Expanding the Chemical Diversity of the Antitumoral Compound Mithramycin by Combinatorial Biosynthesis and Biocatalysis: The Quest for Mithralogs with Improved Therapeutic Window

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Abstract

Mithramycin is an antitumor compound of the aureolic acid family produced by *Streptomyces argillaceus*. It has been used to treat several types of cancer including testicular carcinoma, chronic and acute myeloid leukemia as well as hypercalcemias and Paget's disease. Although the use of mithramycin in humans has been limited because its side effects, in recent years a renewed interest has arisen since new uses and activities have been ascribed to it. Chemically, mithramycin is characterized by a tricyclic aglycone bearing two aliphatic side chains attached at C3 and C7, and disaccharide and trisaccharide units attached at positions 2 and 6, respectively. The mithramycin gene cluster has been characterized. This has allowed for the development of several mithramycin analogs ("mithralogs") by combinatorial biosynthesis and/or biocatalysis. The combinatorial biosynthesis strategies include gene inactivation and/or the use of sugar biosynthesis plasmids for sugar modification. In addition, lipase-based biocatalysis enabled selective modifications of the hydroxyl groups, providing further mithramycin analogs. As a result, new mithramycin analogs with higher antitumor activity and/or less toxicity

have been generated. One, demycarosyl-3D- β -D-digitoxosyl-mithramycin SK (EC-8042), is being tested in regulatory preclinical assays, representing an opportunity to open the therapeutic window of this promising molecular scaffold.

Abbreviations

CNS:	central nervous system
DIG-MTM-SK:	demycarosyl-3D- β -D-digitoxosyl-MTM-SK
EWS-Flt1:	Ewing sarcoma-Flt1 fusion gene
FIH:	first-in-human (clinical trials)
GIST:	gastrointestinal tumors
NSCLC:	non-small cell lung carcinoma
MTM:	mithramycin
MTM-SA:	mithramycin short side chain acid
MTM-SDK:	mithramycin short side chain diketone
MTM-SK:	mithramycin short side chain ketone
NCI:	National Cancer Institute
NDP:	nucleotide diphosphate
PK:	pharmacokinetic
TDP:	thymidine diphosphate

Introduction

MTM is an antitumor compound of the aureolic acid family produced, among other bacteria, by *Streptomyces argillaceus*. It has been used to treat several types of cancer in the 1970 s, and later, hypercalcemias and Paget's disease until its discontinuation at the turn of the 21st century. Although the use of MTM in humans has been limited because of the lack of a therapeutic window, recent literature evidence linking its mechanism of action to both antitumoral and other therapeutic activities has fueled a renewed interest in this

privileged natural scaffold. The growing body of literature on MTM (more than 400 PubMed-indexed articles since 2000 only) showing promising biological activity, especially in oncology [1], reflects the potential of this molecular class for the treatment of different types of cancer. This trend is crowned by two recent MTM clinical trials sponsored by the NCI in two unrelated indications: Ewing sarcoma (NCT01610570) and NSCLC (NCT01624090), which address two different targets in each histology (EWS-FLI1 fusion gene in sarcoma [2] and ABCG2 transporter in lung cancer [3]). More recently, the potential of MTM for can-

Fig. 1 Genetic organization (A) and biosynthesis pathway (B) of mithramycin in *S. argillaceus*. **1** MTM; **2** 4-demethylpremithramycinone; **3** premithramycinone; **4** premithramycin A1; **5** 9-demethyl-premithramycin A2; **6** 9-demethyl-premithramycin A3; **9** premithramycin A3; **12** 3A-deolivosylpremithramycin B; **13** premithramycin B.

cers depending on constitutively activated targets, for example, GIST, a gastrointestinal tumor that depends on the continuous expression of the oncogenic c-kit kinase, has been reported using an unbiased compound screening [4]. Also, it has been found that MTM targets the chemotherapy resistant SOX2+ cell population in the sonic hedgehog subgroup medulloblastoma. Evidence of the ability of the molecule to target propagating tumor subpopulations (cancer stem cells) represents yet another high-profile example of MTM as a potential anticancer drug [5].

Despite the promising biological activity of MTM, which is generally related to its ability to modulate transcription, especially genes regulated by the Sp1 transcription factor, the drug industry still regards Sp1 as an undruggable target, at least by the drug discovery approaches currently in place. Aside from the (mostly distant) clinical experience with MTM, there has been only one attempt at the development of a Sp1-targeted drug, named terameprocol, whose development apparently stopped in 2007 after the sponsor completed FIH phase I clinical trials [6]. All these facts have stimulated an interest in MTM analogs (mithralogs) with improved properties, with a focus on those with lower toxicity, thus having better clinical chances than the parental natural product.

This review describes the results of the efforts to expand the chemical space of MTM and the biological properties of the analogs generated in a quest to identify a viable candidate for clinical development. Chemically, MTM (**1** Fig. 1B) is characterized by an aglycone with three rings and two side chains, which is glycosylated by a disaccharide and a trisaccharide chain. The MTM gene cluster has been cloned and characterized, and the biosynthesis pathway has been established. This has allowed the development of several MTM analogs by combinatorial biosynthesis and/or biocatalysis. Different strategies of combinatorial biosynthesis have been used to generate new MTM derivatives, including gene inactivation, gene expression, the use of sugar biosynthesis plasmids for sugar modification, and combinations of both approaches. In addition, biocatalysis has been used to further diversify the MTM scaffold. Since MTM is a polyhydroxylated molecule, the use of hydrolases, especially lipases, to selectively modify some of the hydroxy groups is a suitable approach to expand the chemical space both from the wild-type product and the combinatorial biosynthesis analogs.

As a result of all these strategies, new mithralogs with higher antitumor activity and/or less toxicity have been generated, and the efficacy of the selected analogs has been tested in murine models of human cancer. DIG-MTM-SK (EC-8042) is the lead can-

Fig. 2 MTM derivatives generated by gene inactivation: tetracyclic compounds. **2** 4-Demethylpremithramycinone; **3** premithramycinone; **4** premithramycin A1; **5** 9-demethyl-premithramycin A2; **6** 9-demethyl-premithramycin A3; **7** 9-C-methyl-premithramycin A1; **8** premithramycin A2; **9** premithramycin A3; **10** 4A-keto-9-demethylpremithramycin A2; **11** 4A-ketopremithramycin A2; **12** 3A-deoliviosylpremithramycin B; **13** premithramycin B; **14** premithramycinone G.

didate in the quest for mithralogs with an improved therapeutic window, since it is one order of magnitude less toxic than MTM *in vivo*, including regulatory species (rats and dogs). Importantly, it also shows a more superior PK profile than MTM because it is active both in cell assays and in xenograft tumor models [7].

Mithramycin Gene Cluster and Biosynthesis Pathway in *Streptomyces argillaceus*

The aim of combinatorial biosynthesis is to produce novel compounds by generating recombinant microorganisms containing combinations of biosynthesis genes that are not found in nature. The use of combinatorial biosynthesis requires knowledge of both the gene cluster and the pathway for the biosynthesis of the target compound. The MTM gene cluster of *S. argillaceus* ATCC 12956 has been cloned and characterized [8]. It comprises 34 genes and is flanked by two repeated DNA sequences of 241-bp [9] (Fig. 1A). Functions to these genes were assigned by comparing their gene products with similar proteins in databases, generating mutants by targeted gene inactivation, expres-

sion of sets of genes, and/or *in vitro* assays of the corresponding enzymes [8]. The pathway for MTM (Fig. 1B) starts by the condensation of ten malonyl-CoA units by the type II polyketide synthase MtmPKS to render a 20-carbon chain that undergoes regioselective cyclizations by aromatase MtmQ and cyclases MtmX and MtmY, oxygenation by MtmOII, and reductions to form the first isolable intermediate **2** [10–13]. Then, **2** is converted into **3** by *O*-methylase MtmMI [14], and in turn is sequentially glycosylated by glycosyltransferases MtmGIV, MtmGIII, and MtmGIV to render **6**, which contains a trisaccharide of D-olivose-D-olivose-D-mycarose incorporated at position C12a-O [15,16]. This compound is *C*-methylated at the C9 position by methylase MtmMII [14,17], and further glycosylated with two D-olivoses by the action of glycosyltransferases MtmGII and MtmGI to render **13** [18,19]. Then, the oxygenase MtmOIV opens the fourth ring via a Baeyer-Villiger reaction to generate MTM's characteristic tricyclic aglycone with a 5-carbon side chain [20–22]. This oxygenase plays a key role in the biosynthesis of MTM since it converts biologically inactive tetracyclic intermediate into active tricyclic MTM. Finally, the highly functionalized pentyl side chain generated after MtmOIV is reduced by ketoreductase MtmW to render

Fig. 3 MTM derivatives generated by gene inactivation: tricyclic compounds. **15** Premithramycin A4; **16** 4C-keto-demycarosyl-MTM; **17** 4E-keto-MTM; **18** 7-demethyl-MTM; **19** MTM-SK; **20** demycarosyl-MTM-SK; **21** MTM-SA; **22** MTM-SDK.

the final compound MTM [23], which is secreted outside the cell by the ABC transporter MtrAB [24].

Mithramycin Analogs by Gene Inactivation

Several strategies of combinatorial biosynthesis can be used to generate new derivatives of a target compound. One of these strategies is gene inactivation [25]. By generating mutants in *mtm* genes (*mtmMI*, *mtmMII*, *mtmD*, *mtmU*, *mtmC*, *mtmTIII*, *mtmGIII*, *mtmGII*, *mtmGI*, *mtmOII*, *mtmOIV*, *mtmW*), several new compounds were identified that showed either a tetracyclic (Fig. 2) or a tricyclic core structure (Fig. 3). Bioassays of these compounds established the first structure-activity relationships for mithralogs. That pointed out that antitumor activity required glycosylated derivatives to contain both a tricyclic core and a methyl group at the C7 position. Some of these compounds showed high antitumor activity. Thus, by inactivating *mtmC* that codes for an enzyme that catalyzes the 4-ketoreduction and the C3-methyl transfer reactions during the biosynthesis of TDP-D-olivose and TDP-D-mycarose, respectively [16,26], three new compounds were generated (**10** and **11** in Fig. 2; **16** in Fig. 3), all lacking D-mycarose and containing an unreduced C-4 carbonyl group at the D-olivose of the trisaccharide [27]. Compound **16** (Fig. 3) showed antitumor activity against human tumor cell lines of lung and breast cancer. Moreover, by inactivating the ketoreductase gene *mtmW* responsible for reducing the 3-carbon side chain, four new compounds were generated (**19–22**, Fig. 3), all containing unreduced and shorter carbon side chains [23,28]. All four compounds showed high antitumor activity, with **19** (MTM-SK) (up to 9 times higher than MTM, particularly against melanoma, leukemia, ovarian, and CNS cancer cells) and **22** (MTM-SDK) (up to 2 times higher than MTM and MTM-SK against several ovarian cancer cell lines) [23,28] being the most active ones.

Generation of Mithramycin Derivatives by Modifying the Tricyclic Core of the Aglycone by Gene Expression

Another strategy for combinatorial biosynthesis is the use of genes from pathways involved in the biosynthesis of structurally related compounds. The tetracyclic aglycone of MTM (premithramycinone) structurally resembles that of antitumorals nogalamycin and tetracenomycin C. By introducing a plasmid containing genes for nogalamycin aglycone (including the ketoreductase *snoaD* and the aromatase *snoaE*) into *S. argillaceus*, four new compounds were generated lacking the C8 hydroxyl group and consequently the disaccharide chain (compounds **23** to **26** in Fig. 4A) [29]. On the other hand, by expressing the oxygenase *tcmH* from the tetracenomycin gene cluster in the mutant strain *S. argillaceus* M702 (lacking oxygenase *mtmOII*) [12], the new hybrid compound **27** (Fig. 4B) was generated [30]. This compound showed changes in the structure of the aglycone that prevented its further glycosylation, however, it showed anticancer activity in the same range as MTM against tumor cell lines of squamous cell carcinoma, melanoma, NSCLC, and breast carcinoma [30].

Generation of Mithramycin Derivatives with Altered Glycosylation Profile

As mentioned above, MTM is a glycosylated compound with an upper disaccharide chain of D-olivose-D-olivose, and a lower trisaccharide chain of D-olivose-D-olivose-D-mycarose. All these sugars are 2,6-deoxyhexoses synthesized from glucose-1-phosphate through four common enzymatic steps to give rise to the intermediate NDP-4-keto-2,6-dideoxy-D-glucose, which is further methylated and/or reduced to render the final activated deoxysugars that are substrates for glycosyltransferases [16,26,31]. Gene inactivation experiments of genes involved in the biosynthesis of MTM sugars (*mtmD*, *mtmU*, *mtmV*, *mtmC*, and *mtmTIII*)

Fig. 4 MTM derivatives with modified aglycone generated by expressing (A) the ketoreductase *snoaD* and the aromatase *snoaE* into *S. argillaceus*, or (B) the oxygenase *tcmH* into *S. argillaceus* M7O2. **23** 8-Dehydroxyl-premithramycinone; **24** 8-dehydroxyl-premithramycin A1; **25** 8-dehydroxyl-9-demethyl-premithramycin A2; **26** 8-dehydroxyl-9-demethyl-premithramycin A3; **27** premithramycinone H.

Fig. 5 MTM derivatives glycosylated at the C9 position generated by expressing heterologous glycosyltransferases. **28** 9-C-Olivosyl-4-O-demethylpremithramycinone; **29** 9-C-oliviosyl-premithramycinone; **30** 9-C-mycarosyl-4-O-demethylpremithramycinone; **31** 9-C-mycarosyl-premithramycinone; **32** 9-C-di-oliviosyl-premithramycinone.

or in their transfer to the aglycone (mtmGI, mtmGII, mtmGIII, and mtmGIV) indicated that these sugars are key for antitumoral activity [15, 18, 26, 27]. Therefore, several attempts were carried out in order to modify the glycosylation pattern of MTM to generate new potentially bioactive derivatives.

Urdamycin A is a glycosylated angucycline polyketide produced by *Streptomyces fradiae* Tü2717 that contains the D-olivose-L-rhodinose-D-olivose trisaccharide linked by a C-glycosidic bond to the aglycone, which resembles, to some extent, MTM intermediates **2** and **3** (Fig. 2). By expressing glycosyltransferase UrdGT2 that catalyzes the C-glycosylation of the urdamycin aglycone [32] in mutants *S. argillaceus* M3G4 (a mtmGIV-minus mutant, blocked at the first glycosylation step of MTM [15]) and *S. argillaceus* M3ΔMG (lacking all glycosyltransferases, mtmGI-IV, and methylases mtmMI and MtmMII [20]), four novel C-glycosylated compounds were generated (**28–31**, Fig. 5) that contained a D-oliviosyl or a D-mycarosyl residue attached through a C-glycosidic bond to either **2** or **3** [33]. In addition, the co-expression of glycosyltransferases UrdGT2 and LanGT1 (the last from the angucycline landomycin gene cluster of *Streptomyces cyanogenus* S136 [34]) in *S. argillaceus* M3G4 resulted in the formation of the novel derivative **32** (Fig. 5) with the D-olivose-D-olivose disaccharide attached by a C-glycosidic bond to the aglycone [33].

On the other hand, new mithralogs with different glycosylation profiles were generated by endowing the producer strain with the capability to synthesize new sugars. To achieve this aim, several “sugar plasmids” coding for the biosynthesis of different NDP-deoxysugars were constructed (L-rhamnose, D- or L-olivose, D-oliosyl, D- or L-digitoxose, D-boivinose, D- or L-amicetose, L-rhodinose, L-mycarose, and L-chromose B) [35–38]. By individually introducing these plasmids into the *S. argillaceus* wild-type strain, *S. argillaceus* M7U1 (blocked in the biosynthesis of D-olivose [26]), or *S. argillaceus* M7C1 (blocked in D-mycarose biosynthesis [26]), 15 new compounds were generated (**33–47**, Fig. 6) differing from MTM in the glycosylation pattern [7, 39, 40]. Most of the compounds reflected high antitumor activity at micromolar concentrations against a panel of three tumor cell lines, with **33**, **35**, and **36** being the most active ones [39, 40]. Moreover, an apoptosis TUNEL assay revealed that **33** and **35** showed improved activity against the estrogen receptor (ER)-positive human breast cancer cell line MCF-7 compared with the parent drug MTM. In addition, **33** and **36** showed significant effects on the ER-negative human breast cancer cell line MDA-MB-231, which is not inhibited by the parent drug MTM [39].

Fig. 6 MTM derivatives with altered glycosylation patterns generated by introducing “sugar plasmids” into (A) a wild-type strain of *S. argillaceus* and into mutants (B) *S. argillaceus* M7U1 and (C) *S. argillaceus* M7C1. **33** Demycarosyl-3D- β -D-digitoxyl-MTM; **34** deoliosyl-3C- α -L-digitoxyl-MTM; **35** deoliosyl-3C- β -D-mycarosyl-MTM; **36** 3A-deoliosyl-MTM; **37** demycarosyl-MTM; **38** dideoliosyl-6- β -D-amicetosyl-MTM; **39** deoliosyl-demycarosyl-3C- β -D-boivinosyl-MTM; **40** deoliosyl-demycarosyl-3C- β -D-olivosyl-3D- β -D-digitoxyl-MTM; **41** dideoliosyl-6- β -D-amicetosyl-deoliosyl-3C- β -D-olivosyl-MTM; **42** dideoliosyl-6- β -D-amicetosyl-deoliosyl-demycarosyl-3C- β -D-olivosyl-3D- β -D-digitoxyl-MTM; **43** dideoliosyl-6- β -D-amicetosyl-deoliosyl-demycarosyl-3C- β -D-boivinosyl-MTM; **44** dideoliosyl-6- β -D-amicetosyl-demycarosyl-2-O- β -D-olivosyl-3C- β -D-olivosyl-MTM; **45** dideoliosyl-6- β -D-amicetosyl-demycarosyl-MTM; **46** deoliosyl-demycarosyl-3C- β -D-amicetosyl-MTM **47** dideoliosyl-6- β -D-amicetosyl-deoliosyl-demycarosyl-3C- β -D-amicetosyl-MTM.

Mithramycin Derivatives with Acetylated Sugars

MTM is structurally related to the antitumor compound chromomycin A_3 produced by *Streptomyces griseus* subsp. *griseus*, differing in the configuration of some of the sugars and in the absence of a methyl and the presence of two acetyl groups in three chromomycin sugars. These differences impact their antitumor activity; chromomycin A_3 is one order of magnitude more active than MTM [41]. The chromomycin biosynthesis gene cluster contains

the acyltransferase *cmmA* involved in acetylating chromomycin sugars. Inactivation of this gene resulted in the production of a compound approximately 100 times less active, which highlights the importance of acetyl groups for the antitumor activity of chromomycin A_3 [42]. To produce new acetylated MTM derivatives with potentially high antitumor activity, the mutant strain *S. griseus* C10GIV blocked in the first glycosylation step (*cmmGIV*-minus mutant [43]), but expressing *cmmA*, was fed with MTM and different MTM derivatives. In this way, seven

new MTM derivatives were generated that differ from MTM in the presence of one, two, or three acetyl groups attached at specific positions of different sugars of the saccharide chains as well as in the glycosylation pattern or in the structure of the aglycone

Fig. 7 Acetylated MTM derivatives generated by the bioconversion of MTMs in *S. griseus* C10GIV. **48** 4E-O-Acetyl-MTM; **49** demycarosyl-3D-β-D-digitoxosyl-3E-O-acetyl-MTM; **50** demycarosyl-3D-β-D-digitoxosyl-4D-O-acetyl-MTM; **51** demycarosyl-3D-β-D-digitoxosyl-4E-O-acetyl-MTM; **52** demycarosyl-3D-β-D-digitoxosyl-3E,4D-O-diacetyl-MTM; **53** demycarosyl-3D-β-D-digitoxosyl-3E,4E,4D-O-triacetyl-MTM; **54** 4D-O-acetyl-MTM-SK.

side chain (**48–54**, **Fig. 7**) [44]. All these new mithralogs showed antitumor activity at micromolar or lower concentrations against a panel of four tumor cell lines. For example, **51** and **49** showed improved activity against glioblastoma and colon cancer cells respectively, while **49**, **50**, and **53** showed improved activity against pancreatic cancer tumor cells [44]. Since acetylated derivatives could be hydrolyzed in aqueous conditions compatible with biological activity, no further efforts were dedicated to this series of compounds.

Mithramycin Derivatives with Modifications Both at the Aglycone Side Chain and in the Sugar Profile

From the abovementioned novel MTM derivatives generated by combinatorial biosynthesis, both **33** (**Fig. 6**) [39,40] and those containing a short 3-carbon side chain, **19–22** (**Fig. 3**), were of special interest since they showed very high antitumor activity [23,28]. Therefore, by providing mutant *S. argillaceus* M3W1 [23] with the capability to synthesize D-digitoxose [38], three novel derivatives were generated that differed from the parental compound in both the glycosylation pattern and the structure of the 3-carbon side chain, DIG-MTM-SK **55** and the MTM-SDK analogs **56** and **57** (**Fig. 8**) [7]. All three compounds showed high antitumor activity in an NCI-60 anticancer drug screening, with GI₅₀ values between 10 nM and 1 μM for most cell lines. DIG-MTM-SK and **57** showed the highest antitumor activities, being particularly active against ovarian tumor IGROV1 and breast tumor MDA-MB-231 cell lines [7]. In a hollow fiber assay, which is an *in vivo* test of antiproliferative activity in multiple cancer cell lines, DIG-MTM-SK was more potent than MTM, indeed one of the most potent ever registered on the NCI records, emphasizing its potential as a broad antitumor agent. *In vivo* xenograft data, using colon (COLO-205) and melanoma (SK-MEL-2) models, showed efficacy, especially in melanoma, indicating the compound deserves further development. Comparison of administration schedules every other day or every three days resulted in better efficacy in the more spaced, higher dose groups, indicating DIG-MTM-SK action might depend on maximum plasma concentration rather than on plasma half-life.

Generation of Mithramycin Derivatives by Biocatalysis

Biocatalysis has become, in recent times, a powerful tool for optimizing complex bioactive compounds isolated from natural sources. Chemical modification of lead molecules for drug development remains challenging in structurally complex natural products, limiting the potential of organic synthesis to expand their chemical space, and is often exacerbated by the chemical fragility

Fig. 8 MTM derivatives with both a modified 3-carbon side chain and an altered glycosylation pattern, generated by introducing a plasmid for D-digitoxose into *S. argillaceus* M3W1. **55** Demycarosyl-3D-β-D-digitoxosyl-MTM-SK; **56** demycarosyl-MTM-SDK; **57** demycarosyl-3D-β-D-digitoxosyl-MTM-SDK.

A

58 $R_1 = \text{COCH}_3$	$R_2 = \text{H}$	$R_3 = \text{H}$
59 $R_1 = \text{H}$	$R_2 = \text{COCH}_3$	$R_3 = \text{H}$
60 $R_1 = \text{COCH}_3$	$R_2 = \text{COCH}_3$	$R_3 = \text{H}$
61 $R_1 = \text{COCH}_2\text{Cl}$	$R_2 = \text{H}$	$R_3 = \text{H}$
62 $R_1 = \text{H}$	$R_2 = \text{COCH}_2\text{Cl}$	$R_3 = \text{H}$
63 $R_1 = \text{COCH}_2\text{Cl}$	$R_2 = \text{COCH}_2\text{Cl}$	$R_3 = \text{H}$
64 $R_1 = \text{COCH}_2\text{CH}_2\text{CH}_3$	$R_2 = \text{H}$	$R_3 = \text{H}$
65 $R_1 = \text{H}$	$R_2 = \text{COCH}_2\text{CH}_2\text{CH}_3$	$R_3 = \text{H}$
66 $R_1 = \text{COCH}_2\text{CH}_2\text{CH}_3$	$R_2 = \text{COCH}_2\text{CH}_2\text{CH}_3$	$R_3 = \text{H}$
67 $R_1 = \text{COCH}=\text{CHCH}_3$	$R_2 = \text{H}$	$R_3 = \text{H}$
68 $R_1 = \text{H}$	$R_2 = \text{COCH}=\text{CHCH}_3$	$R_3 = \text{H}$
69 $R_1 = \text{H}$	$R_2 = \text{COCH}=\text{CHPh}$	$R_3 = \text{H}$
70 $R_1 = \text{COPh}$	$R_2 = \text{H}$	$R_3 = \text{H}$
71 $R_1 = \text{H}$	$R_2 = \text{COPh}$	$R_3 = \text{H}$
72 $R_1 = \text{CO}(\text{CH}_2)_8\text{CH}_3$	$R_2 = \text{H}$	$R_3 = \text{H}$
73 $R_1 = \text{H}$	$R_2 = \text{CO}(\text{CH}_2)_8\text{CH}_3$	$R_3 = \text{H}$
74 $R_1 = \text{H}$	$R_2 = \text{H}$	$R_3 = \text{CO}(\text{CH}_2)_8\text{CH}_3$

B

75 $R_1 = \text{H}$	$R_2 = \text{COOCH}_2\text{CH}=\text{CH}_2$	$R_3 = \text{H}$
76 $R_1 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$	$R_2 = \text{H}$	$R_3 = \text{H}$
77 $R_1 = \text{H}$	$R_2 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$	$R_3 = \text{H}$
78 $R_1 = \text{H}$	$R_2 = \text{H}$	$R_3 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$
79 $R_1 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$	$R_2 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$	$R_3 = \text{H}$
80 $R_1 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$	$R_2 = \text{H}$	$R_3 = \text{COCH}_2\text{CH}_2\text{CO}_2\text{H}$

C

81 $R_1 = \text{COPh}$	$R_2 = \text{COCH}_3$	$R_3 = \text{H}$
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Fig. 9 Acylated MTM derivatives generated by lipase catalyzed reactions employing (A) vinyl esters, (B) anhydrides and carbonates, or (C) by lipase sequential acylation. **58** 4'-O-Acetyl-MTM; **59** 3B-O-acetyl-MTM; **60** 4',3B-O-diacetyl-MTM; **61** 4'-O-chloroacetyl-MTM; **62** 3B-O-chloroacetyl-MTM; **63** 4',3B-O-di-chloroacetyl-MTM; **64** 4'-O-butanoyl-MTM; **65** 3B-O-butanoyl-MTM; **66** 4',3B-O-dibutanoyl-MTM; **67** 4'-O-crotonyl-MTM; **68** 3B-O-crotonyl-MTM; **69** 3B-O-cinnamoyl-MTM; **70** 4'-O-benzoyl-MTM; **71** 3B-O-benzoyl-MTM; **72** 4'-O-decanoyl-MTM; **73** 3B-O-decanoyl-MTM; **74** 4B-O-decanoyl-MTM; **75** 3B-O-[(allyloxy)carbonyl]-MTM; **76** 4'-O-succinyl-MTM; **77** 3B-O-succinyl-MTM; **78** 4B-O-succinyl-MTM; **79** 4',3B-O-disuccinyl-MTM; **80** 4',3B-O-disuccinyl-MTM; **81** 4'-O-benzoyl-3B-O-acetyl-MTM.

of some of the functional groups. Biocatalysts can circumvent most of the aforementioned problems, as they exhibit high selectivity and operate under mild conditions in both aqueous and organic media. Particularly, the regioselective enzymatic acylation was one of the earliest biocatalytic transformations practiced in natural products since many of them are polyhydroxylated molecules [45]. Accordingly, MTM, which bears 9 hydroxyl groups in both the oligosaccharide and aglycone domains, was selectively acylated by lipases A and B from *Candida antarctica* (CAL-A and CAL-B) to provide 22 new mithralogs, **58–80** (Fig. 9A, B) [46]. Specifically, CAL-B was highly regioselective towards the 4'-hydroxyl group of the aglycone, and sterically hindered or poorly reactive esters provided the corresponding 4'-monoacyl derivatives in excellent yields. On the other hand, the use of more reactive acyl donors led to mixtures of mono- and diacylated derivatives in the 4' and 3B-hydroxyl groups. CAL-A, meanwhile, showed regioselectivity towards the disaccharide domain, acylating the 3B- or 4B-hydroxyl groups exclusively. As a result, and just by changing the acylating agent, a plethora of mono- and diacylated mithralogs were obtained (Fig. 9). Furthermore, the different regioselectivity of the lipases allowed their use in a se-

quential mode to produce mixed diacylated products, such as **81** (Fig. 9C). Consequently, the structure of MTM can be tuned by a rational design of the acylation sequence, multiplying the number of possible derivatives and expanding the molecular diversity of this aureolic acid scaffold.

Similarly, MTM-SK and MTM-SDK (Fig. 3) were also submitted to enzymatic acylation leading to novel mithralogs by combining genetic engineering and biocatalysis. These compounds were acylated by CAL-B and CAL-A in the hydroxyl groups of the 3B and 4B positions of the disaccharide, with the aglycone remaining unaltered, providing several acylated MTM derivatives, such as **82–90** (Fig. 10). It is worth highlighting that the biocatalytic acylation is complementary to the genetic engineering approach described above for the generation of MTM derivatives with acetylated sugars (Fig. 7). In that case, by expressing the acyltransferase *cmmA*, it was possible to acylate hydroxyl groups of sugars D and E only (Fig. 7), while lipases can act over both aglycone and sugar B (Figs. 9 and 10).

Along these libraries of acyl derivatives (Figs. 9 and 10), some exhibited potency comparable to, or slightly greater than, the parent drugs. For example, **59** (Fig. 9) was better than MTM

Fig. 10 Acylated derivatives of MTM-SK (**A, B**) and MTM-SDK (**C, D**) generated by lipase catalyzed acylation employing vinyl esters (**A, C**) or carbonates (**B, D**). **82** 3B-O-Acetyl-MTM-SK; **83** 4B-O-acetyl-MTM-SK; **84** 3B-O-crotonyl-MTM-SK; **85** 3B-O-benzoyl-MTM-SK; **86** 3B-O-[(allyloxy)carbonyl]-MTM-SK; **87** 3B-O-acetyl-MTM-SDK; **88** 3B-O-crotonyl-MTM-SDK; **89** 3B-O-benzoyl-MTM-SDK; **90** 3B-O-[(allyloxy)carbonyl]-MTM-SDK.

Fig. 11 Semisynthetic MTM-SA derivatives generated chemically by coupling with hydrazine derivatives (**91**), amines (**92**), diamines (**93**), or amino acid methyl esters (**94–98**).

against lung (A549), colon (CoLo 205), and breast (MCF7) tumor cells, and **72** (● Fig. 9) was the most effective against A498 renal tumor cells. Also, **87** (● Fig. 10) showed notably higher activity than the parental MTM-SDK, as judged by GI_{50} values 2 to 4 times lower in colon (CoLo 205), ovary (OVCAR-3), lung (A549), and renal (A498) cancer cell lines [46]. On the other hand, acetylated compounds in the sugar domain are prone to hydrolysis in aqueous conditions, which limits their use *in vivo* due to instability reasons [46].

Semisynthetic Approaches

Due to the high level of functionalization of aureolic acids, it has been difficult to modify their structure by semisynthesis. Thus, most of the chemically modified aureolic acid derivatives have been reported for olivomycin A, with the changes focusing only on the side chain of the aglycone moiety [47–49]. Regarding MTM, the only reported example is the modification of **21** (MTM-SA), which bears a short 3-carbon side chain with a carboxylic acid, a negative charge that causes repulsion with the DNA phosphate backbone, which is presumably the reason for the poor activity of MTM-SA. The acid function reacted with several primary amines to provide amide analogs **91–98** (● Fig. 11), and their activity was measured in A549 lung tumor cells. Some derivatives showed a notably improved activity with respect to MTM-SA, particularly, the derivative coupled with methyl glycine **95** showed comparable activity to that of MTM-SK [50].

Bioactivity of New Mithramycin Derivatives

Further studies regarding the mechanism of action or *in vivo* activity were carried out on selected mithralogs, mainly MTM-SK, MTM-SDK, and DIG-MTM-SK. The initial driver to focus on these three compounds was the differential properties with respect to other analogs. In the case of MTM-SDK, it was because of its increased antitumor activity, and in the case of MTM-SK and DIG-MTM-SK, it was because of the much lower toxicity *in vivo* [7]. ● Table 1 summarizes the results of the studies carried out on these mithralogs.

Early studies showed that changes in the aglycone caused MTM analogs to lose their ability to bind DNA, except the analog modified in the side chain, MTM-SK, that showed the same DNA binding specificity but with lower binding affinity than MTM [51]. Its weaker interaction with DNA hinted at the possibility of being much less toxic than MTM. This fact was supported by comparing the cytotoxicity of MTM and MTM-SK using 3T3 mouse fibroblasts, resulting in MTM-SK being 1500-fold less toxic than MTM [23]. *In vitro* studies of MTM-SK in colon cancer cell lines, both wild type or with inactivation of p53 and p21, contribute to understanding the mechanism of action of the drug, especially regarding cell cycle and cell death. Treatment of wild-type HCT-116 (p53^{+/+}) colon carcinoma cells with MTM-SK results in polyploidization and mitotic catastrophe, which occurs after a transient halt in the G1 phase followed by the overtaking of the G2-M checkpoint upon a washout period. Cells died mainly by necrosis, involving caspase-3. However, p53^{-/-} cells died mainly following G2-M arrest by p53-independent apoptosis, which appeared to be mediated by caspase-2 [52]. In a follow-up study, treatment of HCT-116 p21^{-/-}, a p21 deficient human colon carcinoma cell line, caused transient arrest in G2-M, with some cells entering a faulty mitotic cycle without cytokinesis that resulted

Table 1 Studies on the biological effects and mechanism of action of selected mithralogs.

Mithralog [reference]	Major results
MTM-SK [51]	<ul style="list-style-type: none"> ▶ MTM-SK is comparable to MTM in promoter reporter, gene expression, and DNA binding specificity ▶ MTM-SK shows lower DNA binding affinity than MTM, which might hint at a lower toxicity than MTM
MTM-SK [23]	<ul style="list-style-type: none"> ▶ MTM-SK is up to 9× more active than MTM in some cell lines, MTM-SK is 1500× less toxic than MTM in 3 T3 mouse fibroblasts; improved <i>in vitro</i> therapeutic index
MTM-SDK, MTM-SK [28]	<ul style="list-style-type: none"> ▶ Antiproliferative activity on seven ovarian cancer cell lines; MTM-SDK twice as active as MTM-SK or MTM ▶ MTM-SDK is a potent inducer of apoptosis and it has minimal effects on normal cells ▶ MTM-SDK is a potent inhibitor of Sp1 reporter activity and interferes minimally with other transcription factors ▶ MTM-SDK gene expression microarray (A2780 cell line) shows repressed transcription of genes implicated in cell cycle, apoptosis, migration, invasion, and angiogenesis
MTM-SK [52]	<ul style="list-style-type: none"> ▶ MTM-SK treatment causes mitotic catastrophe and polyploidy in HCT-116 (p53^{+/+}) cells ▶ HCT-116 (p53^{+/+}) colon carcinoma cells are committed to die by necrosis rather than apoptosis even in presence of active caspase-3, but p53^{-/-} cells die by apoptosis mediated by caspase-2
MTM-SK [53]	<ul style="list-style-type: none"> ▶ Apoptosis in MTM-SK-treated HCT-116 p21^{-/-} colon cancer cells involves caspase-2 rather than caspase-3 ▶ The absence of p21^{WAF1} makes HCT116 cells more sensitive to MTM-SK than to MTM ▶ MTM-SK treatment activates caspase-3 in wild-type HCT116 ▶ MTM-SK is more active than MTM on HCT116 cells lacking the p53 gene
MTM, MTM-SK	<ul style="list-style-type: none"> ▶ DNA binding of MTM (UV melting and competition dialysis) is tighter than that of MTM-SK at 25 °C ▶ DNA binding of MTM and MTM-SK is entropically driven; hydrophobic transfer from solution to binding site
MTM, MTM-SK	<ul style="list-style-type: none"> ▶ DNA binding shows differences depending on the alkyl chains linked to C-3 in the mithramycin analogs
MTM-SDK, MTM-SA [55]	<ul style="list-style-type: none"> ▶ MTM-SDK has the highest binding affinity to DNA
MTM, MTM-SK, MTM-SDK, MTM-SA [56]	<ul style="list-style-type: none"> ▶ Analogs differentially inhibit the rate of cleavage by restriction enzymes that recognize C/G-rich tracts ▶ Correlation between the strength of binding to DNA and antiproliferative activity ▶ Differences in the uptake and retention correlate to improved antiproliferative activities
MTM-SDK, MTM-SK [57]	<ul style="list-style-type: none"> ▶ Expression of Sp1 and VEGF are frequently increased in human epithelial ovarian cancers ▶ MTM-SDK and MTM-SK act as potent inhibitors of Sp1-dependent transcription both <i>in vitro</i> and <i>in vivo</i> ▶ Both are well tolerated in mice and delay growth of subcutaneous ovarian tumor xenografts ▶ MTM-SDK is effective in orthotopic tumors; significant delay of tumor progression
MTM-SDK, MTM-SK [58]	<ul style="list-style-type: none"> ▶ Both are highly effective <i>in vitro</i> in inhibiting proliferation of prostate cancer cells and transcription of Sp-regulated genes ▶ Both are well tolerated with maximum tolerated doses 4× (MTM-SDK) and 32× (MTM-SK) higher than MTM ▶ Both compounds are cleared rapidly from the bloodstream in mice, but maintain plasma levels well above the concentrations required <i>in vitro</i> for inhibition of Sp activity and cell proliferation ▶ MTM-SDK and MTM-SK inhibit transcription of Sp-regulated genes in prostate tumor xenografts ▶ MTM-SDK and MTM-SK exhibit potent antitumor activity in a prostate tumor model ▶ MTM-SDK and MTM-SK inhibit metastasis to the lung in a metastatic prostate tumor model
DIG-MTM [40]	<ul style="list-style-type: none"> ▶ Antiproliferative assays: IC₅₀ for DIG-MTM is low double-digit nanomolar, similar to MTM ▶ Compounds lacking a trisaccharide unit are 5- to 100-fold less active
DIG-MTM [39]	<ul style="list-style-type: none"> ▶ DIG-MTM induces higher apoptosis than MTM in ER-human breast cancer cell lines ▶ DIG-MTM, but not MTM, induces apoptosis in ER+ human breast cancer cell lines
Biocatalysis mithralogs [44, 46]	<ul style="list-style-type: none"> ▶ Antiproliferative assays; data similar to MTM ▶ Stability in physiological saline at 37 °C; acetylated compounds hydrolyze significantly
DIG-MTM-SK [7]	<ul style="list-style-type: none"> ▶ Antiproliferative activity by NCI-60 panel comparison shows 2–7× improvement of DIG-MTM-SK over DIG-MTM ▶ DIG-MTM-SK maximum intravenous tolerated dose (MTD) in mice is 64 mg/Kg. For repeated doses (up to 8 times) every other day, MTD is 12 mg/Kg, and every 3 days MTD is 24 mg/Kg ▶ Similar PK profile to MTM and MTM-SK in mice after intravenous administration, but maximum concentration achieved is 10× higher than in MTM ▶ Significant growth inhibition in subcutaneous xenografts of colon and melanoma cell lines, better response with a high, more spaced dose
MTM-SK, DIG-MTM-SK [60]	<ul style="list-style-type: none"> ▶ DIG-MTM-SK has a higher effect than MTM-SK on gene transcription of 89 cell cycle genes in colon cancer cells; only 5 genes down-regulated by both drugs ▶ p21^{WAF1}/CDKN1 A, involved in halting cells at the G1 and G2/M, is significantly upregulated
DIG-MTM-SK [61]	<ul style="list-style-type: none"> ▶ DIG-MTM-SK is being explored in the treatment of Ewing sarcoma tumor cells
MTM-SK, DIG-MTM-SK [62]	<ul style="list-style-type: none"> ▶ DIG-MTM-SK is a potent inhibitor of both basal and induced expression of Sp1 ▶ DIG-MTM-SK is stronger than MTM as an inhibitor of Sp3-driven transcription and endogenous Sp3 gene expression ▶ MTM, MTM-SK, and DIG-MTM-SK accumulate rapidly in ovarian cancer cell lines
DIG-MTM-SK [63]	<ul style="list-style-type: none"> ▶ Gene expression array; nanomolar levels of DIG-MTM-SK abrogate expression of genes involved in transcription regulation and tumor development in A2780 ovarian cancer cells ▶ Other non-Sp binding sites identified: CREB, E2F, and EGR1

in G1-like cell arrest, which consisted of post-mitotic aneuploid G1 cells. Apoptosis in p21^{-/-} cells involved caspase 2 rather than caspase 3, as in the wild-type cells [53].

Another mithralog that deserved early attention was MTM-SDK, since it was more potent than MTM and MTM-SK according to antiproliferative assays, including the NCI-60 panel. In ovarian

cancer cell lines, the mechanism of action was consistent with the repression of transcription of multiple genes implicated in critical aspects of cancer development and progression, including cell cycle, apoptosis, migration, invasion, and angiogenesis, which is in agreement with the pleiotropic role of Sp1 family transcription factors [28]. MTM-SDK inhibited proliferation and was a po-

tent inducer of apoptosis in ovarian cancer cells, while it had minimal effects on the viability of normal cells.

Both MTM-SK and MTM-SDK have been the subject of several comparative studies. The thermodynamic profile of MTM binding to DNA was compared to that of MTM-SK, showing that the binding to DNA is entropically driven and dominated by the hydrophobic transfer of the antibiotics from the solution to the DNA binding site [54]. In a follow-up study, comparing the binding of MTM-SK, MTM-SDK, and MTM-SA, including a molecular modeling analysis of the impact of the side chain on binding, it was found that MTM-SDK has the highest binding affinity [55]. Differential cleavage at three restriction enzyme sites was used to determine the specific binding to DNA of MTM and shorter side chain analogs. Together with changes in the DNA melting temperature and cellular uptake, these experimental approaches provided mutually consistent evidence of a correlation between the strength of binding to DNA and the antiproliferative activity of the chromophore-modified molecules. Interestingly, the authors reported a link between antiproliferative activity and intracellular accumulation (measured in HCT-116 and PC3 cell lines), since the most bioactive compounds are also the ones accumulated and retained over 72 h periods [56].

In addition, MTM-SDK and MTM-SK acted as potent inhibitors of Sp1-dependent transcription both *in vitro* and in tumor xenografts. Both compounds were well tolerated even after prolonged administration and delayed growth of ovarian tumor xenografts. MTM-SDK was particularly effective against orthotopic tumors, leading to a significant increase of survival and delay of tumor progression [57]. Regarding prostate cancer, MTM-SDK and MTM-SK were highly effective *in vitro* in inhibiting the proliferation of prostate cancer cells and transcription of Sp-regulated genes by blocking the binding of Sp proteins to the gene promoters. When administered to mice, both compounds were well tolerated and were cleared rapidly from the bloodstream, but they maintained plasma levels well above the active concentrations required *in vitro* for the inhibition of Sp activity and cell proliferation. Consistently, MTM-SDK and MTM-SK inhibited the transcription of Sp-regulated genes in prostate tumor xenografts and exhibited potent antitumor activity in both subcutaneous as well as metastatic tumor xenograft models with no or minimal toxicity [58]. The pharmacokinetic profile is similar to MTM and other mithralogs in terms of half-life, however, the maximum plasma concentration is much higher, one order of magnitude higher than MTM. This is very important, since in humans, the reported maximum plasma concentration for MTM is <20 nM, insufficient to achieve therapeutic action, according to the IC₅₀ for most cancer cell lines [59].

DIG-MTM-SK is the most promising mithralog since it has the highest *in vivo* maximum tolerated dose in mice, and thus the lowest toxicity among all mithralogs tested [7]. Since the *in vitro* and *in vivo* activity on cell lines or xenotransplants was similar or better than MTM, DIG-MTM-SK represents a real opportunity to increase the therapeutic window of MTM-based drugs.

The influence of DIG-MTM-SK and MTM-SK on the transcription of 89 genes mainly involved in cell cycle control in human HCT116 colon carcinoma cells by qRT-PCR was analyzed. Each compound downregulated a different set of genes. Indeed, only five genes were downregulated by both compounds. Other genes were significantly upregulated, among them p21^{WAF1}/CDKN1A, which is involved in halting cells at the G1 and G2-M checkpoints. It is of note that just the minor structural change from DIG-MTM-SK to MTM-SK produces such a clear dissimilarity in

their “anti-transcriptional” activity. DIG-MTM-SK seems to be a superior molecule, since abrogation of a larger number of genes was observed (meaning there is a disruption of more interactions between transcription factors and their consensus binding sites in a tumor cell). Also, DIG-MTM-SK seems to have a stronger effect in terms of the upregulation of genes responsive to cell damage [60].

The activity of DIG-MTM-SK is related to its binding to DNA, cellular accumulation, and inhibition of Sp1-driven gene transcription, as shown in ovarian cancer cell lines *in vitro*. The binding of DIG-MTM-SK to DNA shares the general features of other mithralogs, such as the preference for C/G-rich tracts, but there are some differences in the strength of binding and the DNA sequence preferentially recognized by DIG-MTM-SK. Similar to MTM, both MTM-SK and DIG-MTM-SK accumulated rapidly in A2780, IGROV1, and OVCAR3 human ovarian cancer cell lines. Also, DIG-MTM-SK was a potent inhibitor of both basal and induced expression of an Sp1-driven luciferase vector. Furthermore, DIG-MTM-SK was stronger than MTM as an inhibitor of Sp3-driven transcription and endogenous Sp3 gene expression [62].

Using a genome-wide approach, gene expression in A2780 human ovarian carcinoma cells treated with DIG-MTM-SK was studied. Nanomolar concentrations of DIG-MTM-SK abrogated the expression of the genes involved in a variety of cell processes, including transcription regulation and tumor development. Some of those genes have been associated with cell proliferation and poor prognosis in ovarian cancer. Sp1 regulated most of the genes that were downregulated by the drug. The effect of DIG-MTM-SK in the control of gene expression by other transcription factors was also explored. Some of them, such as CREB, E2F, and EGR1, also recognize C/G-rich regions in gene promoters, which encompass potential DIG-MTM-SK binding sites [63].

DIG-MTM-SK, among other mithralogs, is being evaluated to identify a candidate for Ewing sarcoma, a particularly deadly form of cancer [61]. MTM was identified as a candidate out of 50 000 molecules at the NCI and is being tested in phase I clinical trials [2].

Conclusion and Future Directions

The growing body of literature on MTM and its main target Sp1 transcription factor is mostly related to novel mechanisms of action relevant to several diseases, mainly cancer. This fact, together with the recent launch of MTM clinical trials in certain tumors for the first time in decades, has fueled a renewed interest in this promising natural product scaffold.

In the quest for novel mithralogs, the most successful criteria to date has been to identify analogs that allow higher tolerated doses to circumvent the main problem reported in MTM development history, toxicity. Since these analogs are also selected based in their *in vitro* and *in vivo* activity, they might have a chance of expanding the therapeutic window of MTM. Moreover, such higher tolerated doses are also linked to better plasma levels in animals, which could potentially circumvent the recently identified problem in MTM of low circulating levels in humans (reflecting the same situation in animals).

Another area of future research is to identify molecular characteristics that would render certain cancer cells more sensitive to the mithralogs. Typically, these would be overexpressed genes or protein targets, and the advances in proteomics and genomics

will help to unravel the pleiotropic mechanism of action of these kinds of drugs. It is of note that complex natural products, like mithralogs, are designed by nature to interact with membranes through transporters, so a future direction is the study of the differential accumulation in cancer cells, which, if found relevant, is likely to be mediated by active rather than passive transport. The main priority in the development of mithralogs is to verify the promising toxicity and PK data in humans. For this purpose, an FHI trial will be designed to identify doses, schedules, and plasma levels in order to optimize human doses with the potential of being pharmacologically active in patients.

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Conflict of Interest

F.M. and J.G.S. are employees of EntreChem S.L., and C.M., F.M., and J.A.S. hold shares of EntreChem S.L.

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