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Thiazinogeldanamycin, a New Geldanamycin Derivative Produced by *Streptomyces hygroscopicus* 17997

Ni, Siyang[†], Linzhuan Wu[†]*, Hongyuan Wang, Maoluo Gan, Yucheng Wang, Weiqing He, and Yiguang Wang*

Key Laboratory of Biotechnology of Antibiotics of Ministry of Health, Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing 100050, China

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A new geldanamycin (GDM) derivative was discovered and isolated from the fermentation broth of *Streptomyces hygroscopicus* 17997. Its chemical structure was elucidated as thiazinogeldanamycin by LC–MS, sulfur analysis, and NMR. The addition of cysteine to the fermentation medium significantly stimulated the production level of thiazinogeldanamycin, suggesting cysteine as a precursor of thiazinogeldanamycin production. Although showing a decreased cytotoxicity against HepG2 cancer cells, thiazinogeldanamycin exhibited an improved water solubility and photostability. Thiazinogeldanamycin may represent the first natural GDM derivative characterized so far that uses GDM as its precursor. Its appearance also clearly indicates that an appropriate end-point of fermentation is of critical importance for the maximal production of GDM by *Streptomyces hygroscopicus* 17997.

Keywords: Thiazinogeldanamycin, *Streptomyces hygroscopicus* 17997

Geldanamycin (GDM) is a benzoquinone ansamycin produced by *Streptomyces hygroscopicus* [3] that can bind to heat shock protein 90 (Hsp90), a potential cellular target for antitumor agents in humans [12, 16]. Nonetheless, despite exhibiting remarkable cytotoxicities against tumor cells, GDM is only used as a promising lead compound owing to its poor water solubility, photosensitivity, and severe hepatotoxicity.

Hundreds of semisynthetic GDM analogs have already been developed, where 17-AAG (17-allylamino-17-demethoxygeldanamycin) and 17-DMAG [17-(dimethylaminoethylamino)-17-demethoxygeldanamycin] are two examples of potential antitumor agents [8, 13, 14]. In contrast, less than 30 natural GDM analogs/derivatives, including those obtained by genetic manipulation of the biosynthetic gene cluster of geldanamycin and mutasynthesis, have been discovered or created, and very few have shown any promise for further development. Among these, several biosynthetic intermediates have been generated by disruption of the post-PKS tailoring genes of geldanamycin biosynthesis. Structure elucidation of these intermediates, including 17-demethoxy-reblastatin, 4,5-dihydro-7-descarbamoyl-7-hydroxygeldanamycin, and 4,5-dihydrogeldanamycin from *gdmM*-, *gdmN*-, and *gdmP*-inactivated mutants, respectively, has led to a better understanding of the post-PKS processing steps in geldanamycin biosynthesis [5, 11, 15]. Moreover, a refined picture of GDM biosynthesis could be useful for obtaining more natural GDM analogs/derivatives.

Accordingly, based on interest in obtaining novel natural GDM derivatives with improved pharmacological profiles, the potential of *Streptomyces hygroscopicus* 17997, a GDM producer, was explored for novel natural GDM derivative(s), resulting in the discovery of thiazinogeldanamycin from its fermentation broth. Therefore, this note explains the discovery, structure elucidation, and some physicochemical properties of thiazinogeldanamycin.

First, frozen stock spores of *Streptomyces hygroscopicus* 17997 were thawed, spread onto ISPII medium plates (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%, agar power 1.5%), and incubated at 28°C for 8–10 days for mycelium growth and sporulation. A slice of the seed culture was then picked up and inoculated into a fermentation medium (starch 2%, cotton seed power 0.5%, glucose 0.5%, cornsteep liquor 1.0%, yeast powder 0.5%, $CaCO_3$ 0.2%) for shaking (200 rpm) at 28°C for 48–144 h.

For time-course monitoring of the above fermentation supernatants of *Streptomyces hygroscopicus* 17997, the supernatants were extracted at different times (48 h, 72 h, 96 h, 120 h, and 144 h) with equal volumes of ethyl acetate

*Corresponding authors
L. Wu
Phone: +86-10-63165283; Fax: +86-10-63017302;
E-mail: wulinzhuan@yahoo.com.cn
Y. Wang
Phone: +86-10-63038137; Fax: +86-10-63017302;
E-mail: wangyh456@yahoo.com.cn

[†]These two authors contributed equally to this work.

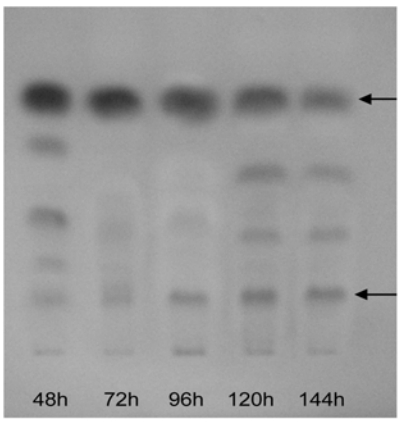

Fig. 1. Silica gel TLC (GF_{254}) of EtOAc extract of fermentation cultures of *Streptomyces hygroscopicus* 17997 (after color reaction by 2 mol/l NaOH).

(EtOAc). The organic layers were then concentrated and applied to silica gel TLC plates for a chromatograph [developed with a mobile phase of EtOAc/ CH_2Cl_2/ hexane/ methanol, 9:6:6:2 (v/v)] and alkaline (2 mol/l NaOH) color reaction for the preliminary discrimination of GDM and its derivatives [9]. An orange band appeared in the lower part of the silica gel TLC plate upon spraying 2.0 mol/l NaOH, (Fig. 1), while a blue band appeared at the same position upon spraying $FeCl_3$ (for specific detection of compounds with phenolic moiety). The band was more evident in the EtOAc extracts from the older fermentation supernatants of *Streptomyces hygroscopicus* 17997.

The compound(s) in the silica gel TLC plate, corresponding to the orange band but without alkaline treatment, was eluted by methanol for LC–MS analysis. A major peak appeared in the LC, and its principal compound had an *m/z* of 656.0 ($[M+Na]^+$) according to ESI(+)–MS (Fig. S1). The MS^2 of the *m/z* 656.0 displayed a fragmentation pattern typical of GDM analogs ($[M+Na–HOCONH_2]^+$, base peak; $[M+Na–CO_2]^+$; $[M+Na–HOCONH_2–HOCH_3]^+$; *etc*.) [7]. Therefore, the compound was believed to be a GDM derivative, and a novel GDM derivative based on a comparison of its molecular mass with known ones.

The compound was then purified for structure elucidation. An equal volume of EtOAc was used to extract the fermentation supernatant (11.5 l) of *Streptomyces hygroscopicus* 17997 (with a fermentation period of 120–144 h), which was then dried to a crude solid (3.6 g) by rotary evaporation at 37°C. After fractionation by silica gel chromatography, the pool containing the compound of interest [eluted by petroleum ether–EtOAc, 5:95 (v/v)] was dried (322 mg) and redissolved in methanol for preparative Sephadex LH-20 (φ1.8 cm×150 cm) fractionation to obtain a refined preparation of the compound (75 mg). This refined preparation was then used for semi-preparative HPLC [Shimadzu LC-10ATVp; Agilent ZorBax SB-C18, 5 μm, φ9.4 mm×250 mm, methanol/water, 45:55 (v/v), 1.5 ml/min], yielding a pure preparation of the compound (32 mg; purity≥93%, calculated by area % of HPLC analysis at 254 nm). Finally, the pure preparation, an amorphous colorless powder, was used for an elemental analysis of sulfur, HR-ESI–MS, and ^1H and ^{13}C NMR analyses.

The accurate mass of the compound was 656.26028 ($[M+Na]^+$) according to HR-ESI(+)–MS. Moreover, the compound contained *ca*. 4.77% sulfur based on the elemental analysis, indicating the presence of a sulfur atom in the molecule (calculated: 5.00%, without Na^+). Therefore, the molecular formula of the compound was deduced to be $C_{31}H_{43}O_9N_3S$ (exact mass, $[M+Na]^+$, 656.26122). The NMR data of the compound indicated that the only structure

Table 1. NMR data of thiazinogeldanamycin[a].

Carbon no.	δ_H (Mult, J^b)	δ_C
1		173.75
2		132.32
3	5.87 (1H, t, 9)	121.55
4	6.25 (1H, d, 11.5)	128.11
5	4.94 (1H, over)	128.61
6	3.70 (1H, t, 3)	72.48
7	4.76 (1H, d, 9)	80.01
8		129.25
9	5.03 (1H, over)	129.67
10	2.26 (1H, d, 6.6)	38.847
11	3.53 (1H, over)	71.65
12	2.81 (1H, over)	79.92
13	1.38/1.66 (2H, over)	30.68
14	1.95 (1H, over)	30.11
15	2.43/ 2.78 (2H, over)	29.44
16		119.42
17		145.60
18		123.93
19		115.98
20		121.75
21		146.72
22	3.203/3.471 (2H, d)	29.45
23		163.72
2-CH_3	1.89 (3H, s)	13.66
6-OCH_3	2.91 (3H, s)	55.28
7-O**C**ONH_2		155.92
8-CH_3	1.22 (3H, s)	11.49
10-CH_3	0.82 (3H, d, 5.9)	19.07
12-OCH_3	3.18 (3H, s)	55.57
14-CH_3	0.65 (3H, d, 5.9)	12.50
17-OCH_3	3.55 (3H, s)	60.62
18-N**H**-	9.67 (1H, s)	
20-N**H**-	8.73 (1H, s)	

[a] ^1H and ^{13}C NMR spectra were obtained at 600 and 125 MHz on a VNS-600 with TMS as internal standard, and 2D NMR spectra were obtained at 600 MHz on VNS-600, respectively, and measured in DMSO-d_6 at room temperature.
[b] Coupling constants are presented in Hz.

variations in comparison with GDM were in the benzoquinone ring [5]. In particular, the ^{13}C NMR and DEPT spectra showed an absence of the C19 methine carbon and the emergence of a new methylene carbon (C22, δ_C 29.45) and two new quaternary carbons (C19, δ_C 115.98; C23, δ_C 163.72). Additionally, instead of chemical shifts 184.13 and 184.97 in GDM (in $CDCl_3$), C18 and C21 appeared in the upfield with chemical shifts 123.93 and 146.72, respectively, suggesting that the benzoquinone moiety had changed to a hydroquinone or phenolic form (positive color reaction by $FeCl_3$ provided additional proof for this conclusion). Furthermore, the 2H of C22 coupled with C23 and C19, yet the most important signals were the coupling of a newly appeared H (18-N**H**-, δ_H 9.67) with C17, C18, C19, C22, and C23. Therefore, these data/ results suggested the formation of a thiazino- ring based on the phenolic moiety. The NMR chemical shifts of the compound were completely assigned from ^1H-^1H COSY, HSQC, and HMBC (Table 1, Fig. 2). The chemical structure of the GDM derivative was elucidated as thiazinogeldanamycin (Fig. 2 and 3).

GDM acts as the biosynthetic precursor of thiazinogeldanamycin. Therefore, to prove this, the bioconversion of GDM to thiazinogeldanamycin by a GDM polyketide synthase gene disruption mutant of *Streptomyces hygroscopicus* 17997 (designated as GDM-*pks*$^-$, which has a full complement of post-PKS processing genes for GDM biosynthesis, yet cannot produce GDM because of its disrupted PKS gene for GDM biosynthesis) was performed. Thiazinogeldanamycin was only detected after the addition of GDM (Fig. S2), proving that thiazinogeldanamycin came from GDM.

The thiazino moiety of thiazinogeldanamycin was quite similar to the thiazin-2-one moiety of rifamycin Verde in chemical structure. As the thiazino moiety of rifamycin Verde comes from cysteine [2], it was presumed that the thiazino moiety of thiazinogeldanamycin may also come from cysteine. To prove this, an additional bioconversion

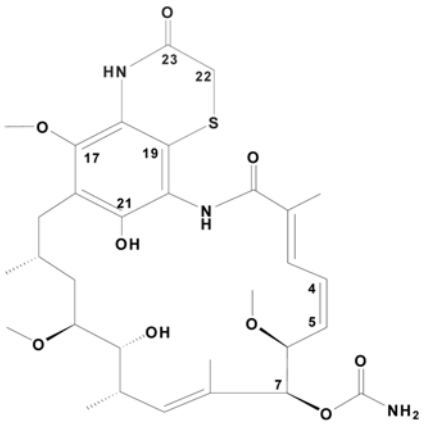

Fig. 2. The chemical structure of thiazinogeldanamycin.

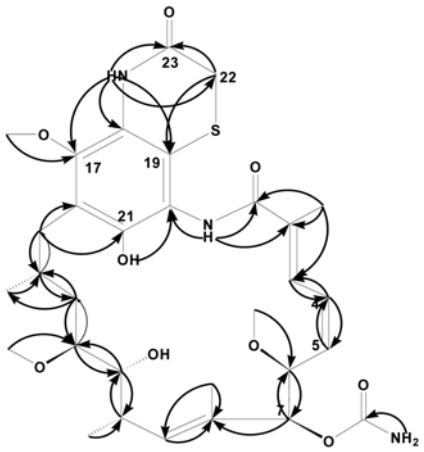

Fig. 3. Key long-range correlations of thiazinogeldanamycin in HMBC ($^1H \rightarrow ^{13}C$).

experiment was performed, in which GDM and cysteine were added simultaneously to a culture of GDM-*pks*$^-$. As expected, cysteine greatly increased the level of thiazinogeldanamycin bioconversion (Fig. S2). In addition, feeding cysteine to the fermentation culture of *Streptomyces hygroscopicus* 17997 significantly increased the production level of thiazinogeldanamycin (Fig. 4). Therefore, these results indicated that cysteine can act as the direct/immediate precursor for the formation of the thiazino moiety of thiazinogeldanamycin. Thiazinotrienomycins A and B [6] and TMC-135 A and B [10] are all triene-ansamycins that

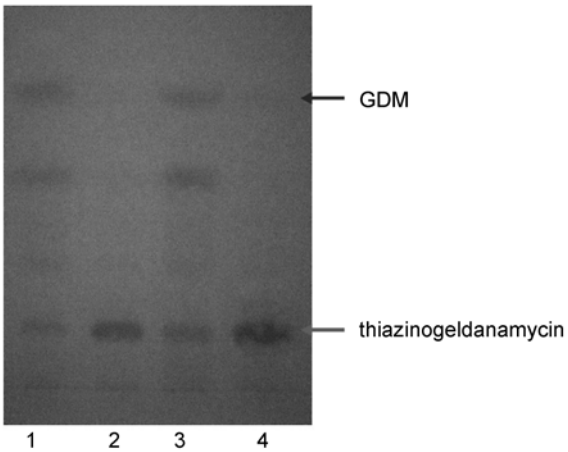

Fig. 4. Feeding of cysteine into the fermentation cultures of *Streptomyces hygroscopicus* 17997 improved significantly the production level of thiazinogeldanamycin.

A final concentration of 150 mg/l or 250 mg/l cysteine was feeded into the fermentation cultures of *Streptomyces hygroscopicus* 17997 at a fermentation time of 84 h, with shaking for another 36 h. The fermentation supernatant(s) was extracted with EtOAc, separated by silica gel TLC, and then subjected to alkaline (2 mol/l NaOH) spraying for comparing the levels of thiazinogeldanamycin produced. 1 & 3, without cysteine; 2 & 4, with 150 mg/l and 300 mg/l cysteine, respectively.

have been isolated from *Streptomyces* sp. and include a thiazino moiety in their molecules, making them very similar to thiazinogeldanamycin in chemical structure. Thus, it is reasonable to believe that cysteine may also act as the direct/immediate precursor for the formation of the thiazino moiety of these compounds.

When compared with GDM, thiazinogeldanamycin showed a remarkably increased water solubility (1.165 mg/ml for thiazinogeldanamycin, <1 μg/ml for GDM, at 25°C), plus it was more polar than GDM. In particular, the 18-NH and 21-OH of thiazinogeldanamycin may form hydrogen bonds with water molecules, thereby enhancing its water solubility. Nonetheless, the water solubility of thiazinogeldanamycin is still smaller than that of 17-DMAG, which is about 10 mg/ml. When compared with GDM, thiazinogeldanamycin also exhibited an increased photostability (Fig. S3). As far as is known, there is no conclusive explanation about the photodegradation mechanism of GDM. However, it is possible that the thiazino-ring moiety, which keeps thiazinogeldanamycin in a locked hydroquinone state, may help to increase its photostability [4].

Molecular docking with human Hsp90 by SYBYL (Tripos, a Certara Company) showed that the binding of thiazinogeldanamycin with Hsp90 differed from that of GDM (Fig. S4), with a CScore of 6.01 and 8.22, for thiazinogeldanamycin and GDM, respectively, suggesting that thiazinogeldanamycin may have a significantly reduced affinity for Hsp90 (CScore is an algorithm to estimate the binding affinity of a given protein–ligand complex with a known three-dimensional structure [1, 17]). In particular, the pattern of the hydrogen bonds formed between thiazinogeldanamycin and Hsp90 was quite different from that between GDM and Hsp90 (Fig. S4); for example, the carbamoyl moiety (critical for its cytotoxicity against cancer cells) of thiazinogeldanamycin forms no hydrogen bond at all with Hsp90, whereas the carbamoyl moiety of GDM forms two hydrogen bonds with Hsp90. A preliminary cytotoxicity assay indicated that thiazinogeldanamycin had an IC_{50} of 307 μM for HepG2 cells (GDM, 0.06 μM). This drastic decrease, yet still evident of the cytotoxicity of thiazinogeldanamycin against the hepatoma cells, confirmed the above molecular docking predictions.

In conclusion, the discovery of thiazinogeldanamycin indicates that GDM is not a closed product of biosynthesis, but can act as a precursor for other GDM derivative(s). Thiazinogeldanamycin may also represent the first natural GDM derivative characterized so far that uses GDM as its precursor. The discovery of thiazinogeldanamycin explains, at least in part, that an appropriate end-point of fermentation is of critical importance for the maximal production of GDM by *Streptomyces hygroscopicus* 17997 or other GDM producers, as it was confirmed that *Streptomyces hygroscopicus* subsp. *geldanus* ATCC 55256 (a GDM producer derived from *Streptomyces hygroscopicus* NRRL 3602) was also able to produce thiazinogeldanamycin.

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