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Origin and bioactivities of thiosulfinated FK228†

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During a large laboratory-scale purification of FK228 from the fermentation broth of *Burkholderia thailandensis* MSMB43, a small amount of thiosulfinated FK228 (TS-FK228) was unexpectedly purified only after the broth was mixed with silica gel. Evidence supports the postulations that TS-FK228 was derived from FK228 through spontaneous chemical reaction with silica gel, and TS-FK228 existed as two isomers 1 and 2. TS-FK228 demonstrated similar inhibitory activity and profile against human class I histone deacetylases but exhibited a much higher antiproliferative activity against representative human cancer cell lines when compared to FK228.

Introduction

Human histone deacetylases (HDACs) are validated drug targets for cancer chemotherapy. Four HDAC inhibitors, namely vorinostat, romidepsin, panobinostat, and belinostat, have been approved in the US, and chidamide approved in China, for the treatment of primarily hematologic cancers.^{1,2} Romidepsin, also known as FK228 (Scheme 1), a natural product of *Chromobacterium violaceum* No. 968,^{3–5} was first approved in 2009 for the treatment of cutaneous T-cell lymphoma,⁶ and then in 2011 for the treatment of peripheral T-cell lymphoma.⁷ It is being aggressively pursued in numerous combination therapies against various types of cancer and other disease conditions.^{8–12} FK228 has a bicyclic peptide framework produced biosynthetically by a hybrid nonribosomal peptide synthetase (NRPS)-polyketide synthase (PKS) assembly line with multiple accessory enzymes.^{13–17} A signature disulfide linkage in FK228 is critical for structural stability when FK228 normally exists as a prodrug, and for bioactivity as a potent HDAC inhibitor when FK228 is reduced *in vivo* to afford two sulfhydryl (–SH) groups. Mechanistically, a –SH group on the long aliphatic chain of reduced FK228 (red-FK228) is able to chelate with zinc ion in the catalytic center of class I HDACs, thus inhibiting the enzyme activity, which leads to a cascade of epigenetic consequences.¹⁸ In addition, the *in vivo* reduction of FK228 produces a reactive oxygen species (*i.e.* H₂O₂), which exerts its cytotoxicity against cancer cells and augments the overall cytotoxicity of FK228 (Scheme 1).¹⁹

Chemical synthesis of FK228 is difficult and current commercial supply of FK228 is made possible through microbial

fermentation of *C. violaceum* No. 968, although the exact yield and conditions of industrial production of FK228 remain proprietary.²⁰ An original report of FK228 production up to 19 mg L^{–1} from a pilot industry-scale fed-batch bacterial fermentation was achieved.⁵ Recently we reported *Burkholderia thailandensis* MSMB43 as a new and significantly better source of FK228;²¹ we showed that this bacterial strain produced authentic FK228 up to 115.9 mg L^{–1} in a laboratory-scale fed-batch fermentation, mechanistically due to a high-level expression of the FK228 biosynthetic genes. During a batch preparation of gram level FK228 from the new fermentation, we noticed and then isolated one labile compound, thiosulfinated FK228 (TS-FK228; likely exists as a mixture of two isomers 1 and 2) (Fig. 1). Here we report our findings about the origin and bioactivities of this new TS-FK228.

Experimental section

Isolation and purification of FK228 and TS-FK228

At the end of a fed-batch 2 × 12 L fermentation of *B. thailandensis* MSMB43,²¹ fermentation broth was cleared by

Scheme 1 Structure and mechanisms of the HDAC inhibitory activity and cytotoxicity of FK228. The dotted frame represents an intracellular environment.

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Fig. 1 Detection of FK228 (upper trace in panel A) and TS-FK228 (lower trace in panel A) by HPLC, and TS-FK228 by LC-MS (insert next to the lower trace in panel A), and the proposed structures of TS-FK228 isomers (1 and 2 in panel B).

centrifugation and the resulting supernatant was passed three times through a large glass column (i.d. 10 × 100 cm) packed 1/3 full with a mixture of Amberlite XAD16 resin (20–60 mesh, Sigma-Aldrich, USA) and Diaion HP-20 resin (Sigma-Aldrich) in a 1:1 ratio. Resin mixture was further washed with abundant water, drained and eluted repeatedly with three volumes of ethyl acetate. Elution fractions were pooled and concentrated with a rotary evaporator to yield 145 g of the crude extract, which was mixed with 150 g of Purasil silica gel (Fisher Scientific, 60 Å pore size) and air-dried in a glass tray in fume hood in three days.

The dried silica gel-crude extract mixture was split into two portions and each portion was loaded to a 450 mL silica gel column (Ø4 × 35 cm) on a flash chromatography system (Yamazen AI-580S) equipped with a UV detector. The column was eluted sequentially with 1 L of hexane, 1 L of hexane:ethyl acetate (3:1), 1 L of hexane:ethyl acetate (1:1), 1 L of ethyl acetate, and 1 L of ethyl acetate:acetone (1:1), all at a flow rate of 50 mL min⁻¹ and monitored at 210 nm. FK228 and TS-FK228 were co-eluted in the 100% ethyl acetate fraction, which was further fractionated with a 300 mL silica gel column (Ø4 × 25 cm) on the same flash chromatography system. The column was eluted sequentially with 1 L of 1%, 5%, 10%, 15%, 20%, and 25% acetone in chloroform, and finally with 1 L of acetone. The fractions of 15% and 20% acetone in chloroform were combined and dried under vacuum, and the residue was resuspended in acetonitrile at 45 mg mL⁻¹. Final separation and purification of FK228 and TS-FK228 was achieved on an Agilent Semi-Prep HPLC system equipped with a PrepStar 218 solvent delivery system, an Infinity 1260 DAD detector and an Agilent prep-C18 column (Ø21.2 × 250 mm, 10 µm). Each 0.2 mL aliquot of the above suspension was loaded to the column which was eluted isocratically with 40% acetonitrile at a flow rate of 8 mL min⁻¹.

Analytical instruments

LC-MS analyses were performed on an Agilent 1100 series LC/MSD Trap SL mass spectrometer equipped with an Agilent Zorbax ODS C18 column (3.0 × 10 mm) and a UV detector. High

resolution mass spectrometry (HR-MS) was performed on an Agilent G1946A LC/MSD TOF instrument. Proton (¹H) and carbon (¹³C) nuclear magnetic resonance (NMR) spectra were determined on a 500 MHz Bruker Avance DRX NMR instrument.

HDAC inhibitory assay and cytotoxicity assay

The HDAC inhibitory activity of FK228 and TS-FK228 were assayed in parallel as described.²² HDAC-GloTM I/II assay and screening system was purchased from Promega and recombinant human HDAC1, HDAC4 and HDAC6 were from BPS Bioscience Inc. The cytotoxicity of FK228 and TS-FK228 against three representative human cancer cell lines (HCT-116 colorectal carcinoma, MDA-MB-231 breast adenocarcinoma, and NCI-H232A non-small cell lung carcinoma) were assayed according to our previously described method to determine the IC₅₀ values.²³

Results

Isolation and characterization of TS-FK228

During a large laboratory-scale preparation of FK228, 1.2 g of FK228 was finally obtained and the purified FK228 was eluted at 30.6 min from an analytical HPLC for quality control (Fig. 1A upper trace). A small amount (~5 mg) of an unknown compound was also purified during the course of FK228 purification and eluted at 19.3 min from a confirmative analytical HPLC (Fig. 1A lower trace; note another tiny peak eluted at 21.2 min). This compound was suspected to be a degradation product of FK228. HR-MS analysis of this unknown compound suggested a molecular formula of C₂₄H₃₇N₄O₇S₂ for the molecular ion species of 557.2108 *m/z* (calculated 557.2098 *m/z*), which is indicative of one oxygen atom more than the molecular formula of FK228 (C₂₄H₃₇N₄O₆S₂; calculated [M + H]⁺ of 541.2155 *m/z*), and collectively suggests an [M + H]⁺ signal of TS-FK228 (Fig. 1A insert). Further NMR analyses showed that this TS-FK228 possesses ¹H and ¹³C NMR profiles highly similar to the historical data of FK228,³ except for the chemical shifts of 14-CH₂ (56.6 ppm vs. 38.8 ppm) with down-shielded ¹H chemical shifts (3.55 ppm, 3.50 ppm) when compared with

those of FK228 (3.00 ppm, 3.14 ppm) (Table 1). This suggests that one oxygen atom is conjugated with 14-C sulfur atom in TS-FK228 (isomer 1 in Fig. 1B).

Origin of TS-FK228

Re-analysis of the purified TS-FK228 on LC-MS exhibited two separate peaks with the same extracted ion signal of 557 *m/z* (Fig. 2D). Those signals were not found in the original ethyl acetate extract of bacterial fermentation broth (Fig. 2A), but in the ethyl acetate extract after having mixed with silica gel for flash chromatography (Fig. 2B), and in the fractions of flash chromatography (Fig. 2C). Those observations led us to postulate that TS-FK228 existed as a mixture of two isomers 1 and 2 (Fig. 1B); they were not biosynthesized by the FK228 producer, but rather were derived chemically from FK228 through spontaneous reaction with silica gel (Fig. 3).

HDAC inhibitory activities and cytotoxicity of TS-FK228 in comparison with FK228

HDAC inhibition assays showed that TS-FK228 exhibited a similar pattern and magnitude of inhibition of representative

HDACs as did FK228 (Table 2A), suggesting that thio-sulfonation of the critical disulfide bond in FK228 does not affect the potency or specificity of HDAC inhibition. A simple interpretation of this observation is that both FK228 and TS-FK228 produce the same red-FK228 upon reduction by glutathione within cells (Fig. 3). In contrast, cytotoxicity assays revealed an average 9-fold increase of antiproliferative activities toward representative cancer cell lines upon thiosulfonation of FK228 (Table 2B). The enhanced cytotoxicity of TS-FK228 through thiosulfonation is likely due to the formation of additional reactive oxygen species (ROSs) perhaps superoxide anion, in addition to hydrogen peroxide (Fig. 3).

Discussion

Only a few naturally produced thiosulfonated or thio-sulfonated natural products were reported in the literature, including cysteine thiosulfonate observed in mammalian cysteine metabolism,²⁴ *S*-methyl methane thiosulfonate from cauliflower,²⁵ and propyl propane thiosulfonate and thiosulfonate from garlic.^{26–28} In this paper, we provided evidence to support a postulation that the observed TS-FK228

Table 1 A comparison between newly acquired ¹³C- and ¹H-NMR data of TS-FK228 and the historical ¹³C- and ¹H-NMR data of FK228.³ Except for the major value differences at C14 position (highlighted in bold), the other minor value differences are likely due to the use of different solvents and instruments, and under slightly difference experimental conditions

Position	δ_C (ppm)			δ_H (ppm)		
	TS-FK228 ^a	FK228 ^b		TS-FK228 ^a	FK228 ^b	
1	170.7	172.5	C=O			C=O
2	170.5	172.1	C=O			C=O
3	168.9	169.9	C=O			C=O
4	167.4	168.8	C=O			C=O
5	164.3	165.6	C=O			C=O
6	128.7	131.6	C			C
7	130.4	131.5	CH	5.71		CH
8	129.0	130.4	CH	5.90		CH
9	133.4	127.8	C	6.75		C
10	69.3	71.5	CH	5.72		CH
11	61.4	63.0	CH	4.18		CH
12	58.4	59.1	CH	4.63		CH
13	51.9	57.7	CH	5.40		CH
14	56.6	38.8	CH₂	3.55, 3.50		CH₂
15	39.5	38.4	CH ₂	2.87, 2.65		CH ₂
16	31.5	36.1	CH ₂	3.28, 3.80		CH ₂
17	31.8	32.0	CH	2.39		CH
18	27.4	31.0	CH ₂	2.80		CH ₂
19	29.5	29.6	CH	2.42		CH
20	19.5	19.5	CH ₃	1.10		CH ₃
21	18.5	19.4	CH ₃	1.08		CH ₃
22	18.5	18.9	CH ₃	1.02		CH ₃
23	18.4	18.8	CH ₃	1.08		CH ₃
24	13.9	13.4	CH ₃	1.75		CH ₃
			NH	6.31		NH
			NH	6.90		NH
			NH	7.12		NH
			NH	7.74		NH

^a TS-FK228 was obtained in this work from the fermentation broth of *B. thailandensis* MSMB43 after having mixed with silica gel and dissolved in CDCl₃. ¹³C NMR and ¹H NMR data were obtained at 125 MHz and at 500 MHz, respectively. ^b FK228 was historically obtained from the fermentation broth of *C. violaceum* No. A968 and dissolved in DMF-*d*₇. ¹³C NMR and ¹H NMR data were obtained at 100 MHz and at 400 MHz, respectively.

Fig. 2 Extracted ion chromatograms of the original ethyl acetate extract of fermentation broth (A), ethyl acetate extract after having mixed with silica gel (B), a fraction from flash chromatography (C), and the purified TS-FK228 (D), showing the presence of signals of TS-FK228 isomers 1 and 2.

was not produced biosynthetically, but rather derived from FK228 during interaction with silica gel matrix (Fig. 3). The similar chemical process happened to natural methoxylated phenylpropenes, where methoxylated phenylpropenes are directly transformed into corresponding dienones (1,5-diaryl-penta-2,4-dien-1-ones) and enones (chalcones and cinnamic esters) *via* allylic oxidation–condensation or allylic oxidation–esterification sequences.²⁹

On another note, the disulfide linkage in FK228 is asymmetrical. During oxidation of asymmetrical disulfide bond, the more electron-rich sulfur atom of disulfide is often oxidized first, followed by the remaining sulfur atom, thus to generate a

Fig. 3 Proposed mechanism for the nonenzymatic formation of TS-FK228 in the presence of silica gel (SiO_2) and the enhanced anti-proliferative activity of TS-FK228 through the production of additional reactive oxygen species (perhaps superoxide anion O_2^-) upon reduction by glutathione (GSH) inside cells.

Table 2 HDAC inhibitory activity (A) and cytotoxicity (B) of FK228 and TS-FK228

	HDAC enzymes	IC_{50} (μM)	
		FK228	TS-FK228
A	HDAC1	0.0024 ± 0.0002	0.0049 ± 0.0003
	HDAC4	4.462 ± 0.302	6.593 ± 0.59
	HDAC6	0.0091 ± 0.0012	0.011 ± 0.002
	Cell lines	GI_{50} (nM)	
		FK228	TS-FK228
B	HCT-116	11.6 ± 0.24	1.3 ± 0.44
	MDA-MB-231	11.5 ± 1.57	1.1 ± 0.69
	NCI-H232A	8.9 ± 1.11	1.1 ± 0.39

mixture of two thiosulfonate isomers.^{30,31} As such, the two extracted ion peaks we observed during re-analysis of purified TS-FK228 by LC-MS (Fig. 2D) supports our notion that TS-FK228 may exist as a mixture of two isomers 1 and 2 (Fig. 1B).

Finally, it is biochemically and genetically proven that the formation of a disulfide bond in FK228 after the biosynthesis of a macrolactam precursor requires a dedicated enzyme DepH;¹⁴ a similar enzymatic process occurs highly likely as well during the biosynthesis of other FK228-family of compounds including thailandepsins,^{22,32} spiruchostatins A and B,^{33,34} and FR901375.³⁵ However, caution is needed to interpret the formation of additional modifications on the sulphur atoms in those natural products as we have experienced with the accidental chemical thiosulfination of FK228. Consequently, if the accidental oxidation of FK228 and related natural products is not desirable, it can be remedied by changing the purification strategy.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- S. Yoon and G. H. Eom, *Chonnam Med. J.*, 2016, 52, 1–11.
- P. S. Suresh, V. C. Devaraj, N. R. Srinivas and R. Mullangi, *Biomed. Chromatogr.*, 2017, 31, e3807.
- N. Shigematsu, H. Ueda, S. Takase, H. Tanaka, K. Yamamoto and T. Tada, *J. Antibiot.*, 1994, 47, 311–314.
- H. Ueda, T. Manda, S. Matsumoto, S. Mukumoto, F. Nishigaki, I. Kawamura and K. Shimomura, *J. Antibiot.*, 1994, 47, 315–323.
- H. Ueda, H. Nakajima, Y. Hori, T. Fujita, M. Nishimura, T. Goto and M. Okuhara, *J. Antibiot.*, 1994, 47, 301–310.
- FDA, *J. Natl. Cancer Inst.*, 2010, 102, 219.

- 7 R. W. Robey, A. R. Chakraborty, A. Basseville, V. Luchenko, J. Bahr, Z. Zhan and S. E. Bates, *Mol. Pharmaceutics*, 2011, **8**, 2021–2031.
- 8 H. Habibi, A. Atashi, S. Abroun and M. Noruzinia, *Cell J.*, 2019, **20**, 576–583.
- 9 B. C. Valdez, Y. Li, D. Murray, Y. Liu, Y. Nieto, R. E. Champlin and B. S. Andersson, *Oncotarget*, 2018, **9**, 3908–3921.
- 10 Y. Kim, J. L. Anderson and S. R. Lewin, *Cell Host Microbe*, 2018, **23**, 14–26.
- 11 A. Petrich and C. Nabhan, *Leuk. Lymphoma*, 2016, **57**, 1755–1765.
- 12 H. Losson, M. Schnekenburger, M. Dicato and M. Diederich, *Molecules*, 2016, **21**, e1608.
- 13 Y.-Q. Cheng, M. Yang and A. M. Matter, *Appl. Environ. Microbiol.*, 2007, **73**, 3460–3469.
- 14 C. Wang, S. R. Wesener, H. Zhang and Y.-Q. Cheng, *Chem. Biol.*, 2009, **16**, 585–593.
- 15 S. R. Wesener, V. Y. Potharla and Y.-Q. Cheng, *Appl. Environ. Microbiol.*, 2011, **77**, 1501–1507.
- 16 V. Y. Potharla, S. R. Wesener and Y.-Q. Cheng, *Appl. Environ. Microbiol.*, 2011, **77**, 1508–1511.
- 17 Y. Qiao, T. Tong, J. Xue, W. Lin, Z. Deng, Y.-Q. Cheng and D. Zhu, *PLoS One*, 2018, **13**, e0196173.
- 18 R. Furumai, A. Matsuyama, N. Kobashi, K.-H. Lee, M. Nishiyama, H. Nakajima, A. Tanaka, Y. Komatsu, N. Nishino, M. Yoshida and S. Horinouchi, *Cancer Res.*, 2002, **62**, 4916–4921.
- 19 H. Mizutani, Y. Hiraku, S. Tada-Oikawa, M. Murata, K. Ikemura, T. Iwamoto, Y. Kagawa, M. Okuda and S. Kawanishi, *Cancer Sci.*, 2010, **101**, 2214–2219.
- 20 K. M. VanderMolen, W. McCulloch, C. J. Pearce and N. H. Oberlies, *J. Antibiot.*, 2011, **64**, 525–531.
- 21 X. Liu, F. Xie, L. B. Doughty, Q. Wang, L. Zhang, X. Liu and Y.-Q. Cheng, *Synth. Syst. Biotechnol.*, 2018, **3**, 268–274.
- 22 C. Wang, C. J. Flemming and Y.-Q. Cheng, *MedChemComm*, 2012, **3**, 976–981.
- 23 X. Liu, S. Biswas, M. G. Berg, C. M. Antapli, F. Xie, Q. Wang, M. C. Tang, G. L. Tang, L. Zhang, G. Dreyfuss and Y.-Q. Cheng, *J. Nat. Prod.*, 2013, **76**, 685–693.
- 24 J. H. Fellman and N. A. Avedovech, *Arch. Biochem. Biophys.*, 1982, **218**, 303–308.
- 25 Y. Nakamura, T. Matsuo, K. Shimoi, Y. Nakamura and I. Tomita, *Biol. Pharm. Bull.*, 1993, **16**, 207–209.
- 26 M. J. Peinado, R. Ruiz, A. Echavarri and L. A. Rubio, *Poult. Sci.*, 2012, **91**, 2148–2157.
- 27 P. Mellado-Garcia, S. Maisanaba, M. Puerto, A. I. Prieto, R. Marcos, S. Pichardo and A. M. Camean, *Food Chem. Toxicol.*, 2017, **99**, 231–240.
- 28 A. Sorlozano-Puerto, M. Albertuz-Crespo, I. Lopez-Machado, J. J. Ariza-Romero, A. Banos-Arjona, M. Exposito-Ruiz and J. Gutierrez-Fernandez, *BioMed Res. Int.*, 2018, **2018**, 7861207.
- 29 A. Sharma, N. Sharma, A. Shard, R. Kumar, D. Mohanakrishnan, Saima, A. K. Sinha and D. Sahal, *Org. Biomol. Chem.*, 2011, **9**, 5211–5219.
- 30 S. Oae, T. Takata and Y. H. Kim, *Bull. Chem. Soc. Jpn.*, 1982, **55**, 2484–2494.
- 31 N. M. McNeil, C. McDonnell, M. Hambrook and T. G. Back, *Molecules*, 2015, **20**, 10748–10762.
- 32 C. Wang, L. M. Henkes, L. B. Doughty, M. He, D. Wang, F. J. Meyer-Almes and Y.-Q. Cheng, *J. Nat. Prod.*, 2011, **74**, 2031–2038.
- 33 Y. Masuoka, A. Nagai, K. Shin-ya, K. Furihata, K. Nagai, K. Suzuki, Y. Hayakawa and H. Seto, *Tetrahedron Lett.*, 2001, **42**, 41–44.
- 34 V. Y. Potharla, C. Wang and Y. Q. Cheng, *J. Ind. Microbiol. Biotechnol.*, 2014, **41**, 1457–1465.
- 35 S. Fuse, K. Okada, Y. Iijima, A. Munakata, K. Machida, T. Takahashi, M. Takagi, K. Shin-ya and T. Doi, *Org. Biomol. Chem.*, 2011, **9**, 3825–3833.