	09 ± 0.1	1.2 ± 0.4	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.6 ± 0.1
PG-11144 5 μM	1.2 ± 0.1	0.9 ± 0.3	1.9 ± 0.8	4.8 ± 0.5	0.7 ± 0.1	0.7 ± 0.1
PG-11150 5 μM	0.6 ± 0.3	0.3 ± 0.1	2.4 ± 0.7	25.2 ± 2.8	2.5 ± 1.0	1.3 ± 0.9
PG-11157 5 μM	2.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.5	1.6 ± 0.4	1.2 ± 0.1	0.7 ± 0.1
PG-11158 5 μM	2.3 ± 1.0	1.8 ± 0.5	1.1 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2
PG-11159 5 μM	1.6 ± 0.4	1.0 ± 0.1	0.6 ± 0.4	3.0 ± 0.9	0.3 ± 0.0	0.2 ± 0.0
PG-11179 5 μM	1.2 ± 0.1	1.5 ± 0.3	2.7 ± 0.6	2.2 ± 0.3	1.6 ± 0.1	0.5 ± 0.2
Tranylcypromine 5 μM	0.3 ± 0.1	0.9 ± 0.3	4.9 ± 1.1	1.3 ± 0.4	1.3 ± 0.1	0.4 ± 0.1
DFMO 5 mM + PG-11144 5 μM	2.3 ± 0.7	1.6 ± 0.3	5.6 ± 1.9	120.6 ± 37.8	2.1 ± 0.3	0.9 ± 0.2
DFMO 5 mM + PG-11150 5 μM	4.4 ± 0.5	2.0 ± 0.9	8.3 ± 1.8	260.4 ± 89.0	7.1 ± 0.7	3.6 ± 0.4
DFMO 5 mM + PG-11157 5 μM	2.8 ± 0.4	2.2 ± 0.1	1.2 ± 0.4	154 ± 9.3	2.2 ± 0.3	2.4 ± 2.0
DFMO 5 mM + PG-11158 5 μM	1.5 ± 0.2	1.6 ± 0.6	1.3 ± 0.4	195.4 ± 59.5	1.6 ± 0.1	1.1 ± 0.3
DFMO 5 mM + PG-11159 5 μM	2.7 ± 1.0	2.3 ± 0.7	3.3 ± 1.3	263.8 ± 56.8	3.7 ± 0.7	2.9 ± 0.8
DFMO 5 mM + PG-11179 5 μM	2.4 ± 0.3	1.5 ± 0.4	18.1 ± 5.7	35.2 ± 6.4	4.8 ± 1.2	1.4 ± 0.1
DFMO 5 mM + Tranylcypromine 5 μM	0.9 ± 0.2	0.7 ± 0.1	2.3 ± 0.5	0.7 ± 0.3	0.8 ± 0.1	0.2 ± 0.1

(b)

Treatment	CDKN2A	GATA4	SFRP2	SFRP4	SFRP5
DFMO 5 mM	0.8 ± 0.2	1.3 ± 0.2	0.8 ± 0.2	1.1 ± 0.3	5.3 ± 0.2
PG-11144 5 μM	1.2 ± 0.1	2.6 ± 0.4	1.8 ± 0.2	1.4 ± 0.4	22.3 ± 5.2
PG-11150 5 μM	1.0 ± 0.6	1.3 ± 0.3	0.9 ± 0.0	0.5 ± 0.1	3.1 ± 3.1
PG-11157 5 μM	3.7 ± 2.3	1.1 ± 0.2	1.6 ± 0.3	1.6 ± 0.8	4.6 ± 1.6
PG-111458 5 μM	2.4 ± 0.5	0.9 ± 0.1	1.4 ± 0.3	1.2 ± 0.5	2.9 ± 0.4
PG-11159 5 μM	4.2 ± 1.5	1.5 ± 0.1	1.5 ± 0.2	0.7 ± 0.4	3.4 ± 0.4
PG-11179 5 μM	6.5 ± 2.1	0.9 ± 0.2	3.9 ± 1.0	0.9 ± 0.1	2.1 ± 0.6
Tranylcypromine 5 μM	9.3 ± 8.1	0.9 ± 0.3	2.0 ± 0.3	0.8 ± 0.1	3.8 ± 1.0
DFMO 5 mM + PG-11144 5 μM	14.6 ± 6.1	2.1 ± 0.2	372.2 ± 45.2	6.4 ± 0.2	5.1 ± 0.9
DFMO 5 mM + PG-11150 5 μM	7.6 ± 1.1	1.3 ± 0.1	51.3 ± 5.0	1.2 ± 0.1	6.8 ± 2.0
DFMO 5 mM + PG-11157 5 μM	38.2 ± 5.8	1.9 ± 0.6	5.2 ± 0.5	2.2 ± 0.1	10.9 ± 2.9
DFMO 5 mM + PG-11158 5 μM	11.3 ± 1.4	1.3 ± 0.1	19.0 ± 1.4	0.8 ± 0.3	3.6 ± 1.8
DFMO 5 mM + PG-11159 5 μM	15.2 ± 4.3	2.1 ± 1.0	250.2 ± 33.7	3.9 ± 0.3	4.3 ± 1.2
DFMO 5 mM + PG-11179 5 μM	43.5 ± 5.2	1.6 ± 0.2	52.7 ± 4.6	0.6 ± 0.2	10.5 ± 1.8
DFMO 5 mM + Tranylcypromine 5 μM	14.8 ± 4.6	1.2 ± 0.2	0.8 ± 0.1	0.3 ± 0.2	4.9 ± 1.0

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