Discovery of a Fluorinated Enigmol Analog with Enhanced *in Vivo* Pharmacokinetic and Anti-Tumor Properties

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Supporting Information

ABSTRACT: The orally bioavailable 1-deoxy-sphingosine analog, Enigmol, has demonstrated anticancer activity in numerous *in vivo* settings. However, as no Enigmol analog with enhanced potency *in vitro* has been identified, a new strategy to improve efficacy *in vivo* by increasing tumor uptake was adopted. Herein, synthesis and biological evaluation of two novel fluorinated Enigmol analogs, CF₃-Enigmol and CF₂-Enigmol, are reported. Each analog was equipotent to Enigmol *in vitro*, but achieved higher plasma and tissue levels than Enigmol *in vivo*. Although plasma and tissue exposures were anticipated to trend with fluorine content, CF₂-Enigmol absorbed into tissue at strikingly higher concentrations than CF₃-Enigmol. Using mouse xenograft models of prostate cancer, we also show that CF₃-Enigmol underperformed Enigmol-mediated inhibition of tumor growth and elicited systemic toxicity. By contrast, CF₂-Enigmol was not systemically toxic and demonstrated significantly enhanced antitumor activity as compared to Enigmol.

KEYWORDS: Sphingolipids, Enigmol, fluorination, prostate cancer

Sphingolipids are a structurally diverse¹ class of lipids containing a common scaffold that is exemplified by sphingosine (Figure 1a), a ubiquitous intermediate in the biosynthesis of a wide array of sphingolipids, many of which are second messengers.² For example, amino group acylation by ceramide synthase (CS) produces ceramides,³ which facilitate signaling events from lipid rafts. Alternatively, phosphorylation of the primary hydroxyl group by sphingosine kinase (SK) generates sphingosine-1-phosphate⁴ (S1P), the endogenous agonist for seven-transmembrane S1P receptors. The sphingolipid biosynthetic and metabolic pathways include numerous pleiotropic signaling molecules involved in complex equilibria. Notable among these are the opposing roles of sphingosine and S1P: S1P promotes cell survival, whereas sphingosine promotes cell death.⁵ While healthy cells benefit from a proper balance of S1P and sphingosine, many cancer cell types overexpress SK,⁶ which tips the balance in favor of S1P. Since the cytotoxicity of

endogenous sphingoid bases⁷ is limited by phosphorylation, inhibition of SK represents a promising anticancer strategy.⁸

By contrast, we envisioned that a sphingosine mimic incapable of phosphorylation could reestablish the proper sphingosine/S1P balance, and thereby, suppress cancer growth. Specifically, sphingoid base analogs lacking the C-1 hydroxyl group were hypothesized to adopt the cytotoxic signaling profile of sphingosine without the counterweight of S1P metabolites. Evaluation of 1-deoxy-sphingoid bases^{9,10} led to the identification of Enigmol (Figure 1b), which exhibited broad-spectrum cytotoxicity ($0.4 \mu\text{M} \leq \text{IC}_{50} \leq 14 \mu\text{M}$) against 57 human cancer cell lines,¹¹ in addition to LNCaP ($\text{IC}_{50} = 9.7 \pm 0.3 \mu\text{M}$) and PC-3 ($\text{IC}_{50} = 10.3 \pm 0.2 \mu\text{M}$) human prostate

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Figure 1. Sphingosine-S1P axis and fluorinated Enigmol analogs. cLogP was calculated using QikProp in Maestro.

cancer cell lines,¹² *in vitro*. This activity profile was attributed to (1) inhibition of SK,¹¹ CS,¹⁰ and protein kinase C⁹ (PKC), (2) “immunity” to SK and other kinases,¹¹ and (3) slow CS-mediated *N*-acylation.¹⁰ Sluggish metabolism endows Enigmol with a longer lifetime than its endogenous counterparts, thereby extending the duration of its therapeutic action. While most compounds with micromolar anticancer activity *in vitro* cannot achieve effective tumor concentrations *in vivo*, Enigmol robustly partitions into tissue, building up drug reservoirs after multiple oral (p.o.) doses.¹² This combination of long lifetime and extensive tissue uptake enabled moderately cytotoxic Enigmol to significantly inhibit tumor growth in multiple cancer models *in vivo* without causing systemic toxicity.¹¹ In a mouse xenograft model of prostate cancer, for example, Enigmol exhibited comparable efficacy to surgical hormone deprivation and to docetaxel, which are the current clinical standards of care for androgen-dependent and androgen-independent prostate cancer, respectively.¹²

As evidenced by striking *in vivo* efficacy and low systemic toxicity, Enigmol's modest *in vitro* potency is leveraged by favorable pharmacokinetic (PK) properties *in vivo*. These results encouraged synthesis and evaluation of various Enigmol analogs,^{12,13} a process facilitated by two diastereoselective approaches to Enigmol developed in our laboratory.^{14,12} However, because the maximum potential of these compounds could only be determined with time- and resource-intensive *in vivo* studies, we decided to increase lipophilicity with the expectation of enhanced anticancer activity due to improved oral absorption and elevated tumor exposure. Specifically, replacing selected hydrogens in Enigmol (cLogP = 3.55) with fluorines was anticipated to increase hydrophobicity without substantially perturbing other physicochemical properties.^{15,16} Accordingly, we prepared two analogs¹³ (Figure 1b), CF₃-Enigmol (cLogP = 4.25, trifluorination at C-18) and CF₂-Enigmol (cLogP = 3.88, difluorination at C-6), and evaluated: (1) cytotoxicity against cultured human prostate cancer cells, (2) plasma and tissue PK properties, and (3) antitumor activity in xenograft models of prostate cancer.

The synthesis of CF₃-Enigmol was accomplished using our previously reported diastereoselective aldol route¹⁴ (Scheme 1a). Homopropargylic alcohol **1** was converted to THP-ether **2** via an alkyne zipper reaction,¹⁷ followed by protection of the primary alcohol. The resulting terminal alkyne was then alkylated¹⁸ with bromide **3**, followed by THP group removal and Dess-Martin oxidation to produce the desired aldehyde.

Scheme 1. Synthesis of (a) CF₃-Enigmol and (b) CF₂-Enigmol

Addition of the aldehyde to a preformed, (–)-DiPCL-stabilized enolate of methyl ketone **5**¹⁴ produced the aldol adduct, which was immediately subjected to reduction with sodium borohydride, yielding *syn*-diol **6** in 50% yield over three steps (*d.r.* = 99:1). Finally, a one-pot alkyne hydrogenation/*N,N*-dibenzylamino hydrogenolysis furnished CF₃-Enigmol. CF₂-Enigmol was prepared using a modified aldol approach involving α,α -difluoro methyl ketone **9** and α -aminoaldehyde **10**¹⁹ (Scheme 1b). Tandem hydrozirconation/iodination²⁰ of alkyne **7** gave the corresponding terminal vinyl iodide, which was subjected to a copper-mediated coupling²¹ with ethyl bromodifluoroacetate to give α,α -difluoro ester **8**. The resulting olefin was reduced via palladium-catalyzed hydrogenation²² and subsequently converted to ketone **9** using a two-step Weinreb ketone synthesis.²³ Achiral borane-mediated aldol reaction with aldehyde **10**¹⁹ resulted in a chromatographically separable mixture of diastereomers (*d.r.* = 2:1, 36% and 20% isolated yields). Although TiCl₄ led to improved *syn*-diastereoselectivity (*d.r.* = 4:1, Supporting Information Table 1), isolated yield suffered substantially, and the use of either (+)-DiPCL or (–)-DiPCL did not significantly improve *syn*-stereoselection. Attempted *syn*-reduction of the resulting β -hydroxy ketone using Et₂BOMe and NaBH₄²⁴ gave disappointing selectivity as

Table 1. Cytotoxicity against Cultured Human Prostate Cancer Cells^a

Drug	LNCaP Cells		PC-3 Cells	
	IC ₅₀ (μM)	IC ₉₀ (μM)	IC ₅₀ (μM)	IC ₉₀ (μM)
Enigmol	11.7 ± 1.10	19.6 ± 1.10	12.1 ± 1.02	14.0 ± 1.00
CF ₃ -Enigmol	11.3 ± 1.10	19.2 ± 1.30	21.4 ± 1.17	24.4 ± 1.20
CF ₂ -Enigmol	13.4 ± 1.07	16.7 ± 1.20	11.7 ± 1.05	15.3 ± 1.10

^aLNCaP or PC-3 cells were incubated with compound for 24 h (*n* = 3–4), and viability was assessed using WST-1. Data represent the mean ± SEM.

well (Supporting Information Table 2). Interestingly, while reduction with DiBAL-H (*d.r.* = 1:5) favored the *anti*-diol, DiBAL-H reduction with additive ZnBr₂²⁵ (*d.r.* = 5:2) furnished the desired *syn*-diol **11** in 56% isolated yield. Finally, deprotection of the *N,N*-dibenzylamino group via hydrogenolysis produced CF₂-Enigmol.

CF₃-Enigmol and CF₂-Enigmol were assayed for cytotoxicity toward cultured androgen-dependent (LNCaP) and androgen-independent (PC-3) human prostate cancer cells by measuring mitochondrial activity¹² (Supporting Information Figure 2). As expected, the IC₅₀ values (Table 1) of CF₃-Enigmol and CF₂-Enigmol were comparable to Enigmol, indicating that fluorine incorporation did not substantially alter potency, and suggesting that any differences in *in vivo* activity would stem from altered PK properties. IC₉₀ values were also calculated to indicate therapeutically effective concentrations desired in tumors.

With therapeutic concentrations established, PK studies were designed to evaluate the effect of fluorination on plasma elimination, oral absorption, and tissue distribution. As rapid intravenous (i.v.) injection effectively normalizes plasma absorption profiles, Sprague–Dawley (SD) rats were injected i.v. with a single 2 mg/kg dose of either Enigmol (*n* = 3), CF₃-Enigmol (*n* = 4), or CF₂-Enigmol (*n* = 4), facilitating reliable assessment of terminal half-life (*t*_{1/2}) and rate of clearance (Cl). Analog concentrations in isolated plasma were quantified using liquid chromatography/tandem-mass spectrometry (LC-MS/MS) analysis (Figure 2). Although plasma elimination was

Figure 2. Plasma pharmacokinetics (i.v.). SD rats (*n* = 3–4 per group) were treated with compound (2 mg/kg) via i.v. injection (vehicle = 40:10:3:47 PEG400/EtOH/Tween80/H₂O), and plasma concentration was measured periodically using LC-MS/MS. Data represent the mean ± SEM.

anticipated to trend inversely with cLogP due to plasma protein binding, *t*_{1/2} and Cl values, as well as the maximum plasma concentration (*C*_{max}) and area under the curve (AUC), indicated that CF₃-Enigmol was removed from plasma substantially faster than both Enigmol and CF₂-Enigmol. By contrast, CF₂-Enigmol followed the expected trend, exhibiting lower Cl and higher *t*_{1/2}, AUC, and *C*_{max} than Enigmol.

Subsequent PK experiments were designed to compare plasma absorption and tissue distribution properties after oral administration. Accordingly, SD rats were dosed once via oral gavage with 10 mg/kg of either Enigmol (*n* = 4), CF₃-Enigmol (*n* = 4) or CF₂-Enigmol (*n* = 4), and analog concentrations in isolated plasma and various tissues were quantified using LC-MS/MS.²⁶ Counter to results from i.v. experiments, systemic exposure after p.o. dose, as indicated by *C*_{max} and AUC, trended with cLogP (Figure 3a). Furthermore, CF₃-Enigmol required

Drug	Plasma Pharmacokinetic Parameters (p.o.)				
	<i>T</i> _{max} (hrs)	<i>C</i> _{max} (nM)	<i>t</i> _{1/2} (hrs)	AUC ₀₋₄ (nM*hrs)	%F
Enigmol	8.0 ± 1.4	84.6 ± 2.87	8.3 ± 4.2	832 ± 26.2	11
CF ₃ -Enigmol	12 ± 0.50	193 ± 30.2	5.5 ± 1.4	2340 ± 236	68
CF ₂ -Enigmol	8.0 ± 0.0	119 ± 16.1	9.9 ± 1.6 ^b	1470 ± 175	10

Drug	Tissue Accumulation (μM) after 24 hrs					
	RBC	Brain	Lungs	Liver	Kidney	Prostate
Enigmol	0.041 ± 0.016	3.25 ± 0.179	5.34 ± 0.414	1.06 ± 0.144	1.35 ± 0.095	0.766 ± 0.106
CF ₃ -Enigmol	0.153 ± 0.034	6.01 ± 1.46	18.7 ± 2.83	1.94 ± 0.234	4.74 ± 0.334	3.46 ± 0.627
CF ₂ -Enigmol	0.936 ± 0.039	16.1 ± 1.46	44.5 ± 2.07	9.29 ± 0.816	14.8 ± 1.30	6.94 ± 1.33

Figure 3. Plasma pharmacokinetics and tissue distribution (p.o.). (a) SD rats (*n* = 4 per group) were orally administered 10 mg/kg of either Enigmol (vehicle = 90:10 olive oil/EtOH), CF₃-Enigmol (vehicle = 90:10 olive oil/EtOH), or CF₂-Enigmol (vehicle = 95:5 PEG400/Tween80), and plasma concentration was measured periodically using LC-MS/MS. (b) After 24 h, tissues were harvested, and drug levels were measured by LC-MS/MS. Data represent the mean ± SEM. CF₂-Enigmol *t*_{1/2} is reported with the exclusion of one rat due to inappropriate semilog plots of the terminal 2, 3, or 4 nonzero data points (positive linear slopes or *R*² = 0.038).

the longest amount of time (*T*_{max}) to reach *C*_{max}, and also demonstrated substantially improved oral bioavailability (68%) as compared to Enigmol (11%) and CF₂-Enigmol (10%). Exponential decay of CF₂-Enigmol plasma concentration after *C*_{max} was likely not observed due to a relatively high *t*_{1/2} and a long absorption phase that overlaps with the elimination phase. Notably, Enigmol and CF₃-Enigmol were not observed in plasma for >1 h, nor was CF₂-Enigmol for >2 h. With plasma elimination virtually complete after 24 h, the accumulation of each compound in red blood cells, brain, lung, liver, kidney, and prostate was measured (Figure 3b). Although tissue distribution was anticipated to correlate with cLogP, CF₂-Enigmol (*n* = 4) achieved substantially higher concentrations than both Enigmol (*n* = 4) and CF₃-Enigmol (*n* = 4) in all tissues

Figure 4. CF₃-Enigmol and CF₂-Enigmol in mouse xenograft models of prostate cancer. Nude mice ($n = 10$ – 11 per group) with palpable tumors derived from PC-3 human prostate cancer cells were treated once daily via oral gavage with either vehicle (95:5 olive oil/EtOH), Enigmol (3 or 10 mg/kg), or CF₃-Enigmol (3 or 10 mg/kg) in the first study (a–c), or with either vehicle (95:5 PEG400/Tween80), Enigmol (10 mg/kg), or CF₂-Enigmol (10 or 30 mg/kg) in the second study (d–f). Tumor volume and change in body weight were measured periodically. At the end of each study, tumors were harvested, and accumulation was measured using LC-MS/MS. Data represent the mean $\pm$ SEM. Statistical significance ($*p < 0.05$, $**p < 0.01$, or $***p < 0.001$) of tumor growth inhibition was assessed via a linear mixed model for repeated measurement with the autoregressive covariance structure using Statistical Analysis System (SAS) software. Significance of change in body mass was determined using One-Way ANOVA followed by Sidak's multiple comparison test.

analyzed. While Enigmol never reached its IC₉₀ level ($\geq 14 \mu\text{M}$) in any tissue type, CF₃-Enigmol did so in lung ($\geq 19 \mu\text{M}$). Remarkably, CF₂-Enigmol achieved IC₉₀ concentrations ($\geq 15 \mu\text{M}$) in lung, brain, and kidney. Also notable are the relative drug levels in prostate, where CF₂-Enigmol achieved 2-fold and 9-fold higher concentrations than CF₃-Enigmol and Enigmol, respectively.

Although tissue accumulation did not trend with *cLogP*, fluorinated analogs reached higher tissue levels than Enigmol. Therefore, CF₃-Enigmol and CF₂-Enigmol were expected to achieve elevated tumor concentrations and enhanced antitumor efficacy. To test this hypothesis, nude mice were injected subcutaneously with PC-3 cells, and tumors were established until palpable before beginning once daily oral gavage of either Enigmol (3 mg/kg or 10 mg/kg), CF₃-Enigmol (3 mg/kg or 10 mg/kg), or vehicle (95:5 olive oil/EtOH). Tumor volume was measured to quantify drug-induced growth inhibition (Figure 4a). While 3 mg/kg dosing regimens were ineffective for both Enigmol ($n = 11$) and CF₃-Enigmol ($n = 10$), 10 mg/kg Enigmol ($n = 11$) significantly inhibited tumor growth ($p = 0.02$) versus control ($n = 11$); 10 mg/kg CF₃-Enigmol ($n = 11$) surprisingly did not ($p = 0.17$), despite substantially elevated tissue concentrations. While no weight loss (Figure 4b) was observed among control, 3 mg/kg Enigmol, 10 mg/kg Enigmol, or 3 mg/kg CF₃-Enigmol treatment groups, 10 mg/kg CF₃-Enigmol caused significant weight loss ($11.6 \pm 1.32\%$) over the treatment course ($p = 0.0037$). To evaluate the relationship between efficacy and local drug concentrations, tumor accumulation of Enigmol and CF₃-Enigmol was quantified using LC-MS/MS at the end of the study (Figure 4c). While 10

mg/kg Enigmol did not achieve IC₉₀^{PC-3} concentrations ($=14 \mu\text{M}$) in tumor, 10 mg/kg CF₃-Enigmol (IC₉₀^{PC-3} = $24 \mu\text{M}$) did. A related boost in antitumor activity, however, was unexpectedly not observed.

Thus, focus was shifted to CF₂-Enigmol, which was compared head-to-head with Enigmol in a subsequent xenograft study. After tumors were established, once daily oral gavage of vehicle (95:5 PEG400/Tween80, $n = 9$ or 10 , one mouse died on day 32), 10 mg/kg Enigmol ($n = 9$ or 10 , one mouse died on day 36), 10 mg/kg CF₂-Enigmol ($n = 10$), or 30 mg/kg CF₂-Enigmol ($n = 10$) was initiated (Figure 4d). Gratifyingly, 10 mg/kg CF₂-Enigmol significantly inhibited tumor growth relative to both control ($p = 0.0003$) and 10 mg/kg Enigmol ($p = 0.04$), and neither 10 mg/kg nor 30 mg/kg CF₂-Enigmol caused significant weight loss (Figure 4e). Consistent with tissue distribution, 10 mg/kg CF₂-Enigmol (IC₉₀^{PC-3} = $15 \mu\text{M}$) achieved strikingly higher tumor concentrations ($24 \mu\text{M}$) than 10 mg/kg Enigmol (Figure 4f). Although 30 mg/kg CF₂-Enigmol reached extraordinary tumor levels ($62 \mu\text{M}$), it underperformed relative to 10 mg/kg CF₂-Enigmol, suggesting that a therapeutically saturating concentration in tumor cells can be achieved after 2 weeks of once daily oral dosing at $<10 \text{ mg/kg}$.

CF₂-Enigmol exhibited slower plasma elimination kinetics (i.v.), more robust plasma absorption (p.o.), and more pronounced tissue accumulation than Enigmol and CF₃-Enigmol. Although oral absorption trended with *cLogP*, the effects of fluorination on plasma elimination and tissue accumulation followed entirely different trends. Since *cLogP* can be an insufficient representation of fluorinated small

molecule lipophilicity, an astute reviewer suggested that empirical measurements of LogD would help to clarify the role of lipophilicity. Therefore, LogD (pH = 7.4, $n = 2$, mean $\pm$ SEM) of each analog was measured empirically (Absorption Systems, Exton, PA), indicating an unexpected trend in lipophilicity: Enigmol (3.50 ± 0.09) > CF₃-Enigmol (3.24 ± 0.06) > CF₂-Enigmol (1.73 ± 0.06). Counter to the Hansch hydrophobicity parameters¹⁵ and the average increase in LogD upon fluorine incorporation,¹⁶ fluorination sometimes actually decreases lipophilicity due to the interplay between change in volume and dipole moment.²⁷ For example, trifluoroethanol¹⁵ (LogP = 0.36) is more lipophilic than ethanol (LogP = -0.32) because the volume change upon fluorination overrides the change in dipole moment. Conversely, larger trifluoromethylhexanol (LogP = 1.14) is less lipophilic than hexanol (LogP = 2.03) because the volume change is insignificant compared to the change in dipole moment. In the case of fluorinated Enigmol analogs, an apparent increase in dipole moment upon fluorination appears to override the relatively small increase in volume, leading to decreased lipophilicity. Furthermore, increased acidity of the CF₂-Enigmol C-5 alcohol likely leads to improved hydrogen bonding with water,¹⁶ combining with the inherent polarity of the difluoromethylene unit²⁸ to decrease LogD. Notably, the conventional wisdom that lipophilicity is a strong determinant of systemic exposure after oral administration did not hold up here: tissue accumulation actually trended inversely with LogD.

Ultimately, the flat *in vitro* SAR observed with Enigmol analogs was overcome by modulating PK properties via fluorine installation. Since CF₃-Enigmol and CF₂-Enigmol exhibited similar cytotoxicity to Enigmol against human prostate cancer cells *in vitro*, we conclude that differences in antitumor efficacy resulted from changes in PK. As no trend between LogD and any PK parameter (other than an inverse trend with tissue accumulation) was observed, the PK results presented herein indicate that physicochemical properties other than lipophilicity contribute to the plasma elimination, oral absorption, and tissue distribution of these compounds. Despite achieving IC₉₀ levels in tumor, CF₃-Enigmol did not significantly inhibit tumor growth and caused significant weight loss. Alternatively, CF₂-Enigmol statistically suppressed tumor growth better than Enigmol, likely because of elevated tumor concentrations. Since Enigmol was equally efficacious to docetaxel,¹² CF₂-Enigmol may offer an advantage over taxane-based chemotherapeutics for hormone-refractory prostate cancer, especially given the lack of systemic toxicity. Furthermore, due to enormous CF₂-Enigmol concentrations in lung, brain, and kidney, antitumor efficacy could prove to be even more profound for the treatment of solid tumors residing in these tissues.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.6b00113.

Synthetic procedures utilized for the preparation of CF₃-Enigmol and CF₂-Enigmol, concentration–response curves of Enigmol, CF₂-Enigmol, and CF₃-Enigmol, and experimental methods and raw data from pharmacokinetic and xenograft experiments. (PDF)

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Author Contributions

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■ ABBREVIATIONS

CS, ceramide synthase; SK, sphingosine kinase; S1P, sphingosine-1-phosphate; PK, pharmacokinetic; i.p., intraperitoneal; i.v., intravenous; p.o., per os (oral); NOE, Nuclear Overhauser Effect; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfohenyl)-2H-tetrazolium monosodium salt; SD, Sprague–Dawley; LC-MS/MS, liquid chromatography/tandem-mass spectrometry

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