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Cytotoxicities and Structure–Activity Relationships of Natural and Unnatural Lamellarins toward Cancer Cell Lines

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Twenty-two naturally occurring and three unnatural lamellarins were synthesized and evaluated for their cytotoxicities against cancer cells. Across eleven cancer cell lines derived from six different cancer types, the IC_{50} values of these compounds ranged from sub-nanomolar (0.08 nM) to micromolar (> 97.0 μM). About one-fourth (6/25) and one-half (11/25) of these lamellarins are more potent than the positive control, etoposide, against at least six different cell lines and three different cell types, respectively. In general, lamellarins D, X, ε, M, N, and dehydrolamellarin J are significantly more potent than the other lamellarins. The IC_{50} values were used to perform structure–activity relationship (SAR) studies by comparing the cytotoxic activities of several pairs of lamellarin structures that differ in selected substitution patterns. Our results not only reveal the importance of specific hydroxylation or methoxylation patterns for the first time, but also confirm prior findings and clarify some previous uncertainties.

Introduction

Lamellarins are a group of marine natural products initially isolated from mollusks and subsequently found in ascidians and sponges. Their discovery in 1985 by Faulkner and co-workers[1] prompted research groups worldwide to screen their biological activities, conduct total or partial syntheses, and, more recently, investigate molecular mechanism(s) of their anticancer action. Interestingly, some lamellarins have been found to exhibit a wide array of promising biological activities, which include cytotoxicity, multi-drug resistance (MDR) reversal in some cancer cell lines, HIV-1 integrase inhibition, and immunomodulation.[2,3]

Since 1985, over 35 of these polyaromatic pyrrole alkaloids have been isolated (Figure 1 and Table 1).[1,4] Despite the lack of other crucial information such as toxicity toward normal cell lines, chemical stability, and related pharmacological properties, considerable effort has been expended on the synthesis, biological evaluation, and the study of other biochemical properties on lamellarin D. This compound exhibits potent anticancer activity at nanomolar concentrations.[5,6] Interactions between regions of lamellarin D and specific amino acid residues of the topoisomerase I–DNA ternary complex have been identified from molecular modeling studies.[6] Additionally, the pro-apoptotic activity of lamellarin D has been correlated with its ability to promote DNA cleavage through stabilization of topoisomerase I–DNA covalent complexes and inhibition of the enzyme.[7] Unlike lamellarin D and its structural analogues, the biological profiles—particularly the anticancer activities—of other lamellarins have been studied, but to a lesser extent.

Even though a majority of lamellarins (except lamellarins O–R) contain the same pentacyclic 2-pyrrolo(dihydro)isoquinoline lactone core and only differ in the substituents present on each ring (Figure 1 and Table 1), many exhibit rather diverse cytotoxic activities.[8] Owing to their low natural abundance, total synthesis of the lamellarins is a pivotal alternative for pro-

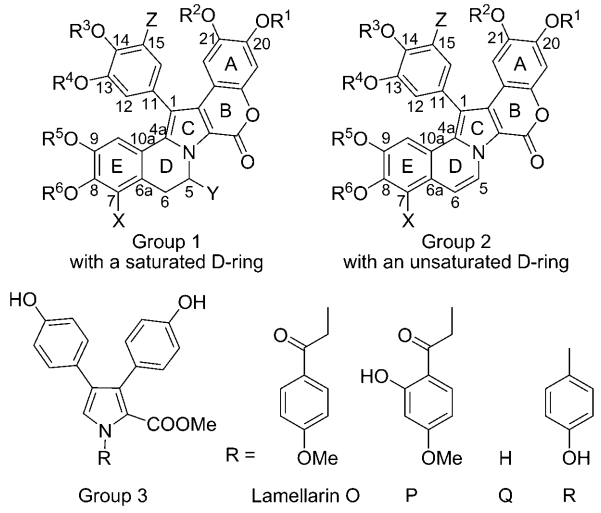

Figure 1. Structure of the lamellarins.

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Table 1. Substituents around the lamellarin core.

Group 1	Group 2	Substituents								
		R^1	R^2	Z	R^3	R^4	R^5	R^6	X	Y
Lamellarin A[a]	–	H	Me	H	H	Me	Me	Me	OMe	OH
Lamellarin C	Lamellarin B	H	Me	H	H	Me	Me	Me	OMe	H
Lamellarin χ	Lamellarin D	H	Me	H	H	Me	Me	H	H	H
Lamellarin E	Lamellarin X	H	Me	H	Me	H	Me	Me	OH	H
Lamellarin F	Lamellarin ε	H	Me	H	Me	Me	Me	Me	OH	H
Lamellarin G	–	Me	H	H	Me	H	Me	H	H	H
–	Lamellarin H[a]	H	H	H	H	H	H	H	H	H
Lamellarin I	Lamellarin ζ	H	Me	H	Me	Me	Me	Me	OMe	H
Lamellarin J	Dehydrolam. J[b]	H	Me	H	Me	Me	Me	H	H	H
Lamellarin K	Lamellarin M	H	Me	H	H	Me	Me	Me	OH	H
Lamellarin L	Lamellarin N	H	Me	H	Me	H	Me	H	H	H
Lamellarin S[a]	–	H	H	H	H	H	Me	H	H	H
Lamellarin T	Lamellarin W	H	Me	H	Me	H	Me	Me	OMe	H
Lamellarin U	Lamellarin α	H	Me	H	Me	H	Me	Me	H	H
Lamellarin V[a]	–	H	Me	H	Me	H	Me	Me	OMe	OH
Lamellarin Y	Dehydrolam. Y[b]	H	Me	H	Me	H	H	Me	H	H
Lamellarin Z[a]	–	Me	H	H	H	H	Me	H	H	H
Lamellarin β[a]	–	H	H	H	Me	H	H	H	H	H
Lamellarin γ[a]	–	H	Me	OMe	–	Me	Me	Me	OH	H
–	Lamellarin ϕ[a]	H	Me	H	H	Me	H	Me	OMe	H
Dihydrolam. η[b,c]	Lamellarin η[c]	H	Me	H	Me	Me	Me	Me	H	H

[a] Synthesis in progress. [b] Unnatural lamellarins. [c] Not tested due to insolubility in DMSO.

viding sufficient quantities for further detailed biological evaluation. Because of their diverse biological activities, it is necessary to establish a comprehensive structure–activity relationship (SAR) for these compounds. Thus, a number of convergent and flexible synthetic routes have been designed and developed for the lamellarin framework.[5,9]

Our research program on the total synthesis and medicinal aspects of lamellarins has focused on the design and execution of efficient synthetic routes.[9f,j,m,o,s] Accordingly, natural and unnatural lamellarins with either a saturated or an unsaturated D-ring have been synthesized.[9s] Herein, we report the evaluation of the cytotoxicities of these lamellarins against 11 cancer cell lines and disclose our observations on SARs that highlight the importance of the C5=C6 double bond in the D-ring and the substitution pattern on the periphery of the lamellarin core in relation to their cytotoxicity toward cancer cells.

Results and Discussion

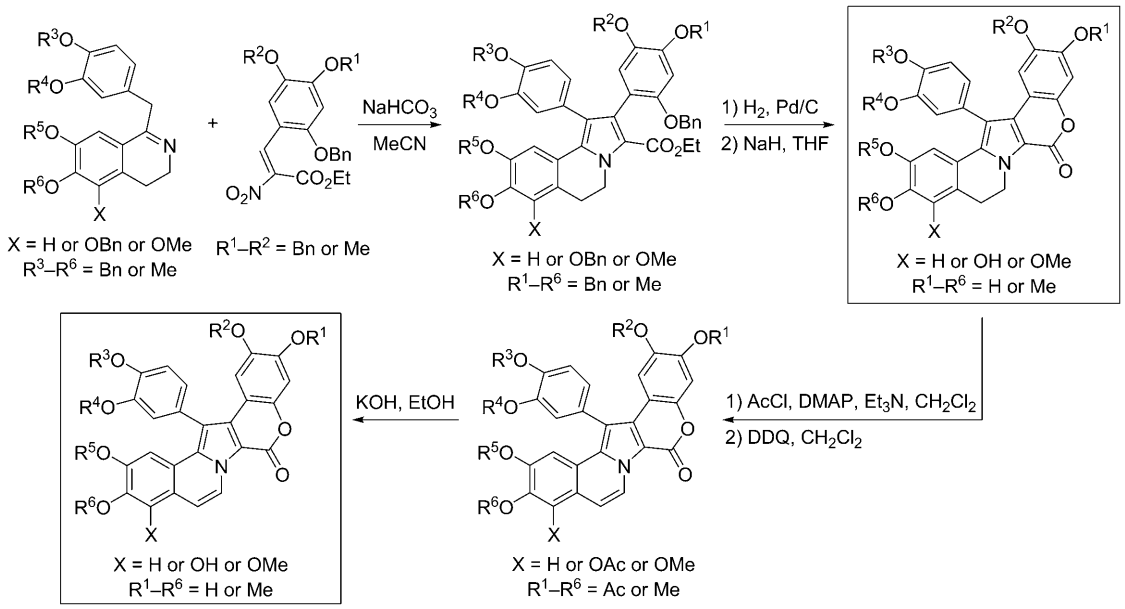
Chemistry

Lamellarins are classified into three structural groups, two of which possess either a "fused" saturated or unsaturated D-ring (groups 1 and 2, respectively; Figure 1). The third group, lamellarins O–R, on the other hand, contain an open structure lacking the B-, D-, and E-rings, and can be classified as 3,4-diaryl-2-carbomethoxypyrrole derivatives. Our modular synthetic strategies[9s] have focused on the preparation of lamellarins with a saturated D-ring, which can then be converted directly into the corresponding lamellarins with an unsaturated D-ring via oxidation, as shown in Scheme 1. The key features of our synthetic strategies are: 1) the efficient Michael addition/ring closure (Mi-RC) condensation between the benzyldihydroisoquinoline and α-nitrocinnamate derivatives to form directly the 3,4-diaryl pyrrole core, 2) the sole use of benzyl and acetate as phenoxy protecting groups, 3) the stability of intermediates under the reaction conditions and to storage, and 4) minimal requirement of purification for each intermediate.

Cytotoxic activities

The cancer cell lines used in this study represent various types of cancer commonly found in a number of major tissues and organs. These include the hepatocellular carcinoma (HepG2 and S102) and cholangiocarcinoma (HuCCA-1) frequently found in Thailand. A multi-drug-resistant small-cell lung carcinoma (SCLC) cell line, H69AR, was also included in order to screen for potential candidates that may help circumvent the problem of multi-drug resistance. The use of human embryonic lung fibroblasts (MRC-5) serves to demonstrate the toxicity of selected lamellarins toward normal cells.

As shown in Table 2, a number of lamellarins exhibit potent anticancer activities, with IC_{50} values in the nanomolar or low-micromolar range. While lamellarins D, K, and M are usually classified among the most cytotoxic molecules in the series,[2] the results show that lamellarin N and dehydrolamellarin J are also promising candidates. These compounds are significantly more potent than the other lamellarins and the positive control, etoposide, in most cancer cell lines. Moreover, both of them exhibit relatively low toxicity toward MRC-5 cells, in contrast to the other compounds tested, especially lamellarins D, M, X, and ε, that have similar cytotoxic activities toward cancer cells.



Scheme 1. Synthetic route used for the preparation of natural and unnatural lamellarins with either a saturated or an unsaturated D-ring.

Table 2. Cytotoxic activities of lamellarins.[a]

Compound	IC_{50} [μM]											
	Oral	Lung		Breast		Liver			Cervix	Blood cell		Fibroblast
	KB	A549	H69AR	T47D	MDA-MB-231	HuCCA-1	HepG2	S102	HeLa	P388	HL-60	MRC-5
Lamellarin C	5.7	3.6	12.1	7.7	8.3	11.5	18.3	4.4	7.9	4.2	5.7	ND
Lamellarin B	4.4	5.4	6.4	0.2	4.4	5.3	0.8	5.9	4.8	6.1	6.2	68.1
Lamellarin χ	2.6	2.0	38.9	3.8	4.8	49.9	0.1	3.4	6.6	1.6	1.8	ND
Lamellarin D	0.04	0.06	0.4	0.00008	0.4	0.08	0.02	3.2	0.06	0.1	0.04	9.2
Lamellarin E	4.0	2.2	7.2	5.3	3.4	9.4	1.0	2.8	5.3	2.6	4.5	ND
Lamellarin X	0.08	0.3	0.3	0.006	0.08	0.04	0.2	1.6	0.09	0.3	0.2	10.1
Lamellarin F	4.2	4.4	10.1	4.6	3.7	8.8	0.5	2.7	6.4	3.1	3.6	ND
Lamellarin ε	0.3	0.3	2.3	0.006	0.3	0.07	0.1	2.1	0.3	0.3	0.1	25.8
Lamellarin G	3.0	4.0	7.4	8.6	15.0	49.9	1.5	9.6	4.2	1.6	7.5	ND
Lamellarin I	6.3	10.6	18.1	9.5	8.6	11.2	1.3	12.4	11.2	3.8	6.9	ND
Lamellarin ζ	4.7	10.6	23.3	0.09	4.7	6.3	0.3	7.9	8.3	7.2	12.3	>89.7
Lamellarin J	>97.0	1.1	>97.0	13.0	7.4	>97.0	0.4	19.4	>97.0	0.8	0.9	ND
Dehydrolam. J	0.08	0.04	0.3	0.0001	0.4	0.006	0.01	2.1	0.08	0.08	0.04	>97.4
Lamellarin K	0.9	4.2	4.3	0.09	0.4	3.4	1.0	4.4	2.8	3.4	3.8	ND
Lamellarin M	0.2	0.04	0.3	0.009	0.1	0.06	0.02	1.9	0.3	0.1	0.06	13.4
Lamellarin L	3.0	0.8	3.0	4.4	1.8	21.9	0.3	1.4	2.8	0.5	1.9	ND
Lamellarin N	0.06	0.04	0.06	0.0006	0.6	0.008	0.02	2.3	0.04	0.08	0.04	>100.1
Lamellarin T	6.4	2.9	13.2	13.2	8.6	14.7	0.6	5.5	9.9	4.8	6.4	ND
Lamellarin W	5.3	5.2	4.4	4.2	5.2	4.2	0.9	5.8	5.0	5.6	6.7	28.5
Lamellarin U	3.9	0.9	8.7	10.3	4.5	44.6	0.6	3.0	5.0	1.8	4.5	ND
Lamellarin α	9.4	1.6	8.0	0.6	3.9	5.8	0.06	5.6	7.6	1.7	10.5	>97.4
Lamellarin Y	5.0	0.9	14.8	7.2	8.0	37.9	0.6	4.3	29.9	1.0	5.0	ND
Dehydrolam. Y	0.8	1.3	7.6	0.08	0.6	1.4	0.4	6.2	1.6	0.9	3.4	31.0
Dihydrolam. η	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Lamellarin η	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Etoposide	0.5	1.1	45.9	0.08	0.2	6.8	0.2	1.5	0.4	0.4	2.3	>85.0

[a] ND = not determined, as these group 1 lamellarins are generally less cytotoxic than their group 2 counterparts, and are therefore less likely to be toxic to normal cells. NA = not available due to insolubility of the compounds in DMSO. Cell lines used (in alphabetical order): A549, human non-small-cell lung carcinoma; H69AR, human multi-drug-resistant small-cell lung carcinoma; HeLa, human cervical adenocarcinoma; HepG2, human hepatocellular carcinoma; HL-60, human promyelocytic leukemia; HuCCA-1, human cholangiocarcinoma; KB, human oral epidermoid carcinoma; MDA-MB-231, human hormone-independent breast cancer 231; MRC-5, human fetal/embryonic lung fibroblast; P388, mouse lymphoid neoplasm; S102, human hepatocellular carcinoma; T47D, human hormone-dependent breast cancer.

Interestingly, lamellarin N and dehydrolamellarin J also demonstrate appreciable cytotoxic activity against the multi-drug-resistant H69AR cell line, relative to etoposide. It has been shown that some lamellarins effectively reverse multi-drug resistance by inhibiting P-glycoprotein.[8] However, this might not be the case for H69AR cells, which, unlike most multi-drug-resistant cell lines, do not overexpress P-glycoprotein.[10] In fact, the alternative resistance mechanisms in this cell line have been shown to involve decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II,[11,12] as well as overexpression of multi-drug-resistance--associated protein (MRP).[13,14]

Additionally, some lamellarins show selective cytotoxicities toward certain cancer cell lines, whereas some are broadly toxic against all cell lines under evaluation. The most prominent selectivity is observed with the human breast cancer T47D and MDA-MB-231 cells, which differ mainly in the presence and absence of estrogen receptors (ER), respectively. One plausible explanation is that these lamellarins may also act as anti-estrogens in inhibiting the proliferation of ER-positive T47D cells, whereas this mechanism may not be possible for ER-negative MDA-MB-231 cells. Nevertheless, the actual reasons for the selectivity remain to be investigated.

SAR studies

In an early attempt to correlate the structures of lamellarins with their cytotoxic activities, Quesada et al.[8] observed that an increase in the number of methylations and/or methoxylations appears to cause a decrease in the antitumor activities of the 13 lamellarins tested in their studies. Most subsequent SAR studies have been directed at derivatives of lamellarin D by using their mechanism-based (i.e., topoisomerase I inhibition) activities in relation to their cytotoxicities against cancer cell lines. It has been demonstrated that the full pentacyclic structure of lamellarins is important for their biological activity. Simplification of the lamellarin D structure by opening the lactone ring results in a significant decrease in cytotoxicity toward certain human tumor cell lines.[9r] Another general observation made from these limited studies focusing on the lamellarin D series is that the planarity of the pharmacophore conferred by the C5=C6 double bond is also essential for cytotoxicity and topoisomerase I inhibition.[6,15] Unfortunately, as reported by Quesada et al.,[8] such a relationship does not hold for lamellarins K and M. These compounds contain the same substituents, but differ in the nature of the C5–C6 bond (see Table 1). However, they possess roughly the same cytotoxicity, and furthermore, lamellarin K triacetate is even more cytotoxic than lamellarin M triacetate.

Additionally, Ishibashi et al.[5] have demonstrated that the hydroxy groups at the C8 and C20 positions of lamellarin D are important structural requirements for cytotoxic activity, whereas neither the hydroxy group at C14 nor the two methoxy groups at C13 and C21 are necessary. However, it was subsequently reported by Tardy et al.[15] that all of the phenolic hydroxy groups at each of the C8, C14, and C20 positions of lamellarin D are important for maintaining the activity against topoisomerase I and potent cytotoxic action. It was also found that these groups could be substituted with positively charged amino acid derivatives without loss of activity. Furthermore, it was pointed out in a review article that almost any modifications of the substitution pattern on lamellarin D decrease the cytotoxicity of the molecule.[2]

The discrepancies of the previous findings observed with lamellarin D derivatives have prompted us to execute a more comprehensive SAR investigation of the lamellarins in groups 1 and 2, beyond the lamellarin D series, to clarify the importance of each substituent on the lamellarin skeleton. The number and diversity of the substitution patterns of the lamellarins investigated in this study allow us to compare the cytotoxic activities of several pairs of lamellarins, which differ only in substitution at the positions of interest.

The first structural element considered was the C5=C6 double bond in the D-ring. The importance of this functionality for the cytotoxic activities of these compounds was studied by comparing the IC_{50} values of 11 pairs of lamellarins, each of which contains exactly the same substituents and differs only in the nature of the C5–C6 bond. As shown in Figure 2, the results unequivocally indicate that, in most cases, the presence of the C5=C6 double bond significantly decreases the IC_{50}

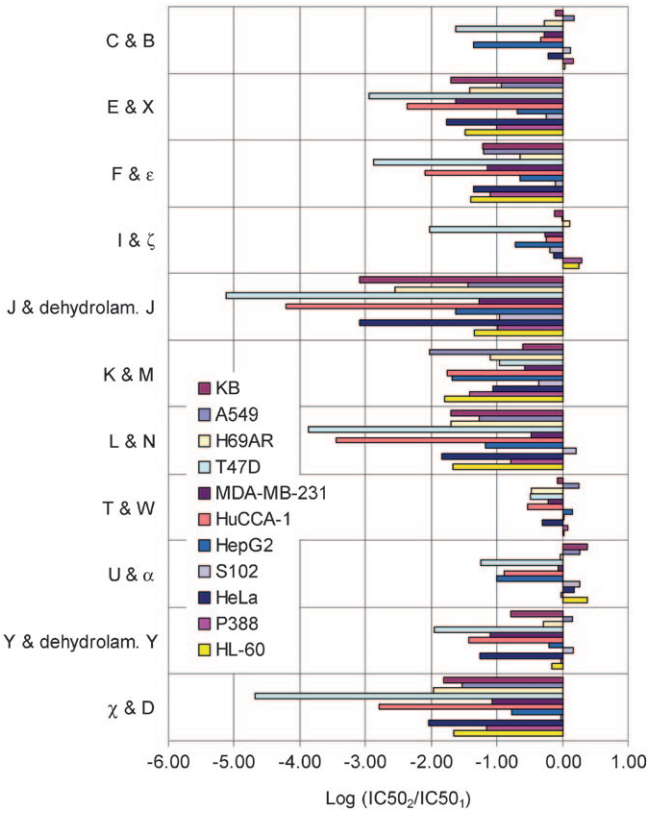


Figure 2. Contributions of the C5=C6 double bond in the D-ring to the cytotoxic activities of various lamellarins toward cell lines (indicated by color). The IC_{50} values of two structures differing in one given position were compared, and the logarithm of the IC_{50} ratio was subsequently determined with the IC_{50} values of the compounds after and before the modification in the numerator and denominator, respectively. Positive log (IC_{50} ratio) values indicate a loss of cytotoxic activity, whereas negative values show an increase in the activity upon the structural modification being considered.

values, that is, increases the cytotoxic activities of the lamellarins.

It has been pointed out previously that, in the case of lamellarin D, the planarity of the pentacyclic lamellarin core is essential for intercalation into DNA and topoisomerase I inhibition.[6] On the other hand, replacement of the C5=C6 double bond in lamellarin D by a single bond in lamellarin 501, subsequently isolated from natural sources and named lamellarin χ, introduces a large kink into the core structure and abolishes the capacity of the molecule to insert between two consecutive base pairs in the DNA.

Among all the lamellarins investigated, the substituent at C7 may be a hydrogen atom, a hydroxy group, or a methoxy group. To our knowledge, the contribution of the substituent at this particular position to the cytotoxic activities of lamellarins has not been reported previously. As shown in Figure 3a, substitution of the hydrogen atom at C7 with a hydroxy group significantly increases the cytotoxicities of lamellarins with a C5=C6 double bond (compare lamellarin α and lamellarin X). On the other hand, methoxylation at this position may only slightly affect the cytotoxic activities of these compounds (Figure 3b). However, it is difficult to evaluate any trend conclusively in this case, as only two pairs of lamellarins were available for comparison.

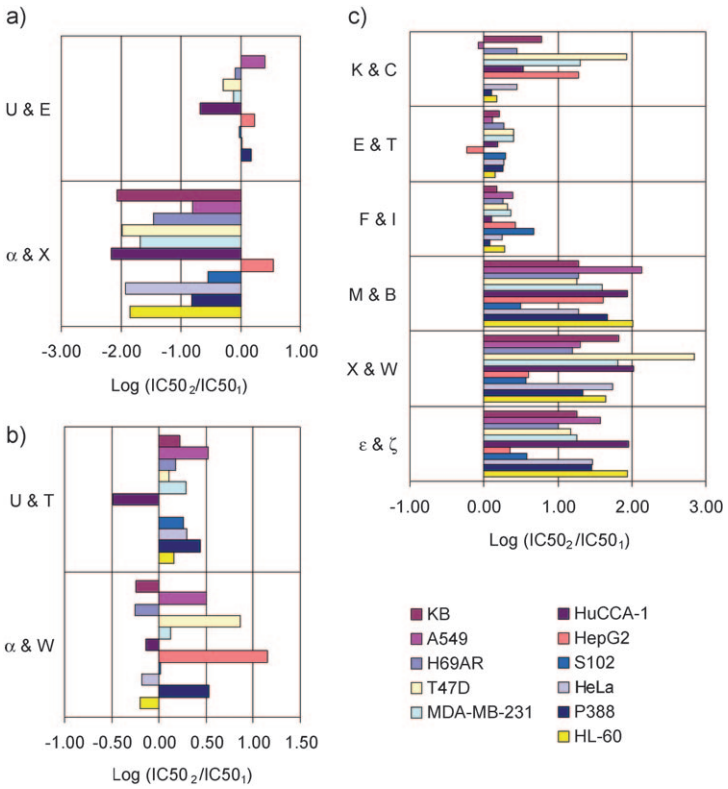


Figure 3. Contributions of the hydroxylation/methoxylation pattern at the C7 position of lamellarins to their cytotoxic activities: a) H vs. OH, b) H vs. OMe, c) OH vs. OMe. Increases or decreases in cytotoxicity were determined as described in Figure 2.

Interestingly, the effect is significantly more pronounced if the C7 hydroxy group is replaced by a methoxy group. This clearly decreases the cytotoxic activities of the lamellarins, especially those containing a C5=C6 double bond (Figure 3c). These results indicate that the hydroxy group at this position is an important structural element that may also feature in an interaction with the putative biological target(s).

In the case of the substituent at C8 in the two pairs of the lamellarins examined in this study, the results clearly show that methylation of the hydroxy group at this position decreases cytotoxic activity (Figure 4). The effect is much more prominent if the C5–C6 single bond in lamellarin L and lamellarin U is replaced by a double bond in lamellarin N and lamellarin α, respectively. However, due to the rather limited number of lamellarins for direct comparison at this position, additional lamellarins that differ from the others exclusively at C8 are targets of our future investigations.

On the other hand, methylation of the hydroxy group at C9, C13, or C14 induces rather more subtle changes in cytotoxic activity (Supporting Information figures S1 and S2). Whereas the significance of the C9 hydroxy group has not been reported before, the relative lack of influence that substituents at C13 and C14 have toward cytotoxic activity agrees well with observations of the lamellarin D derivatives reported by Ishibashi et al.[5] More importantly, switching the hydroxy and methoxy groups between these two positions does not significantly affect cytotoxicity in most cases (Supporting Information figure S3). Nevertheless, additional lamellarin derivatives with modified C13 and C14 substituents are currently being synthesized by our research group for further investigations.

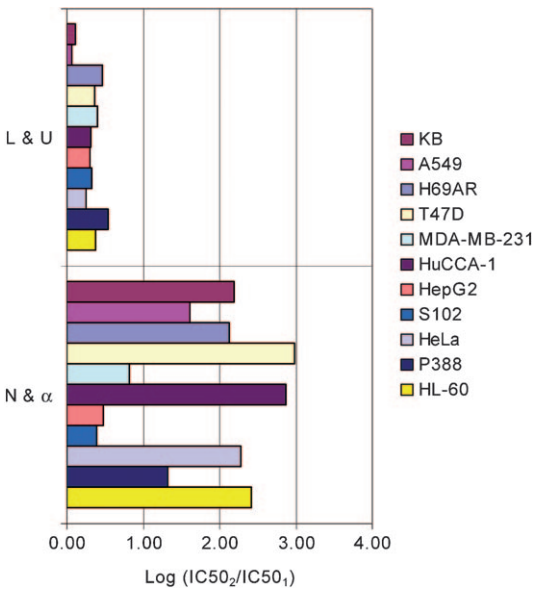


Figure 4. Contributions of the hydroxylation/methoxylation pattern (OH vs. OMe) at the C8 position of lamellarins to their cytotoxic activities. Changes in cytotoxicity were determined as described in Figure 2.

Even though most naturally occurring lamellarins contain a hydroxy group at C20 and a methoxy group at C21, there is

one pair of lamellarins, lamellarin L and lamellarin G, in which these two substituents are reversed. As shown in Table 2, interchange of the C20 hydroxy and C21 methoxy groups of lamellarin L to provide lamellarin G results in a substantial decrease in cytotoxic activity against most of the cell lines tested in this study (Supporting Information figure S4). These results agree well with all previous findings, including the observation that the C20-sulfated form of lamellarins usually show little or no cytotoxicity, in contrast to the non-sulfated analogues with a free C20 hydroxy group. Instead, the presence of a sulfate group at this position appears to be a key structural element for HIV-1 integrase inhibition by lamellarins.[9g]

According to all the results from our SAR studies, there appear to be four important structural elements that determine the cytotoxic activities of lamellarins toward cancer cells. These include the C5=C6 double bond as well as the C7, C8, and C20 hydroxy groups (Figure 5). Based on their cytotoxic activities, the lamellarins with an unsaturated D-ring may be subdivided into two categories (Table 3): those with lower cytotoxicities all contain only the C5=C6 double bond and a C20 hydroxy group, whereas the more potent compounds also contain a hydroxy group at either C7 or C8. Such a group may provide additional interactions with the target biological macromolecules. Notably, the other substituents that play a less important role are located toward the inner part of the lamellarin molecule.

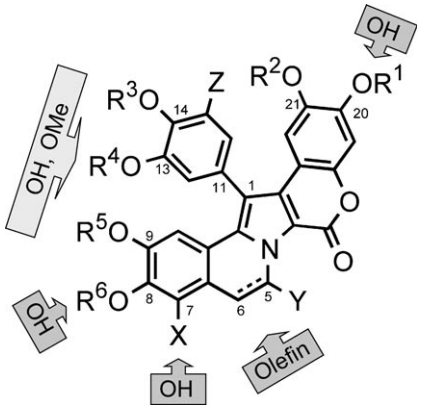


Figure 5. Important structural elements in the lamellarin skeleton.

Molecular modeling studies

The results from our preliminary molecular modeling studies using HyperChem 7.5 show that the C5=C6 double bond ensures planarity of the molecule and also affects the spatial array of the substituents around the periphery of the lamellarin skeleton, especially those on the E-ring (C7, C8, and C9). The group 1 lamellarins bearing a C5–C6 single bond are twisted, as most clearly observed if the molecules are viewed side-on from the E-ring (Figure 6a and 6c compared with Figure 6b and 6d, respectively), resulting in the spatial displacement of the substituents, most notably on the E-ring. As summarized in Table 4, the E-ring of group 1 lamellarins assumes a dihedral angle θ about C6a–C10a–C4a–C1 of much less than 180° (164.7–166.7°), significantly different from the planarity otherwise present in the group 2 lamellarins ($\theta = 179.0$–180.0°).

The orientation of the E-ring may be the factor that accounts for the more pronounced and more easily distinguished effects generally observed in the group 2 lamellarins as substituents are varied. These compounds with the unsaturated D-ring possess a more rigid framework and better alignments of the substituents around the pyrrole core than those in the group 1 lamellarins. Therefore, the C5=C6 double bond may exert its effect not only by making the molecule planar and thus suitable for intercalating the topoisomerase I–DNA complex, but also by spatially aligning the substituents, especially those on the E-ring, to their respective amino acid residues of the enzyme. It has been reported that the hydroxy groups at C8 and C20 are within hydrogen-bonding distance from Asn 722 and Glu 356 of the enzyme, respectively, whereas the ester carbonyl group interacts with the Arg 364 residue.[6]

Conclusions

We have disclosed herein a comprehensive evaluation of the cytotoxic activities of the lamellarins against a number of

Table 3. Classification of the lamellarins with an unsaturated D-ring, according to their cytotoxicities to cancer cells.[a]

Group	Lamellarin	IC_{50} [μM] to MRC-5	Substituents								
			OR^1 (C20)	OR^6 (C8)	X (C7)	OR^2 (C21)	Z (C15)	OR^3 (C14)	OR^4 (C13)	OR^5 (C9)	Y (C5)
Less cytotoxic	Lamellarin B	68.1	**OH**	OMe	OMe	OMe	H	**OH**	OMe	OMe	H
	Lamellarin W	28.5	**OH**	OMe	OMe	OMe	H	OMe	**OH**	OMe	H
	Lamellarin ζ	> 89.7	**OH**	OMe	OMe	OMe	H	OMe	OMe	OMe	H
	Lamellarin α	> 97.4	**OH**	OMe	H	OMe	H	OMe	**OH**	OMe	H
	Dehydrolam. Y[b]	31.0	**OH**	OMe	H	OMe	H	OMe	**OH**	**OH**	H
Highly cytotoxic	Lamellarin D	9.2	**OH**	**OH**	H	OMe	H	**OH**	OMe	OMe	H
	Lamellarin N	> 100.1	**OH**	**OH**	H	OMe	H	OMe	**OH**	OMe	H
	Dehydrolam. J[b]	> 97.4	**OH**	**OH**	H	OMe	H	OMe	OMe	OMe	H
	Lamellarin M	13.4	**OH**	OMe	**OH**	OMe	H	**OH**	OMe	OMe	H
	Lamellarin X	10.1	**OH**	OMe	**OH**	OMe	H	OMe	**OH**	OMe	H
	Lamellarin ε	25.8	**OH**	OMe	**OH**	OMe	H	OMe	OMe	OMe	H

[a] See Table 2. [b] Unnatural lamellarins.

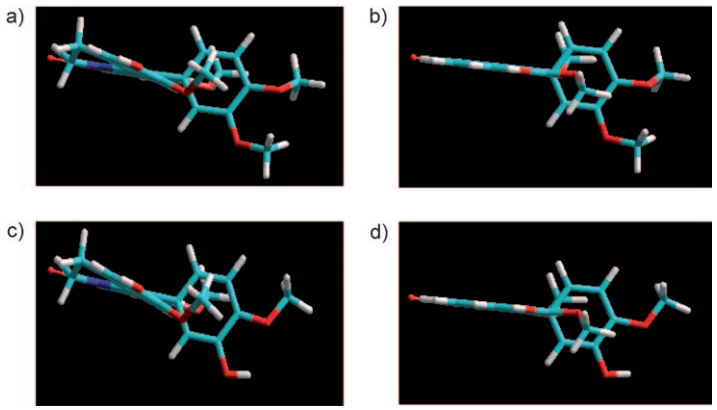


Figure 6. Energy-minimized structures of selected lamellarins calculated by using the AM1 method in HyperChem 7.5: a) lamellarin J (with a dihedral angle θ about C6a–C10a–C4a–C1 of 164.7°), b) dehydrolamellarin J ($\theta = 179.9°$), c) lamellarin L ($\theta = 164.8°$), d) lamellarin N ($\theta = 179.8°$).

Table 4. Dihedral angles in various lamellarins (AM1 method).

Lamellarin	Dihedral angle θ [°]	
	6a–10a–4a–1	4a–1–11–12
Lamellarin C	166.6	88.3
Lamellarin B	180.0	91.8
Lamellarin χ	165.1	89.4
Lamellarin D	180.0	91.6
Lamellarin E	164.9	89.5
Lamellarin X	179.0	90.4
Lamellarin F	164.9	89.3
Lamellarin ε	179.8	91.8
Lamellarin I	166.7	88.6
Lamellarin ζ	179.9	97.4
Lamellarin J	164.7	90.2
Dehydrolam. J	179.9	92.2
Lamellarin K	165.2	88.8
Lamellarin M	179.8	92.3
Lamellarin L	164.8	89.7
Lamellarin N	179.8	90.6
Lamellarin T	166.4	87.8
Lamellarin W	179.9	91.8
Lamellarin U	164.8	89.8
Lamellarin α	179.9	91.0
Lamellarin Y	165.5	86.9
Dehydrolam. Y	179.9	89.1
Dihydrolam. η	165.2	90.0
Lamellarin η	179.9	93.4

cancer cell lines. This study reveals that some of these compounds may be potential candidates for anticancer drug development. In addition, the results of the SAR studies have helped delineate the important structural elements in the lamellarin skeleton that contribute to cytotoxicity. These data will be employed for the structure optimization of these compounds in order to improve their efficacy, safety, as well as their physicochemical and pharmacokinetic properties.

The contributions from the C5=C6 double bond as well as the C8 and C20 hydroxy groups toward the overall anticancer activity are in good agreement with previously reported data. Importantly, this study reveals for the first time a significant contribution from the C7 substituent. Previous SAR studies have focused on lamellarin D and its derivatives that only possess a hydrogen atom at C7. It is noted that because the point-to-point comparisons of substituents at C13 and C14 give unclear results, possibly due to their flexibility, the importance of these positions toward anticancer activity remains to be evaluated.

Information from our current and planned SAR studies is critical for streamlining structural requirements for good anticancer activity. Further structural modifications that increase aqueous solubility or lower toxicity toward normal cells can then be made at those positions known not to have a marked effect on the anticancer activity.

Experimental Section

Syntheses of lamellarins

Two series of natural and unnatural lamellarins, containing either a saturated or an unsaturated D-ring, were previously synthesized and fully characterized using our established methodologies.[9s] All compounds were purified by crystallization from appropriate solvent systems, and the purified lamellarins were stored at 4 °C without solvent until use.

Spectroscopic data (^{1}H NMR, ^{13}C NMR, and HRMS-TOF) were used to confirm the identity of the compounds by comparing them with previously reported data. For unnatural lamellarins, their spectroscopic data were correlated with those of the natural lamellarins for structure elucidation. The purity of compounds was estimated based on their melting point ranges, the cleanliness of both ^{1}H NMR and ^{13}C NMR spectra, as well as their HPLC traces. Only trace amounts of impurities, if any, could be detected by HPLC analyses (Supporting Information table S1).

Cell culture

Four commercially available cancer cell lines (A549, H69AR, HepG2, and T47D) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Additionally, KB and P388 cell lines were obtained from the University of Illinois at Chicago (Chicago, IL, USA), whereas HeLa, HL-60, and MDA-MB-231 cell lines were obtained from the University of Texas M. D. Anderson Cancer Center (Houston, TX, USA). Human cholangiocarcinoma (HuCCA-1) and human hepatocellular carcinoma (S102) cells were obtained from Thai patients as previously reported.[16,17] The human lung fibroblast (MRC-5) cells were generously provided by the Armed Force Research Institute of Medical Sciences (AFRIMS, Bangkok, Thailand).

All cell lines were maintained using standard procedures in the appropriate culture media supplemented with the nutrients essential for each cell line (Supporting Information table S2). All culture media and fetal bovine serum (FBS) were obtained from HyClone Laboratories (Logan, UT, USA), whereas L-glutamine, dimethyl sulfoxide (DMSO), glucose, sodium pyruvate, non-essential amino acids, bovine insulin, crystal violet, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide (XTT) sodium, phenazine methosulfate (PMS), and etoposide were obtained from Sigma (St. Louis, MO, USA). All materials were used as received.

Cytotoxicity assays

All lamellarins were solubilized in DMSO and tested for their cytotoxic activities against a panel of 11 cancerous and one normal (MRC-5) cell lines. Briefly, the cells suspended in the corresponding culture medium (100 μL for adherent cells and 75 μL for suspended cells, i.e., P388 and HL-60 cells) were inoculated in 96-well microtiter plates (Corning Inc., NY, USA) at a density of 10000–20000 cells per well, and incubated at 37 °C in a humidified atmosphere with 95% air and 5% CO_2. After 24 h, an equal volume of additional medium containing either the serial dilutions of the test compounds, positive control (etoposide), or negative control (DMSO) was added to the desired final concentrations, and the microtiter plates were further incubated for an additional 48 h.

The number of surviving cells in each well was determined using either crystal violet staining (for adherent cells) or XTT assay (for suspended cells), as described below, in order to determine the IC_{50}, which is defined as the concentration that inhibits cell growth by 50% (relative to negative control) after 48 h of continuous exposure to each test compound. Within each experiment, determinations were done in triplicate, and each compound was tested in at least two separate experiments. Any experiments with a variation greater than 10% were excluded from the analysis. The results are expressed as the mean IC_{50} value; standard deviations are omitted for visual clarity.

For the crystal violet staining, supernatants were discarded, and the cells were washed twice with 10 mM phosphate-buffered saline (PBS; 100 μL well^{-1}), fixed with 95% ethanol (100 μL well^{-1}) for at least 5 min, and then stained with a solution of crystal violet (0.5%) in 25% methanol (50 μL well^{-1}) for 10 min. Afterward, the cells were air dried and subsequently lysed with a solution of HCl (0.1 N) in absolute methanol (100 μL well^{-1}). The absorbance at 540 nm was measured using a Multiskan Ascent microtiter plate reader (Labsystems, Helsinki, Finland). The percentage of surviving cells was then calculated for each concentration of the test compounds by comparing the absorbance of each sample well to the average absorbance of the negative control wells.

For the XTT assay, 75 μL of the mixture prepared from 1 mg mL^{-1} XTT sodium (5 mL) and 0.383 mg mL^{-1} PMS (100 μL) was added to each well, and the microtiter plates were further incubated for 4 h. The absorbance of the orange formazan compounds formed was measured at both 492 and 690 nm (reference absorbance) using a SPECTRA max PLUS 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The absolute absorbance ($\Delta A = A_{492} - A_{690}$) was then calculated for each well, and the percentage of surviving cells compared with control was determined by comparing ΔA_{sample} with $\Delta A_{control}$.

SAR studies

The mean IC_{50} values obtained from the cytotoxicity assays were used to perform SAR studies in a systematic manner. To determine the importance of a particular structural component for the cytotoxic activity of lamellarins, the IC_{50} values of the two structures differing only in that particular position were compared, and the logarithm of the IC_{50} ratio was subsequently determined with the IC_{50} values of the compounds after and before the modification in the numerator and denominator, respectively. Positive log (IC_{50} ratio) values indicate a loss of cytotoxic activity, whereas negative values show an increase in the activity upon the structural modification being considered. To account for experimental variation, the contribution from the position of interest to the cytotoxic activity was deemed significant only when $|\log(IC_{50}\ \text{ratio})| > 1$, meaning that the IC_{50} value either increased or decreased by at least 10-fold upon modification at that particular position.

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