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Bioorganic & Medicinal Chemistry Letters 14 (2004) 2303–2308
Bioorganic & Medicinal Chemistry Letters

Synthesis, SAR, and antitumor properties of diamino-*C*,*N*-diarylpyrimidine positional isomers: inhibitors of lysophosphatidic acid acyltransferase-β

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Received 21 December 2003; accepted 30 January 2004

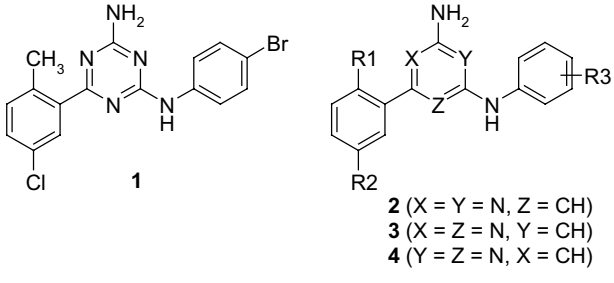
Abstract—2,4-Diamino-N^4,6-diarylpyrimidines were identified as potent, isoform specific inhibitors of lysophosphatidic acid acyltransferase-β (LPAAT-β). Active inhibitors also blocked proliferation of tumor cell lines in vitro. The effect of **2j** in an in vivo tumor model was investigated.

Lysophosphatidic acid acyltransferases (LPAATs) catalyze the *sn*-2 acylation of 1-acyl-*sn*-glycerol-3-phosphate (lysophosphatidic acid, LPA) to produce 1,2-diacyl-*sn*-glycerol-3-phosphate (phosphatidic acid, PA). The majority of LPAAT activity in mammalian cells has been attributed to two isoforms, LPAAT-α and LPAAT-β.[1] Both isoforms are integral membrane proteins. While LPAAT-α is uniformly expressed in all human tissues tested, LPAAT-β displays distinct tissue distribution and is highly expressed in a wide variety of tumor cells and their surrounding stroma.[1–5]

PA is an important lipid cofactor that has been implicated in cell signaling events including Raf translocation to membranes, mTOR activation, epidermal growth factor receptor (EGFR) internalization, and activation of PKCζ.[6–9] Ectopic overexpression of LPAAT-β cooperates in activation of Ras/Raf/Erk pathway in *Xenopus* oocytes and PA produced by LPAAT-β appears to play an important role in signaling pathways involved in tumor cell survival.[10] RNAi knockdown of LPAAT-β blocked tumor cell proliferation.[5] Accordingly, LPAAT-β may provide a novel target for cancer therapy.

A group of 2,4-diamino-N^4,6-diaryltriazines have been reported as isoform specific inhibitors of LPAAT-β.[11] This group is exemplified by **1** (CT-32228, LPAAT-β $IC_{50} = 0.06\,\mu M$) an effective antiproliferative agent toward a variety of tumor cell lines in vitro with IC_{50}s ranging from 0.025 to 0.5 μM.[5,10]

In our search for structural variants of active triazines we synthesized bioisosteric diamino-*C*,*N*-diarylpyrimidine analogs. A pyrimidine, possessing one less ring nitrogen than a triazine, provides the opportunity to examine preferences for LPAAT-β inhibition by three possible pyrimidine positional isomers **2**, **3**, and **4**. This report summarizes the synthesis and elaboration of the SAR for LPAAT-β inhibition by all three structural variants. Also, we investigated the effects of these pyrimidines on proliferation of tumor cell lines in vitro.



2,4-Diamino-N^4,6-diarylpyrimidine **2** was synthesized in two to four steps involving Suzuki coupling for arylation of the pyrimidine ring (Scheme 1).[12,13] Aryl

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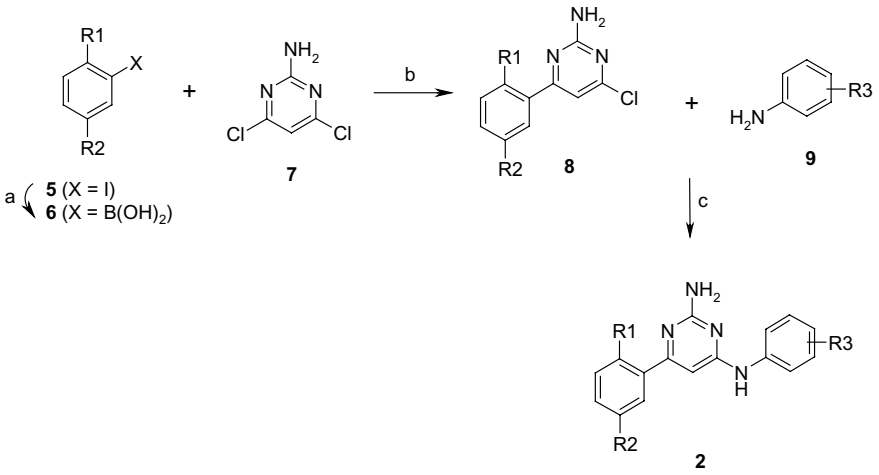
doi:10.1016/j.bmcl.2004.01.104

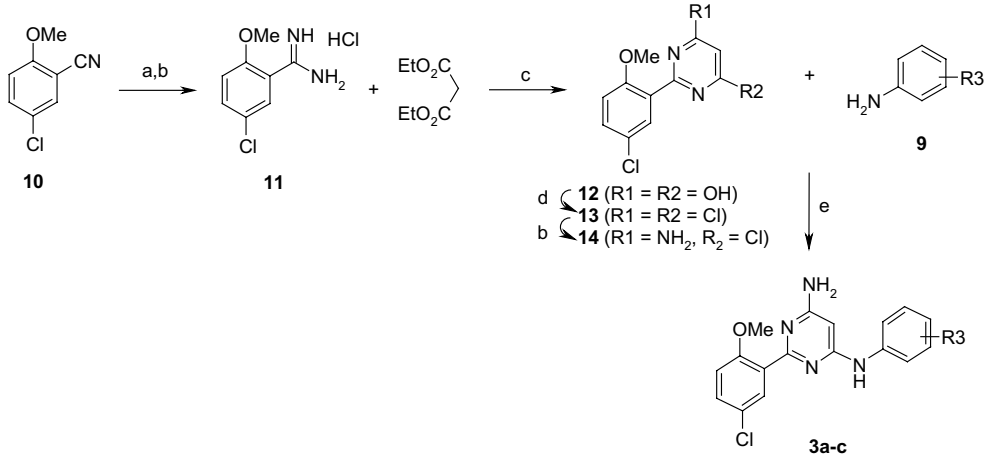


Scheme 1. Reagents and conditions: (a) *i*-PrMgCl, $B(OMe)_3$, THF; (b) $Pd(OAc)_2$, PPh_3, Na_2CO_3, H_2O, monoglyme; (c) HCl–dioxane, EtOH, reflux, 1 h.

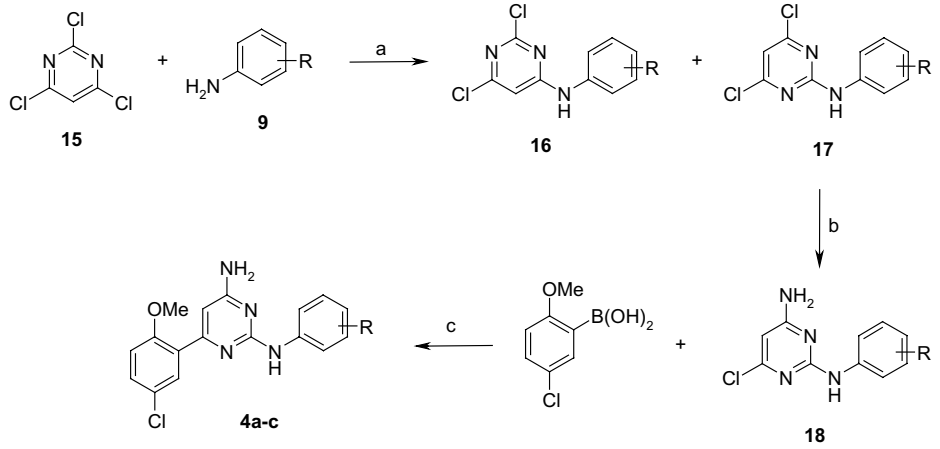
boronic acid **6** was either commercially available or synthesized from the corresponding aryl iodide **5** by iodine–magnesium exchange using *i*-PrMgCl followed by magnesium–boron exchange with $B(OMe)_3$.[14] To obtain aryl iodide **5** (R1 = Me, R2 = Br), 4-bromotoluene was iodinated using $NaIO_4$–H_2SO_4–Ac_2O–AcOH.[15] Palladium catalyzed Suzuki coupling of **6** with 2-amino-4,6-dichloropyrimidine **7** yielded the requisite intermediate 2-amino-6-aryl-4-chloropyrimidine **8**. Phenolic pyrimidines **8** (R1 = Me, R2 = OH), **8** (R1 = OH, R2 = Cl), and **8** (R1 = OH, R2 = Br), used in preparing **2m**, **2n**, and **2o**, respectively, were produced by O-demethylation of their corresponding methyl ethers using BBr_3–DCM. Displacement of the remaining chloro group in **8** with aniline **9** under acidic conditions provided pyrimidine isomer **2**. Apparently, activation of the pyrimidine ring in **8** by protonation is essential for this displacement to proceed since basic conditions failed to yield **2**.[16] The synthesis of **2r** was completed by $LiAlH_4$–THF reduction of **2** (R1 = OEt, R2 = Cl, R3 = CO_2Et).

4,6-Diamino-2,N^4-diarylpyrimidine **3** was synthesized in six steps involving pyrimidine ring construction (Scheme 2). Treatment of benzonitrile **10** with HCl–EtOH provided a benzimidic acid ethyl ester, which upon treatment with NH_3–THF yielded benzamidine **11** as its HCl salt.[17]Condensation of **11** with ethyl malonate in the presence of sodium methoxide as base provided aryldihydroxypyrimidine **12**. Conversion of **12** to the symmetrical dichloropyrimidine **13** was accomplished by heating **12** with neat $POCl_3$.[18] Monoamination of **13** with NH_3–THF in a pressure tube provided aminochloropyrimidine **14**. Displacement of the remaining chloro group in **14** with aniline **9** under acidic conditions generated pyrimidine positional isomer **3** albeit at a slower rate than comparable reaction conditions to produce **2**.

Synthesis of 2,4-diamino-N^2,6-diarylpyrimidine **4** provided additional evidence to support our structure assignment for **3** (Scheme 3). Treatment of trichloropyrimidine **15** with aniline **9** in dioxane under reflux yielded a 1:1 mixture of two dichloropyrimidine positional isomers **16** and **17**, which were efficiently separated by silica gel column chromatography.[19] Purified symmetrical dichloropyrimidine **17** was treated with



Scheme 2. Reagents and conditions: (a) HCl, EtOH; (b) NH_3, THF; (c) NaOMe, MeOH, reflux; (d) $POCl_3$, reflux; (e) HCl, dioxane, EtOH, reflux.

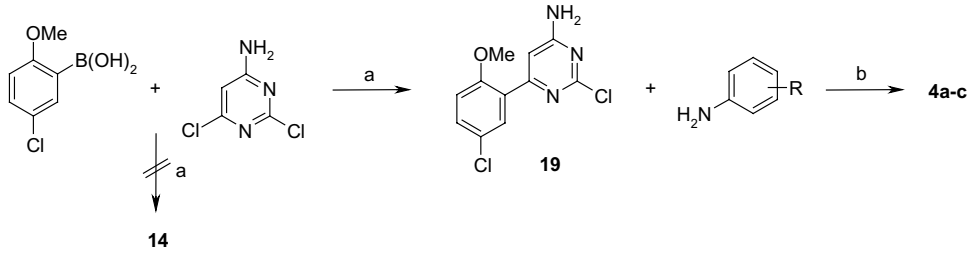


Scheme 3. Reagents and conditions: (a) dioxane, reflux; (b) NH_3, THF; (c) $Pd(OAc)_2$, Ph_3P, Na_2CO_3, H_2O, monoglyme.

NH_3–THF to produce aminochloropyrimidine **18**. Suzuki coupling of **18** with 5-chloro-2-methoxyphenyl boronic acid generated positional isomer **4**.

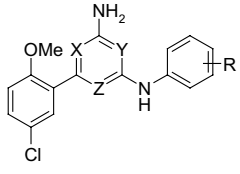
Compound **4** was synthesized more conveniently and in higher yield by the method described in Scheme 4. Suzuki coupling of 5-chloro-2-methoxyphenyl boronic acid with 4-amino-2,6-dichloropyrimidine yielded aminochloropyrimidine **19**. Although we anticipated a mixture of two isomers (**14** and **19**) from this Suzuki coupling, only **19** was observed. Treatment of **19** with aniline **9** under acidic conditions provided **4**. TLC behavior (R_f) and the ^1H NMR spectrum of **4a** synthesized by this method were identical to material produced by the method outlined in Scheme 3 confirming that the Suzuki arylation of 4-amino-2,6-dichloropyrimidine with 5-chloro-2-methoxyphenyl boronic acid occurred at the pyrimidine 6-position to produce **19** rather than at the 2-position to produce **14** (Scheme 4).

The compounds listed in Tables 1 and 2 were tested for their ability to inhibit human LPAAT-β overexpressed



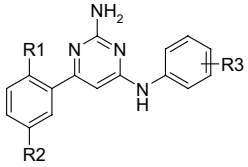
Scheme 4. Reagents and conditions: (a) $Pd(OAc)_2$, Ph_3P, Na_2CO_3, H_2O, monoglyme; (b) HCl, dioxane, EtOH, reflux.

Table 1. LPAAT-β inhibition and antiproliferative activity of diamino-*C*,*N*-diarylpyrimidine positional isomers



Compd	X	Y	Z	R	LPAAT-β (IC_{50}, μM)	Tumor cell proliferation (IC_{50}, μM)	
						K-562	DU145
2a	N	N	CH	4-Cl	0.054	0.15	0.5
2b	N	N	CH	4-Br	0.028	0.15	0.25
2c	N	N	CH	4-CH=N–NH-5	0.080	0.25	1
3a	N	CH	N	4-Cl	7	0.75	5
3b	N	CH	N	4-Br	8	0.15	0.8
3c	N	CH	N	4-CH=N–NH-5	2.1	5.5	>10
4a	CH	N	N	4-Cl	7	3	>10
4b	CH	N	N	4-Br	2.3	3	>10
4c	CH	N	N	4-CH=N–NH-5	0.8	>10	>10

Table 2. LPAAT-β inhibition and antiproliferative activity by 2,4-diamino-N^4,6-diarylpyrimidines



Compd	R1	R2	R3	LPAAT-β (IC_{50}, μM)	Tumor cell proliferation(IC_{50}, μM)	
					K-562	DU145
2d	H	H	4-Cl	1.1		
2e	Cl	Cl	4-Cl	0.02	<0.1	0.19
2f	Me	F	4-Cl	0.15	0.25	>1
2g	Me	Cl	4-Cl	0.022	0.06	0.12
2h	Me	Cl	4-Br	0.034	0.06	0.13
2i	Me	Cl	3-Br	0.61	0.2	0.4
2j	Me	Cl	4-CF_3	0.14	0.14	0.23
2k	Me	Br	4-Cl	0.031	0.07	0.1
2l	Me	Br	4-Br	0.018	0.07	0.11
2m	Me	OH	4-Cl	8.3		
2n	OH	Br	4-Cl	0.45	>1	>1
2o	OH	Cl	4-Cl	10		
2p	OEt	Cl	4-Cl	0.017	<0.012	0.07
2q	OEt	Cl	2-Cl	4.9		
2r	OEt	Cl	4-CH_2OH	0.075	<0.012	0.04
2s	OEt	Cl	4-$(CH_2)_2OH$	0.028	<0.05	0.07
2t	OEt	Cl	4-$(CH_2)_3OH$	0.23	>1	>1
2u	OEt	Cl	4-CH_2CO_2H	0.58	>1	>1
2v	OEt	Cl	4-NO_2	0.010	0.18	0.35
2w	OEt	Cl	3-NO_2	0.64	0.45	>1
2x	OEt	Cl	3-NH=NCH-4	0.024	<0.1	0.2
2y	OEt	Br	H	0.12	0.26	>1
2z	OEt	Br	Cl	0.016	0.05	0.08
2aa	OEt	Br	4-CO_2H	2.7		
2ab	OEt	Br	4-CH_2CO_2H	0.21	>1	>1
2ac	OEt	Br	4-$B(OH)_2$	0.091	0.2	0.5
2ad	OEt	Br	4-CN	0.017	0.07	0.4
2ae	OEt	Br	3-CN	0.72	0.35	>1
2af	OEt	Br	4-C(=O)NH_2	0.75		
2ag	OEt	Br	4-C(=O)NHOH	0.088	0.7	>1
2ah	OEt	Br	4-CH_2C(=O)NHOH	0.4	>1	>1
2ai	OEt	Br	4-C(=NH)NH_2	>5		
2aj	OEt	Br	4-C(C=O)Me	0.034	0.05	0.09
2ak	OEt	Br	4-C(C=NHOH)Me	0.084	<0.05	<0.05
2al	OEt	Br	4-C(C=NHOMe)Me	0.97	>1	>1

in SF9 insect cell membranes.[20] Also, compounds were tested for their ability to inhibit similarly overexpressed LPAAT-α. Only one of the listed compounds measurably inhibited LPAAT-α activity up to a concentration of 40 μM (**2z**, 50% inhibition at 40 μM). In addition, most of the listed compounds were tested for their ability to block proliferation of K-562 leukemia and DU145 prostate carcinoma cell lines in vitro.[21]

Comparing LPAAT-β inhibition by the pyrimidines listed in Table 1 **2a**–**c** were the most potent. Comparison with tumor cell proliferation data in Table 1 indicated correlation between LPAAT-β inhibition and antiproliferative activity of the listed positional isomers. An exception was **3b**, although a weak LPAAT-β inhibitor it was an effective antiproliferative agent.

Because of the exceptional LPAAT-β inhibition and antitumor properties observed with the 2,4-diamino-N^4,6-diarylpyrimidine positional isomers **2a**–**c** subsequent studies were focused on elaboration of the SAR for this series. The 2,5-disubstitution pattern in the 6-aryl ring of **2** was essential for potent inhibition of LPAAT-β. Analogs with altered substitution pattern in this ring or lacking one or both (**2d**) of these substituents were significantly weaker inhibitors (data not shown). Compounds with nonpolar groups at the 2- and 5-positions were active inhibitors, whereas, compounds with polar groups at either of these sites (**2m** and **2n**) were significantly less potent. Compounds with the aryl 5-position substituted with chlorine or bromine and the 2-position substituted with a chloro, alkyl, or alkoxyl group gave the highest LPAAT-β activity.

Analysis of the data in Table 2 indicates that LPAAT-β inhibition is dependent on location of substituents on the N^4-phenyl group. Comparing data for **2h** with **2i**, **2p** with **2q**, **2v** with **2w**, and **2ad** with **2ae** it was evident that

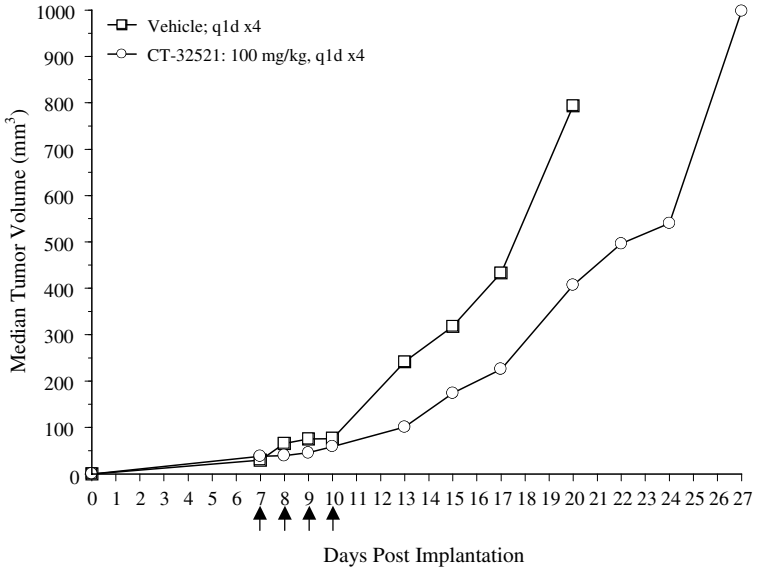


Figure 1. Effect of **2j** (CT-32521) on growth of murine LL2 lung tumors in C57BL/6 mice.

the *para*-position provides more potent inhibitors than compounds substituted at the *meta*- or *ortho*-positions. Analogs with a *para*-bromo or *para*-chloro substituent were potent LPAAT-β inhibitors and effective inhibitors of tumor cell proliferation in vitro. Of the compounds incorporating short chain primary alcohols at the *para*-position (**2r**–**t**), the 1- and 2-carbon alcohols **2r** and **2s** were particularly noteworthy as potent inhibitors of LPAAT-β and highly effective antiproliferative agents in vitro. However, when tested in an in vivo mouse tumor model **2r** and **2s** were inactive because of rapid metabolic degradation of the primary alcohol groups (data not shown). Compounds with electron withdrawing substituents at the *para*-position (**2v** and **2ad**) were also potent LPAAT-β inhibitors and antiproliferative agents. However, compound **2aa** with an electron withdrawing *para*-carboxyl group or its primary amide (**2af**) and amidine (**2ai**) derivatives were only weak inhibitors. Although the hydroxamic acid derivative (**2ag**) of carboxylic acid **2aa** was an active LPAAT-β inhibitor it displayed limited antiproliferative activity. Compound **2ab** with a *para*-carboxyl group attached to the ring through a methylene tether was an effective inhibitor. Acetophenone **2aj** and its oxime derivative **2ak** also were noteworthy for their potent LPAAT-β activity and antiproliferative effects. Although *meta*-substitution was generally not well tolerated, the 3,4-disubstituted analogs **2c** and **2x** with the 3- and 4-positions fused in a second ring were effective inhibitors and antiproliferative agents. For most of the compounds listed in Table 2 antiproliferative activity correlated quite well with LPAAT-β inhibition.

Many of the LPAAT-β inhibitors listed in Table 2 with potent antiproliferative activity in vitro were highly insoluble in aqueous media (data not shown). However, **2j** (CT-32521) was sufficiently soluble in cremophor EL–EtOH–PBS (1:1:10) as vehicle to warrant its testing in an in vivo tumor model. Female C57BL/6 mice were implanted with murine LL2 Lewis lung tumor cells.[22] Compound **2j** (in vitro LL2 $IC_{50} = 0.16\,\mu M$), injected at a dose of 100 mg/kg q1d×4, displayed median tumor growth delay (TGD) to 1000 mm^3 compared to vehicle alone of 6.0 days (Fig. 1). Because tumor growth resumed at an exponential rate a few days following withdrawal of this LPAAT-β inhibitor we surmised that **2j** may function as a cytostatic agent.

In summary, the three diamino-*C*,*N*-diarylpyrimidine positional isomers **2**, **3**, and **4** were synthesized and tested for their ability to inhibit the enzymatic activity of LPAAT-β and the proliferation of tumor cells in vitro. The 2,4-diamino-N^4,6-diarylpyrimidines positional isomer **2** displayed more potent LPAAT-β inhibition compared to positional isomers **3** and **4**. Several examples of **2** were synthesized and found to be both potent inhibitors of LPAAT-β and highly effective antiproliferative agents in vitro. When tested in an in vivo tumor model, the pattern of tumor growth delay displayed by **2j** was consistent with it functioning as a cytostatic agent. A compound like **2j** used in combination with a cytotoxic drug or radiation treatment may provide a novel approach for cancer therapy.

Acknowledgements

We thank Lisa Romero for conducting LPAAT assays and Tod Martin for conducting proliferation assays.

References and notes

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20. LPAAT-α and LPAAT-β assays were conducted using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as the colorimetric reagent according to the method described in Ref. 10.
21. Proliferation assays were conducted using the CyQuant Cell Proliferation Kit (Molecular Probes, Eugene, OR) as described in Ref. 10.
22. Female C57BL/6 mice were injected subcutaneously in the right flank with murine LL/2 Lewis lung (ATTC, CRL-1642) tumor cells (3×10^5 cells in 0.2 mL). When the tumor sizes averaged 50–100 mm^3, groups of 10 mice each were injected intravenously (20 mL/kg) with either vehicle (8.3% cremophor EL and 8.3% EtOH in PBS) or **2j** at 100 mg/kg (5 mg/mL in vehicle) on four consecutive days. Tumor volumes were determined by measuring three orthogonal tumor diameters using a caliper according to the formula: (length × width × height)/2. Mice bearing tumors $\geqslant 1000\,mm^3$ were euthanized. Antitumor efficacy was expressed as absolute tumor growth delay: the median time (in days) for the tumors in the treatment group to reach 1000 mm^3 minus the median time for the tumors in the control group to reach the same volume.