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Novel azulene-based derivatives as potent multi-receptor tyrosine kinase inhibitors

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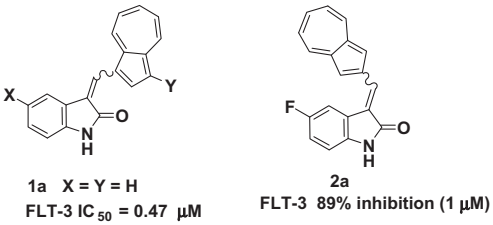
ABSTRACT

A series of azulene-based derivatives were synthesized as potent inhibitors for receptor tyrosine kinases such as FMS-like tyrosine kinase 3 (FLT-3). Systematic side chain modification of prototype **1a** was carried out through SAR studies. Analogue **22** was identified from this series and found to be one of the most potent FLT-3 inhibitors, with good pharmaceutical properties, superior efficacy, and tolerability in a tumor xenograft model.

Receptor tyrosine kinases (RTKs) are a large family of transmembrane receptors with diverse biological activity. At least 19 RTK subfamilies have been identified. One example is the platelet-derived growth factor receptor (PDGFR) subfamily. Members of this family include PDGFRα, PDGFβ, colony-stimulating factor-1 receptor (CSF-1R), FMS-like tyrosine kinase 3 (FLT-3), and c-KIT, and these are believed to promote angiogenesis and tumor cell growth. The constitutive activation of FLT-3 and c-KIT by mutation is directly associated with acute myeloid leukemia (AML) and gastrointestinal stromal tumors (GIST). FLT-3 is expressed on blast cells in most patients with AML, and internal tandem duplication (ITD) of the FLT-3 mutation has been found in up to 30% of all AML patients. The important role played by FLT-3 in the survival and proliferation of blast cells and its overexpression in most patients with AML make FLT-3 an attractive therapeutic target.[1–8] Rational design efforts in our laboratory identified an azulene-oxindole lead compound, designated **1a**, as a submicromolar inhibitor of FLT-3 (Fig. 1). Modifications of this lead structure, either by adding a side chain to an azulene moiety or by altering oxindole into azaoxindole, were investigated in this study.

The synthetic route for preparing azulene-oxindole-based derivatives is shown in Scheme 1. Treatment of commercially available tropolone with *p*-toluene sulfonyl chloride and triethyl amine yielded *p*-(tosyloxy)tropone **4**, whereas treatment of *p*-(tosyloxy)tropone with dimethyl malonate in the presence of sodium methoxide in toluene resulted in **5**.[9] Treatment of **5** with tetrabutyl ammonium hydroxide in alkaline solution produced **6**, which was then treated with dibromoalkane in acetonitrile to yield **7a** ($n = 1$) and **7b** ($n = 2$). Compound **7a** underwent [8+2] cycload-



1a X = Y = H
FLT-3 IC_{50} = 0.47 μM

2a
FLT-3 89% inhibition (1 μM)

Figure 1. Novel azulene-based compounds.

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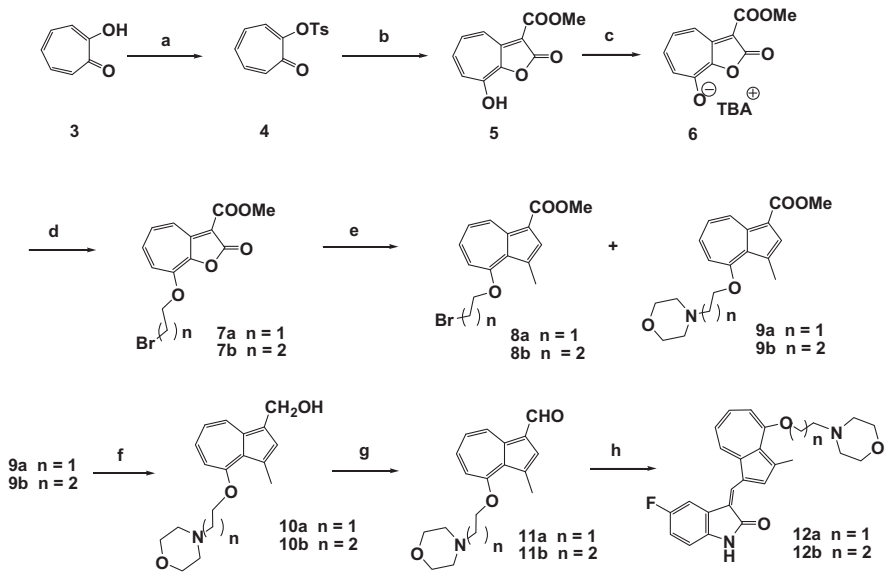


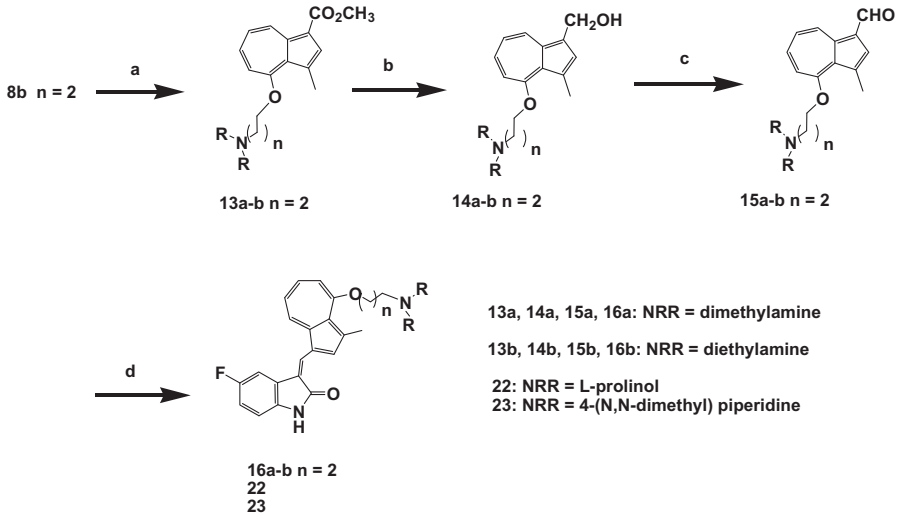
Scheme 1. Synthesis of compounds **12a** and **12b**. Reagents: (a) TsCl, Et_3N/CH_2Cl_2; (b) CH_2 $(COOMe)_2$, MeONa/MeOH, toluene; (c) $NaOH/H_2O$, tetrabutyl ammonium hydroxide; (d) 1,2-dibromoethane ($n = 1$), 1,2-dibromopropane ($n = 2$)/CH_3CN; (e) EtCHO, morpholine/toluene; (f) DIBAL/ether; (g) MnO_2/CH_2Cl_2; (h) 5-fluoro-2-oxindole/ethanol, piperidine.

dition with morpholine and propionyl aldehyde in toluene to produce the bromo compound **8a** and morpholine-substituted compound **9a**. Reduction of the methyl ester **9a** with DIBAL/heptane in ether formed the primary alcohol **10a** and subsequent oxidation with MnO_2/CH_2Cl_2 resulted in the aldehyde **11a**. The subsequent reaction of **11a** with 5-fluoroxindole yielded the desired compound **12a** in the single isomer (*Z*) form. Compound **12b** could be prepared by a procedure similar to that described for **12a**.

The bromo compound **8b** ($n = 2$) was treated with a selective amine to produce **13a–b**, and procedures b, c, and d were used to produce the desired products **16a–b** (Scheme 2). All these final products could be obtained in the single isomer form or as a mixture of the *E*/*Z* isomers. The latter could be separated by chromatography and were assigned by ^1H NMR experiments.[10]

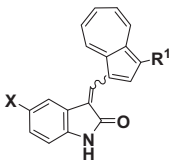
The compounds were assayed for their activity against a panel of receptor tyrosine kinases, including FLT-3, c-KIT, and KDR. Given that FLT-3 plays a pivotal role in AML, our emphasis was on optimizing the potency of the compound against FLT-3. Compound **1a** was used as the lead compound in the series, and it showed moderate inhibitory activity against the FLT-3 enzyme in the submicromolar range (0.47 μM). The results of the SAR studies on **1a** are shown in Tables 1–3. We investigated the effects of different substituents on the oxindole and azulene rings. As shown in Table 1, fluoro substitution at the 5-position of oxindole and incorporation of a fluoro or methyl group at the 3-position of the azulene core moiety was essential for potency in the series (**1h**–**1j**). Replacement of the methyl group of **1i** (*Z*) with hydrogen resulted in loss of potency (**1b** (*Z*) >1000 nM). Removal of the fluoro group from **1i** (*Z*) yielded **1g** (*Z*), which had significantly lower inhibitory activity. This trend was also observed in the case of **1h** (*E*).

To improve the inhibitory activity against FLT-3, we explored the effects of attaching an alkylaminoalkyl ether link to the seven member ring (Table 2). Various terminal alkylamino groups were investigated, including cyclic and noncyclic tertiary amine groups. In most cases (X = 5-F, Y = C), a spacer length of 2–3 methylene units was tolerated (except **12b** (*Z*)) for FLT-3 inhibitory activity.



Scheme 2. Synthesis of compounds **16a-b**, **22**, and **23**. Reagents: (a) Amine/CH_3CN; (b) DIBAL/ether; (c) MnO_2/CH_2Cl_2; (d) 5-fluoro-2-oxindole/ethanol, piperidine.

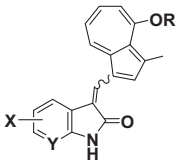
Table 1
SAR at the X/R^1 position

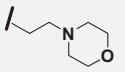
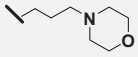
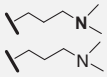
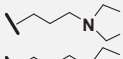
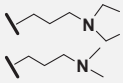
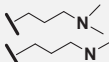
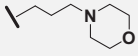
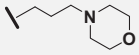
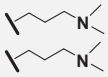
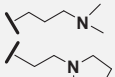
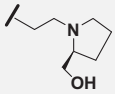
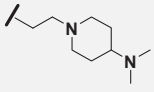


Compound	X	R^1	FLT-3 inhibition IC_{50}[a] (nM)
1a (*E*/*Z* 6:1)	H	H	470
1b (*Z*)	F	H	>1000
1c (*E*)	Br	H	>1000
1d (*Z*)	Br	H	>1000
1e (*Z*)	CH_3	H	>1000
1f (*E*)	H	CH_3	>1000
1g (*Z*)	H	CH_3	>1000
1h (*E*)	F	CH_3	60
1i (*Z*)	F	CH_3	128
1j (*Z*)	F	F	23
1k (*Z*)	$SO_2N(CH_3)_2$	H	>1000
SU11248			3

[a] See Ref. 11 for assay details.

Table 2
SAR of X, Y, and R

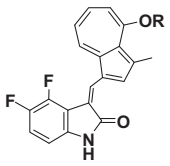


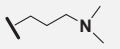
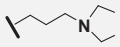
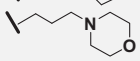
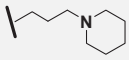
Compound	Y	X	R	FLT-3 inhibition IC_{50}[a] (nM)
12a (*Z*)	C	5-F		19
12b (*Z*)	C	5-F		224
16a (*E*)	C	5-F		25
16a (*Z*)	C	5-F		12
16b (*E*)	C	5-F		30
16b (*Z*)	C	5-F		39
17 (*Z*)	N	H		1460
18 (*Z*)	N	5-F		90
19 (*Z*)	N	5-Br		86
20 (*E*)	C	6-F		742
21 (*E*)	C	7-F		177
22 (*E*/*Z* 1.6:1)	C	5-F		98
23 (*Z*)	C	5-F		18

[a] See Ref. 11 for assay details.

The E/Z isomers **16b** (*E*) and **16b** (*Z*) showed almost equipotent activity, whereas **16a** (*E*) was twofold less potent than **16a** (*Z*). Next, we investigated the effects of C(6) and C(7) substituents on the oxindole ring. Incorporation of a fluoro group at the 6-position in **20** (*E*) led to a 29-fold loss in potency in comparison with **16a** (*E*). Similarly, incorporation of a fluoro group at the 7-position in **21** (*E*) resulted in a sevenfold loss of activity in comparison to **16a** (*E*). We also studied the effect of the 7-azaoxindole moiety on FLT-3 inhibitory activity. Compounds **18** (*Z*) and **19** (*Z*) displayed moderate activity (90 and 86 nM), whereas **17** (*Z*) showed severe loss of potency when the halo group (F, Br) was removed from the 5-position of the azaoxindole moiety. Furthermore, we utilized the 4,5-difluoro substitutions of oxindole to constrain our compound in the *Z* form only. Although these compounds showed potent inhibitory activity against FLT-3, their solubility was poor (data not shown). All of these results are summarized in Table 3.

Table 3
Variation of the R moiety in 4,5-difluoro-oxindole



Compound	R	FLT-3 inhibition IC_{50}[a] (nM)
24 (*Z*)		5
25 (*Z*)		14
26 (*Z*)		12
27 (*Z*)		17

[a] See Ref. 11 for assay details.

Table 4
In vitro cellular assay of compound **22**

In vitro assay	Cells/cellular kinase	**22**	SU11248
Cell proliferation IC_{50}[a] (nM)	MV4-11	87	9
Cellular kinase phosphorylation IC_{50}[b] (nM)	FLT-3-ITD	67	ND
	FLT-3-WT	70	16
	cKIT	44	15
	KDR	186	53
	PDGFRβ	28	17

[a] See Ref. 12
[b] See Ref. 13 and14 for assay details.

Table 5
In vivo pharmacokinetic parameters of **22** (n = 3/dosing route)

Dose route[a]	Parameter[b]	
Intravenous	CL (mL/h/kg)	709.6
	V_{ss} (mL/kg)	1232.4
	$T_{1/2}$ (h)	1.8
	AUC (h ng/mL)	1413.4
Oral	T_{max} (h)	2.0
	C_{max} (ng/mL)	566.3
	AUC (h ng/mL)	3222.1
	F (%)	22.8

[a] Dosing vehicle: 10% *N*-methyl-2-pyrrolidone, 20% cremophor EL, and 70% phosphate-buffered saline. Values represent the mean value of a study with three animals.
[b] See Ref. 15 for assay details.

Western blot analysis was used to test the target compounds for their in vitro ability to inhibit cellular proliferation[12] and inhibit receptor tyrosine kinase autophosphorylation in cellular models.[13,14] Compound **22** exhibited high cytotoxic potency against

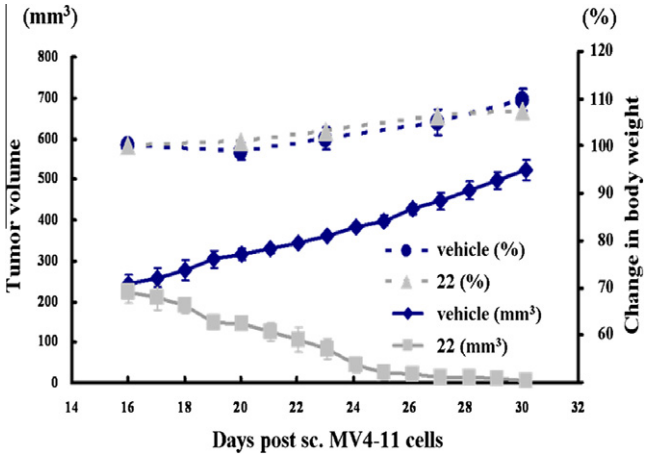


Figure 2. Compound **22** leads to tumor regression in MV4-11 xenograft nude mice. This compound was administered at 50 mg/kg (po, b.i.d.) from day 16 to day 29 after subcutaneous implantation of MV 4–11 cells. No lethality or body loss was observed in any group. The data represent the mean ± SEM ($n = 4$).

MV4-11 with IC_{50} values in the submicromolar range and also showed high inhibitory activity against KDR, cKIT, PDGFRβ, and FLT-3 (Table 4). Taken together, the in vitro and cellular assay results demonstrated that **22** is a potent multi-receptor tyrosine kinase inhibitor that inhibits kinase phosphorylation both in vitro and in cells.

Plasma pharmacokinetic studies were carried out with compound **22** in rats.[15] A single dose of the compound was administered intravenously or orally. As shown in Table 5, compound **22** exhibited moderate clearance (0.7 L/h/kg), a short elimination half life (1.8 h), and a medium volume of distribution (1.2 L/kg). The oral bioavailability was acceptable ($F = 22.8\%$). Compound **22** had a notable antitumor activity in human leukemia MV4-11 xenografts in BALB/c nude mice.[16] Vehicle or 22 was given at 50 mg/kg (twice a day; b.i.ds.) for 14 days per oral (po), and tumor growth inhibition (in %) by 22 treatment was 99 ± 0.82% on day 30 (Fig. 2). During the same time period, no progressive weight loss and lethality were observed during the experimental period. These observations indicated that compound **22** was well tolerated and was highly efficacious in this human leukemia xenograft model.

In conclusion, a novel series of FLT-3 inhibitors were designed and evaluated. Optimization led to the development of compound **22**, which showed biochemical potency against its main target as well as good potency in proliferation and cell-based mechanistic assays. Compound **22** also had an acceptable pharmacokinetic profile, with plasma exposure that was sufficient for obtaining good antitumor activity in vivo. The azulene-based derivatives shown here can potentially be developed as a new class of antileukemia agents. Of particular interest, these compounds have been found to inhibit not only FLT-3 and KDR but also TrkA and pim-1 kinases (data not shown). The benefits of compound **22** in other cancers, such as pancreatic tumors, will be evaluated in the future.

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11. *In vitro kinase assays.* FLT-3 kinase inhibition was determined by a time-resolved fluorescence method. The resulting phosphorylated peptide was measured in streptavidin-coated microtiter plates using the ULTRA Evolution Multi-Detection Microplate Reader from Tecan. Phospho-tyrosine mouse mAb (P-Try-100) and europium-labeled anti-mouse IgG were used for the assay. The IC_{50} value of the compound was calculated by nonlinear regression with Sigma Plot 10.0.
12. *Cell proliferation assays.* MV4-11 cells were plated in 96-well microtiter plates (1×10^4 cells per well), and cell viability was determined using MTT, a tetrazolium dye. The formazan crystals were dissolved in DMSO, and the absorbance at 600 nm was recorded in an ELISA plate reader. The IC_{50} values were calculated by nonlinear regression and defined as the concentration required for 50% reduction in the absorbance of treated versus untreated control cells.
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14. *Western blot analysis.* The cell lines used for measuring autophosphorylation in this study included Kasumi-1(cKIT), HUVEC(KDR), RS4-11(FLT3), and MV4-11(FLT3-ITD); these were obtained from the Bioresource Collection and Research Center (Taiwan) and American Type Culture Collection (USA) and were cultured as described in literature. Receptor stimulation was carried out with each ligand (stem cell factor (SCF), vascular endothelial growth factor (VEGF-162), and FLT-3 ligand (FL)). The probe primary anti-phosphotyrosine antibody (PY99) and reprobe antibodies for c-KIT, KDR, and FLT-3 were obtained from Santa Cruz Biotechnology and R and D Systems. The cells were plated at $2–5 \times 10^5$ cells per well in six-well plates. More details of the experimental procedures are described in Ref. 13.
15. *In vivo pharmacokinetics.* The pharmacokinetic profile of **22** was investigated in overnight-fasted male SD rats after administration of a single dose of the compound intravenous or oral. The blood was collected and processed to plasma by centrifugation and stored at −80 °C. Samples were analyzed by the LC/MS/MS technique. The pharmacokinetics were analyzed by WinNonlin 5.2 (Pharsight) via noncompartmental analysis.
16. *In vivo xenograft tumor model.* The procedures for establishing tumor xenografts and the dosing of **22** were carried out in accordance with the guidelines of the ITRI Institutional Animal Care and Use Committee (IACUC). Female BALB/c nude mice (CAnN.Cg-*Foxn1*^{NU}/Crl, 6–8 weeks old) were purchased from BioLASCO (Taiwan) and acclimated for 1 week in the Animal Laboratory for Biomedical Research, ITRI. Female BALB/c nude mice were implanted subcutaneously in the right flank with 1×10^7 MV4-11 cells per mice. Treatments were initiated when the tumor size was 200–300 mm^3. Mice were randomly assigned to cohorts (four mice per group for efficacy studies). Compound **22** (50 mg/kg, b.i.d.) and vehicle (10% NMP + 20% Cremophor EL + 70% saline) were given via oral gavage for 14 days. Caliper measurements of the tumors were converted into mean tumor volume using the following formula: $0.5 \times [\text{length} \times (\text{width})^2]$.