

Novel Synthesis and Biological Evaluation of Enigmols as Therapeutic Agents for Treating Prostate Cancer

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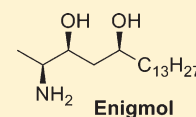
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S Supporting Information

ABSTRACT: Enigmol is a synthetic, orally active 1-deoxysphingoid base analogue that has demonstrated promising activity against prostate cancer. In these studies, the pharmacologic roles of stereochemistry and *N*-methylation in the structure of enigmols were examined. A novel enantioselective synthesis of all four possible 2*S*-diastereoisomers of enigmol (2-amino-octadecane-3,5-diols) from *L*-alanine is reported, which features a Liebeskind–Srogl cross-coupling reaction between *L*-alanine thiol ester and (*E*)-pentadec-1-enylboronic acid as the key step. *In vitro* biological evaluation of the four enigmol diastereoisomers and 2*S*,3*S*,5*S*-*N*-methylenigmol against two prostate cancer cell lines (PC-3 and LNCaP) indicates that all but one diastereomer demonstrate potent oncolytic activity. In nude mouse xenograft models of human prostate cancer, enigmol was equally effective as standard prostate cancer therapies (androgen deprivation or docetaxel), and two of the enigmol diastereomers, 2*S*,3*S*,5*R*-enigmol and 2*S*,3*R*,5*S*-enigmol, also caused statistically significant inhibition of tumor growth. A pharmacokinetic profile of enigmol and *N*-methylenigmol is also presented.



KEYWORDS: Enigmol, palladium cross-coupling, 1-deoxysphingoid bases, Liebeskind–Srogl reaction, prostate cancer therapy, PC-3, LNCaP

Although mortality rates due to prostate cancer have declined slightly in recent decades, it nonetheless remains a significant health threat for men throughout the world. It is currently second only to lung cancer as a cause of cancer-related deaths among men in the United States.¹ Approximately 80% of prostate cancer patients initially respond to androgen deprivation therapy,² but in nearly all cases, prolonged hormone ablation progresses within 18–24 months to hormone-refractory prostate cancer (HRPC), which is resistant to most known chemotherapeutic agents. Docetaxel (Taxotere, Sanofi Aventis) has been found to improve median survival a modest 2.2 months to 18.5 months compared to mitoxantrone/prednisone treatment in patients with HRPC,³ and it is currently considered the best chemotherapeutic option. Neither androgen deprivation nor chemotherapy is highly effective for long-term treatment of prostate cancer, and the vast majority of patients treated with either of these modalities eventually experience disease progression.

Sphingolipids are a diverse class of lipids that, in addition to being components of cell membranes, play important roles in processes related to cancer biology, including, *inter alia*, proliferation, migration, differentiation, and apoptosis.^{4–7} Accordingly, the sphingolipid pathway and its associated constellation of enzymes and receptors provide attractive targets for cancer therapeutics. Enigmols are 1-deoxysphingolipid analogues, discovered at Emory

University, that were designed to mimic the cytotoxic effects of endogenous sphingoid bases (e.g., sphingosine, Figure 1) and to be resistant to catabolism, a process that begins by phosphorylation of the C-1 primary hydroxyl group of endogenous sphingoid bases by sphingosine kinase. Removing the potential for C-1 phosphorylation may also prevent the analogues from behaving similarly to sphingosine 1-phosphate, which is mitogenic and antiapoptotic and promotes angiogenesis.^{8–12} The addition of a hydroxyl group on C-5 allows the 1-deoxy compounds to more closely mimic the hydrophobicity and log *P* characteristics of sphingosine, which easily traverses and diffuses between cell membranes.¹³ This similarity may facilitate drug uptake by and distribution within target tumor cells.

Previous biological studies with this series of compounds have been primarily focused on one stereoisomer, named enigmol (2*S*,3*S*,5*S*-2-amino-3,5-dihydroxyoctadecane, Figure 1, 5a). *In vitro*, enigmol inhibits both sphingosine kinase and ceramide synthase,^{14,15} and it has demonstrated potent anticancer activity in cells derived from multiple types of cancer, including colon, breast, brain, and prostate.¹⁵ Furthermore, in rodent

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models of colon and prostate cancers, enigmol has shown significant oral efficacy, with no evidence of host toxicity at effective doses. Mass spectrometry of lipids extracted from cancer cells treated with enigmol has shown that enigmol is not phosphorylated and, as predicted by our original rationale, was more cytotoxic and more persistent as a free sphingoid base than as an exogenous sphingosine.^{15,16}

Enigmol has stereocenters at C-2, C-3, and C-5, and the effect of stereochemistry on its bioactivity is unknown. Therefore, one objective of these studies was to investigate the impact of manipulation of the stereochemistry at C-3 and C-5 on activity. Additionally, since previous work has shown that enigmol is metabolized to *N*-methylenigmol *in vitro*,¹⁶ which may minimize further drug metabolism and provide greater plasma and tissue exposure to drug substance, we studied the impact of *N*-methylation on enigmol's oral pharmacokinetic parameters and anticancer activity. We report herein a concise synthetic method to access the entire diastereomeric set.^{17–19} The relative oncolytic activities of each diastereomer along with *N*-methylenigmol were evaluated in both *in vitro* and *in vivo* models of prostate cancer. Two separate xenograft models of prostate cancer were used to compare enigmol's efficacy to that of conventional prostate cancer therapies: androgen deprivation for androgen sensitive prostate cancer, and docetaxel for androgen independent cancer. Finally, plasma concentration time profiles and estimation of the pharmacokinetic parameters of enigmol and *N*-methylenigmol after oral dosing in mice were determined in

order to support the interpretation of *in vivo* data and to assess the relative effects of *N*-methylation in this series.

The synthesis of enigmol and its diastereoisomers was largely inspired from a previously described synthesis of sphingosine.^{20,21} We have extended and refined this approach to allow for the preparation of all four of the C3 and C5 diastereomers of enigmol from a single enantiopure precursor **2**. Strategic sequencing of stereoselective reduction and epoxidation protocols provides stereochemical diversification that can be accomplished *via* the stereocontrolled reduction of the desired diastereomeric configuration of the target enigmol with high selectivity. Specifically, formation of alanine thiol ester **1** is achieved using classical peptidic coupling conditions from alanine in excellent yield and with complete retention of enantiopurity (Scheme 1).²² Liebeskind–Srogl cross-coupling²³ then provides the expected ketone **2** in 87% yield and 99.7% ee using DMF as the solvent.²⁴ Then treatment with *L*-Selectride produces the *threo* diastereomer **3a** (dr >95:5), whereas exposure to lithium *tert*-butoxy aluminum hydride leads to *erythro* diastereomer **3b** (dr >95:5).²⁵ α -Epoxy alcohols **4a** and **4d** were then generated using *m*-chloroperbenzoic acid (*m*-CPBA)²⁶ in excellent yields and diastereoselectivities (dr >95:5).²⁷ Conversely, α -epoxy alcohols **4b** and **4c** were produced via Sharpless epoxidation conditions in moderate yields and excellent dr's. Regioselective ring-opening of the epoxides **4a–d** was achieved through Red-Al reduction,²⁸ resulting in exclusive 1,3-diol formation. Removal of the *N*-Boc protective group provided enigmol (**5a**) and its diastereoisomers **5b–d**.²⁹ *N*-Methylenigmol (**8**) was prepared in 85% yield from enigmol (**5a**) via the two step, one pot procedure shown in Scheme 2. First, enigmol was treated with formyl acetate in CH₂Cl₂ (DCM) at room temperature. The resulting *N*-formyl intermediate **7** was then reduced *in situ* with borane–THF complex to afford the *N*-methyl analogue **8**.

The effect of the enigmol stereochemistry and *N*-methylation on prostate cancer cell toxicity was tested *in vitro* using the WST-1 assay. Androgen-independent and androgen-sensitive prostate cancer cells (PC-3 and LNCaP, respectively) were treated for 24 h with enigmol (**5a**), 2*S*,3*S*,5*R*-enigmol (SSR, **5b**),

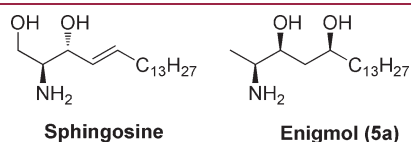
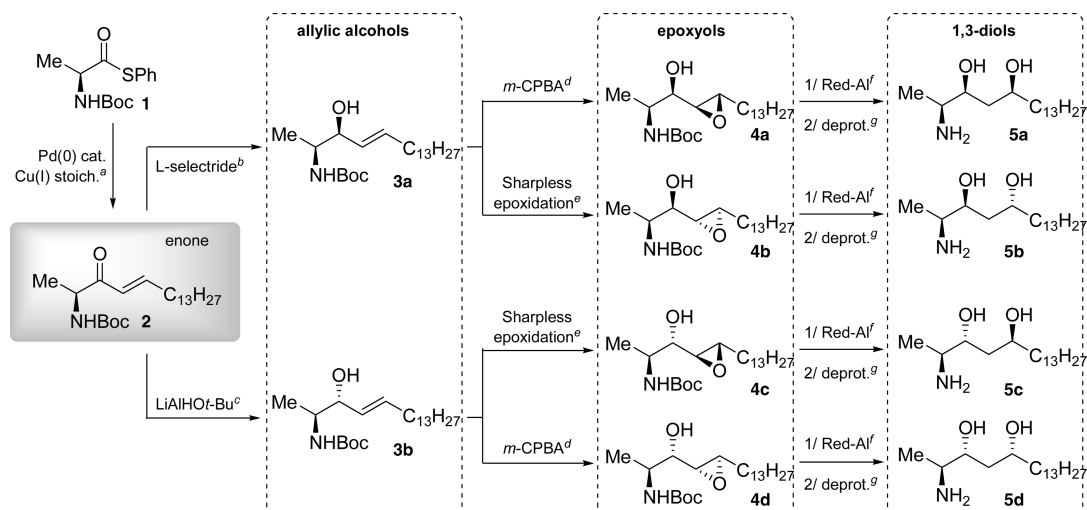
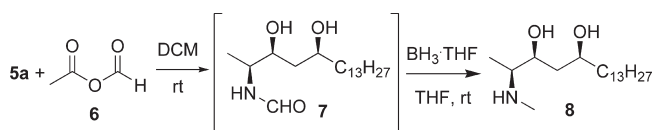


Figure 1. Structures of sphingosine and enigmol.

Scheme 1. Synthesis of Enigmol (**5a**) and Its 2*S*-Diastereomers (**5b–5d**)



Conditions: ^a (*E*)-C₁₃H₂₇-C=C-B(OH)₂, Pd₂(dibenzylideneacetone)₃ 2.5 mol %, Cu(I) thiophene-2-carboxylate, 1.7 equiv, P(OEt)₃ 20 mol %, DMF, 23 °C, 4 h, 87%, 99.7% ee. ^b *L*-Selectride, THF, -78 °C, 3 h, 91%, dr >95:5. ^c LiAlH(O*t*-Bu)₃, EtOH, -40 °C, 3 h, 89%, dr >95:5. ^d *m*-CPBA, CH₂Cl₂, 23 °C, 16 h, 89–92%, dr >95:5. ^e *D*- or *L*-Diethyl tartrate (matched), Ti(O*i*-Pr)₄, *t*-BuOOH, 4 Å molecular sieves, CH₂Cl₂, -20 °C, 2–3 d, 51–60%, dr >95:5. ^f Red-Al, THF, 23 °C, 95–97%, dr >95:5. ^g CF₃CO₂H, 0 °C, then NaOH, 94–99%, dr >95:5.

Scheme 2. Synthesis of *N*-Methylenigmol (8) from Enigmol (5a)

2*S*,3*R*,5*S*-enigmol (SRS, **5c**), 2*S*,3*R*,5*R*-enigmol (SRR, **5d**), or *N*-methylenigmol (NME, **8**) as 1:1 molar complexes with fatty-acid free bovine serum albumin. IC_{50} values calculated from these assays are given in Table 1. In both PC-3 and LNCaP cells, enigmol (**5a**), SSR (**5b**), and SRS (**5c**) exhibited IC_{50} values of approximately 10 μ M. *N*-Methylenigmol also demonstrated cancer cytotoxicity in both cell lines, with IC_{50} values in the 10–25 μ M range. In contrast, the SRR diastereomer (**5d**) demonstrated 2- to 10-fold less *in vitro* cytotoxicity than the other compounds in the study.

The relative pharmacokinetic profiles in mice of enigmol (**5a**) and *N*-methylenigmol (NME, **8**) were evaluated in order to assist in the design and interpretation of our preclinical pharmacology studies. Results suggest that both analogues possess attractive druglike properties and provide an advanced starting point for continued study of the compound class. Enigmol (**5a**) and NME (**8**) were both administered to outbred male, Swiss Webster, or CD-1 mice ($n = 3$ animals per time point) orally at 10 or 30 mg/kg in a vehicle composed of 10% ethanol and 90% olive oil. Animals were dosed at 5 mL/kg, and blood was obtained from the submandibular vein or retroorbital venous plexus at designated time points.

A graphical representation of the resultant plasma concentration profiles and pharmacokinetic parameters can be found in Figure 2. Both enigmol (**5a**) and NME (**8**) were orally available under the dosing conditions, and both demonstrated pharmacokinetics that were roughly dose proportional. The C_{max} values for enigmol were 137 and 705 nM, and AUC values were 1884 and 5962 h·nM at dose levels of 10 and 30 mg/kg, respectively. These parameters were both higher for NME (**8**), for which C_{max} values were 232 and 1115 nM and AUC values were 3860 and 13564 h·nM at 10 mg/kg and 30 mg/kg, respectively. Drug metabolism studies with these two compounds are planned to help provide insight into possible reasons for the different pharmacokinetic profiles observed with the two compounds.

Interestingly, the oral volume of distribution ($V_z \text{ } F_{obs}$) for these compounds (see Figure 2) was very high, thus suggesting a substantial distribution into tissues. This is consistent with previous reports that suggest accumulation of enigmol in various tissues in excess of 10 pmol/mg ($\sim 10 \mu$ M) after a single oral dose in rats of 100 mg/kg including prostate tissue, in which an average level of 39 pmol/mg was achieved.¹⁶ We therefore believe that tissue levels of drug may significantly exceed those observed in plasma. The long plasma half-lives observed with both compounds would be expected to lead to significant accumulation of drug in plasma with multiple days of dosing. Indeed, the data in Figure 2 shows that significant levels of drug are still present 24 h after a single dose, indicating that repeated daily dosing would likely lead to accumulation. This is consistent with the reported *in vivo* efficacy of enigmol in models of prostate and colon cancer,¹⁵ even though the observed single dose plasma levels were significantly lower than the IC_{50} values for the cell lines tested. Additional pharmacokinetic and tissue distribution

Table 1. Enigmol Analogue SAR Data^a

Comp.	Structure	Cytotoxicity, IC_{50} (μ M)	
		PC-3	LNCaP
Enigmol (5a)		10.3 ± 0.2	9.7 ± 0.3
SSR-enigmol (5b)		10.2 ± 0.2	9.7 ± 0.2
SRS-enigmol (5c)		9.7 ± 0.2	5.0 ± 0.2
SRR-enigmol (5d)		49.3 ± 0.3	50.0 ± 0.4
<i>N</i> -methyl-enigmol (8)		10.5 ± 0.2	25.3 ± 0.1

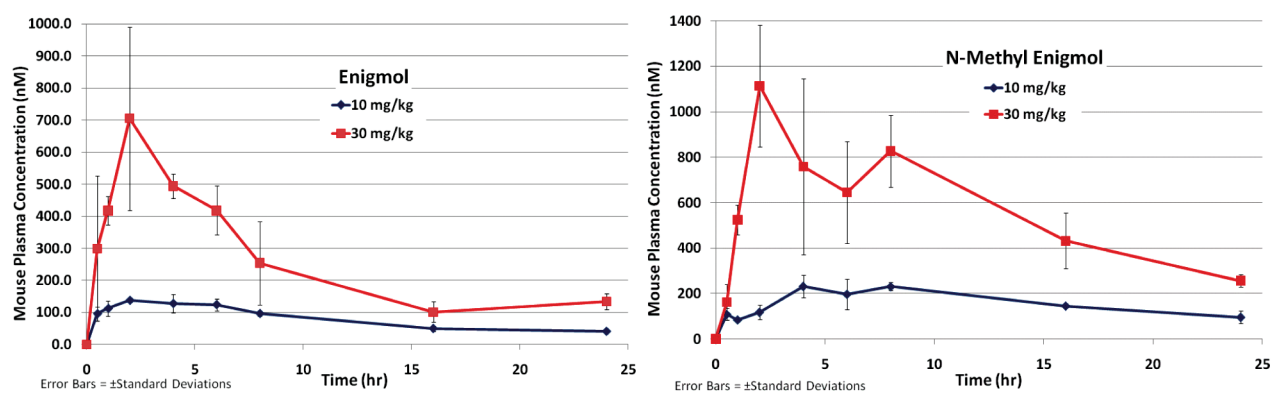
^a Prostate cancer cells were treated with enigmol or analogues for 24 h, and cytotoxicity was assessed by WST-1 assay.

studies are underway in order to better understand the ADME properties of these compounds.

A series of three xenograft studies were carried out in nude mice to investigate the efficacy of this series of 1-deoxy compounds *in vivo*. The oncolytic activity of enigmol was compared to standard prostate cancer therapies using LNCaP (androgen sensitive) and PC-3 (androgen insensitive) cell lines. These studies were designed to probe three specific issues: (1) the oncolytic activity of enigmol relative to androgen deprivation, produced by castration, in a LNCaP xenograft; (2) the oncolytic activity of enigmol compared with docetaxel in a PC-3 xenograft; and (3) the effects of C3–C5 stereochemical changes, as well as *N*-methylation, on the oncolytic activity of enigmol in a PC-3 xenograft.

To address the issue of enigmol efficacy vs castration, a subcutaneous LNCaP xenograft in nude mice was selected as a model of androgen sensitive prostate cancer. In this experiment, mice with palpable LNCaP tumors ($n = 10–12$) were castrated, treated with enigmol gavage, or a combination of the two, and the efficacy of treatments was compared to controls. As a control for effects of surgery or gavage that were unrelated to the effect of treatment, mice that were not castrated were given sham operations, and mice not receiving enigmol were gavaged with vehicle.

Tumor growth curves from the LNCaP study are shown in Figure 3A. Castrations and sham operations were performed on Day 13 after xenograft implantation, and daily oral gavage treatments began on Day 14 (10 mg/kg). Due to excessive tumor burden, all control mice, two mice in the castrated group, and three mice in the enigmol group were sacrificed on Day 36, and two mice in the enigmol + castration (**5a+C**) group were sacrificed on Day 38. Treatments were continued for the remaining mice to investigate whether greater efficacy of one treatment over another would be seen after a longer treatment period. Significance of differences in tumor growth rates was assessed using a mixed model for repeated measurements with covariance structure. Mixed effects models have been recommended for the analysis of tumor growth data for multiple reasons, including the ability to address missing values.³⁰ The use of this model allowed the comparison of tumor growth rates for all mice in this study, even those removed before its termi-



Mean Pharmacokinetic Parameters from Mouse PK Studies

Compound	Dose (mg/kg)	T _{max} (h)	C _{max} (nM)	T _{1/2} (h)	AUC (0-t) (h*nM)	AUC (0-∞) (h*nM)	AUC % Extrap (%)	V _z _F_obs (L/kg)	Cl_F_obs (L/h/kg)
Enigmol (5a)	10	2	137	11	1884	2543	26	211	13
Enigmol (5a)	30	2	705	5	5962	6958	14	107	14
NME (8)	10	4	232	15	3860	5989	36	117	5
NME (8)	30	2	1115	12	13564	17875	24	90	5

Figure 2. Mean enigmol (5a) and *N*-methylenigmol (8) plasma concentrations (nM) after p.o. dosing at 10 and 30 mg/kg in mice and PK parameters calculated from this data.

nation. Statistically significant, slower tumor growth rates were found in the enigmol (5a) and the 5a+C groups compared to controls ($P < 0.05$). Significance was not found between control versus castration ($P = 0.1$) nor in comparisons of noncontrol groups. Notably, two mice in the 5a+C group and one mouse in the 5a group, which initially had palpable tumors (albeit with small volumes, 13.5–32 mm³), experienced complete regression. No complete regressions were seen in mice that did not receive enigmol.

The second experiment probed the anticancer activity of enigmol against androgen insensitive prostate cancer using a PC-3 xenograft model in nude mice. The efficacy of enigmol (5a) was compared to that of docetaxel (D) and to a combination of enigmol and docetaxel (5a+D). In this experiment, oral gavage treatments (10 mg/kg) of mice bearing PC-3 xenografts ($n = 15$) began on Day 22 after implantation and continued until Day 34, with i.p. injections of vehicle or docetaxel administered on Days 22 and 29. The docetaxel dosage level of 15 mg/kg given once weekly was chosen because of positive results from a previous PC-3 xenograft study, and tumor growth in the docetaxel group here was comparable to that study.³¹ Tumor growth curves are shown in Figure 3B. Using the mixed model for repeated measurements, tumor growth rate was found to be significantly slower in the docetaxel, enigmol, and enigmol + docetaxel groups compared to controls ($P < 0.05$), while there was no significant difference between noncontrol groups.

A third *in vivo* study was designed to probe the role that various structural features of enigmol play in the anticancer activity of the agent. Specifically, two of three of the C-3, C-5 diastereomeric analogues, as well as the *N*-methyl analogue, were prepared and tested *in vivo* against androgen independent PC-3 xenografts to compare their relative efficacies with enigmol.³² Nude mice ($n = 10$) bearing established PC-3 tumors were given enigmol (5a), SSR (5b), SRS (5c), or *N*-methylenigmol (8) at 10

mg/kg daily by oral gavage, starting on day 16 after implantation, and tumor growth was measured over time (Figure 3C). Over the duration of treatment, animals receiving 5a, 5b, and 5c experienced significantly slower tumor growth rates than those treated with vehicle ($p = 0.02, 0.01, \text{ and } 0.01$, respectively, using the mixed model for repeated measurements). The degree of tumor growth inhibition by enigmol (5a) was similar to that seen in previous PC-3 xenograft studies,¹⁵ as well as in the PC-3 docetaxel study (Figure 3B), highlighting the reproducibility of *in vivo* efficacy with enigmol. Differences in tumor growth rates were not statistically significant in *N*-methylenigmol versus controls. A summary of p -values from the comparison of tumor growth rates for the three xenograft studies above is presented in the Supporting Information (Table Supp. 1).

In all three xenograft studies, animal weights were monitored regularly as an indicator of drug toxicity (Figure 3D–F). Through the course of treatment in the LNCaP xenograft study, the castration (C), enigmol (5a), and enigmol + castration (5a+C) groups had gained 8.0%, 9.5%, and 26% of pretreatment body weights, respectively, after 22 days of treatments (Day 36 after xenograft implantation). During the same time period, LNCaP control mice lost an average of 34% of body weight, most likely attributable to disease state. After the 22nd day of treatment, mice in the 5a+C group continued gaining weight, while weight change in the castration (C) and enigmol (5a) groups remained within 6% of pretreatment body weights. In the PC-3 studies, weight change in all groups was 0.3–6.6%, and the differences in weight change between groups were not statistically significant. Accordingly, we did not observe weight loss in any of the animal groups treated with enigmol or its analogues, suggesting little, if any, significant drug related toxicity.

Although enigmol (5a) did not generate a statistical advantage over androgen deprivation in androgen-sensitive cancer or in

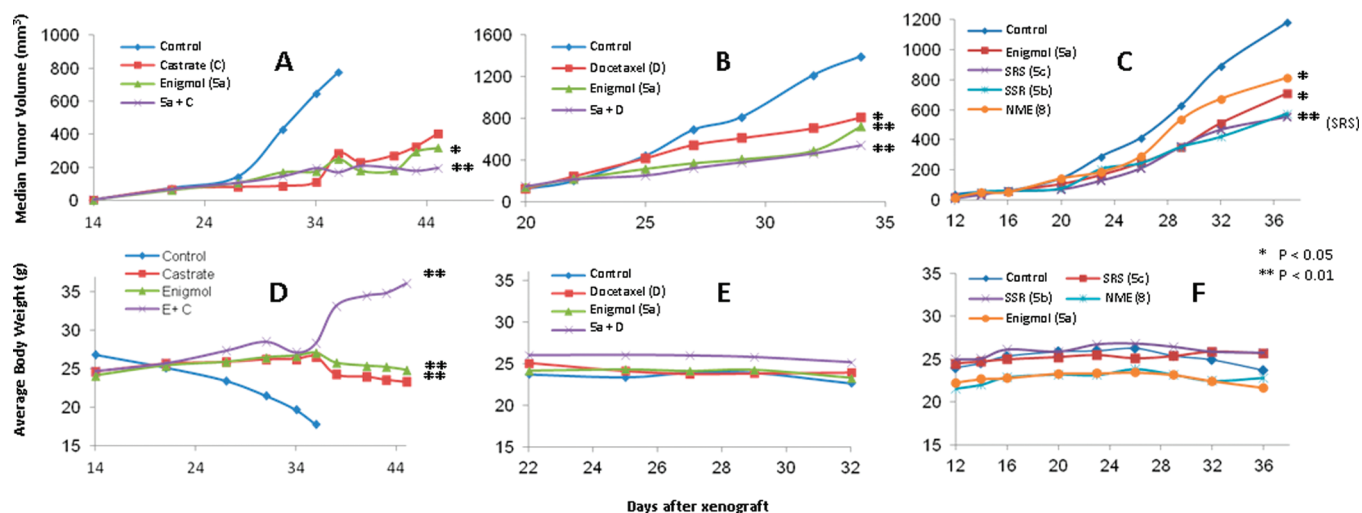


Figure 3. Results from three independent *in vivo* studies of enigmol (**5a**), two of its diastereomers (**5b** and **5c**), and *N*-methylenigmol (**8**). Nude mice with established tumors of LNCaP (A) or PC-3 (B, C) cells were treated with enigmol or enigmol analogues by daily oral gavage (10 mg/kg in all experiments), and tumor growth over time was measured. Efficacy of **5a** was compared to androgen deprivation in part A and to docetaxel in part B, and the relative efficacies of the enigmol analogues were assessed in part C. Animal weights were monitored over the course of treatments (D–F): mice treated with enigmol (**5a**) or analogues did not experience excessive weight loss that would be an indicator of drug-related toxicity. Significance of differences was determined by the mixed model for repeated measurements (A–C) or by ANOVA (D–F): * $p < 0.05$; ** $p < 0.01$ in relation to control groups. **5a**+**D** group, receiving both enigmol and docetaxel therapies; **5a**+**C** group, receiving therapies of both enigmol and androgen deprivation; NME, *N*-methylenigmol (**8**).

docetaxel treatment in hormone refractory cancer in these studies, the potential use of a safe and orally available option for prostate cancer treatment is appealing, particularly in the case of patients with androgen-sensitive prostate cancer facing castration. Furthermore, prostate cancer can have an exceptionally long clinical course: the prostate cancer-specific ten year survival for untreated cancer approaches 85%.^{33,34} Thus, it can be viewed in some senses as a “chronic disease”, which further heightens the need for multiple forms of therapy with safety profiles that allow for long-term dosing. It is not unusual for multiple, long-term therapeutic regimens to be utilized sequentially in individual patients with this disease, and there is substantial evidence that intermittent therapy may actually be preferable to continuous therapy in some patients.³⁵

In conclusion, we have evaluated enigmol, its C3 and C5 diastereomeric analogues and *N*-methylenigmol for efficacy in both *in vitro* and *in vivo* models of prostate cancer. In order to access these compounds synthetically, we developed an efficient, diversity-oriented synthetic approach of sphingolipid analogues, using a common intermediate derived from a Liebeskind–Srogl cross-coupling reaction, that results in both skeletal and stereochemical differences. Of the structural variations studied here, two of the diastereomers of enigmol displayed oncolytic activity similar to the parent compound, while the *N*-methyl analogue showed significantly less oncolytic activity *in vivo*.³² Results from the *N*-methylenigmol study were surprising, given that it had compared favorably with enigmol *in vitro* and had generated significantly higher circulating plasma levels in pharmacokinetic studies. Further studies are ongoing to expand our understanding of the structure activity effects of modifications to the enigmol scaffold. This insight will then be leveraged to optimize key druglike properties, including oncolytic efficacy, pharmacokinetic and tissue distribution profiles, and safety. Similarly, the synthetic approach that is reported here is being further refined and

expanded to provide an increasingly comprehensive access to diverse analogue sets.

■ ASSOCIATED CONTENT

S Supporting Information. Synthetic experimental details, analytical data of compounds, and biological assay protocols. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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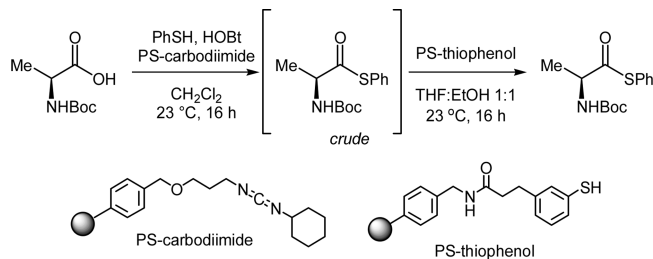
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