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Discovery of a ^{19}F MRI sensitive salinomycin derivative with high cytotoxicity towards cancer cells†

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Salinomycin is a promising anti-cancer agent which selectively targets cancer stem cells. To improve its potency and selectivity, an analog library of salinomycin was generated by site-specific modification and CuAAc derivatization. Through a cytotoxicity analysis of the library, a fluorinated analog with high potency, selectivity, and ^{19}F MRI sensitivity was discovered as a novel theranostic agent.

Salinomycin, a polyether ionophore antibiotic isolated from *Streptomyces albus*, has been commercially used in veterinary medicine for decades. In 2009, salinomycin was identified as a selective breast cancer stem cell inhibitor with activity 100-fold higher than paclitaxel.[1] Since then, its cytotoxicity against a variety of cancer stem cells and cancer cells has been discovered.[2] Although the detailed mechanism of these effects is still unclear,[3] salinomycin is already in clinical trials for a variety of cancers. Therefore, generation of highly potent, highly specific salinomycin analogs is of great interest. Furthermore, it is desired that the analogs can also be used to probe the modes of action of salinomycin.

As a complex natural product with 18 chiral centres, 5 rings, and multiple reactive groups, the site-specific modification of salinomycin is very challenging. Currently, the modification strategies are limited to esterification or amidation of its carboxylic group, acylation of its hydroxyl groups, and hydrogenation of its double bond (Fig. 1).[4] Recently, Strand's group developed a strategy for site-specific acylation of the hydroxyl groups in salinomycin and found that C20-acylated analogs with the least bulky substituents had the highest potency against cancer cells.[4f,5] The X-ray crystal structure and molecular modelling of salinomycin show that the C20 hydroxyl group is not involved in ion chelation, and its acylation actually poses steric hindrance for ion chelation.[6] We envisioned that the inversion of the C20 configuration could relieve the steric hindrance and therefore enhance ion chelation and potency. In this way, a highly valuable conjugation site is also available for targeted delivery of salinomycin without compromising its ion chelation and potency.[7] To this end, inversion of the C20 hydroxyl group with an azido group is preferred because it could provide an easy access to various salinomycin analogs and conjugates using the Cu-catalyzed azide-alkyne cycloaddition (CuAAC) reaction[8] under mild conditions (Fig. 1). Herein, we report a convenient strategy for the C20-specific modification of salinomycin with an azido group and the consequent generation of a library of analogs through the CuAAC reaction, as well as a detailed cytotoxicity assay of the library.

After esterification of salinomycin **1** with TMS$(CH_2)_2$OH using a reversible strategy for carboxylic group masking (Scheme 1),[4f] the selective inversion of the C20 configuration with an azido nucleophile was investigated. Because the allylic C20 hydroxyl

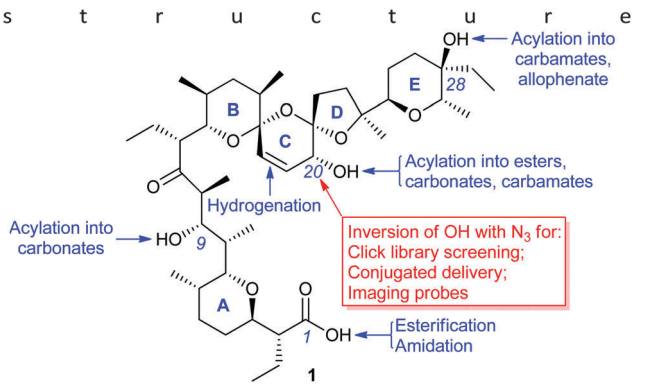

Fig. 1 Methods for modification of salinomycin **1**.

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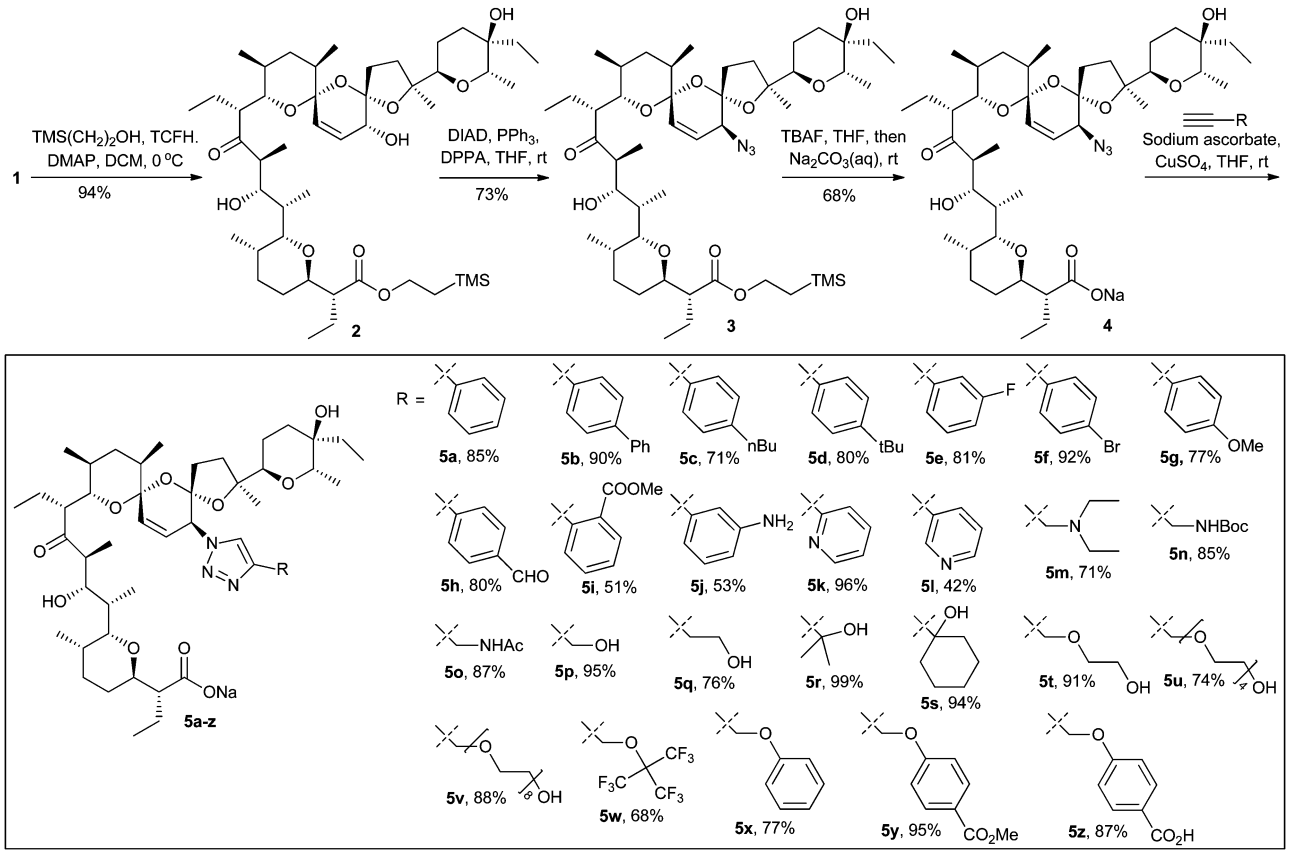
† Electronic supplementary information (ESI) available: Synthesis of library compounds, copies of ^{1}H NMR, ^{13}C NMR, ^{19}F NMR, IR and HRMS of compounds, and single-crystal X-ray diffractograms. CCDC 1448688 (**5f**-Na$^+$) and 1449564 (**5f**-K$^+$). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c6cc01508e



Scheme 1 Synthesis of salinomycin-based click library.

group is more reactive than C9 and C28 ones,[4f,6] selective tosylation of **2** provided C20 tosylate **S1** in high yield (see the ESI†). However, many attempts at nucleophilic substitution of the tosylate group with an azido group resulted in the decomposition of **S1**. Fortunately, an azido group was successfully introduced into **2** at C20 through the Mitsunobu reaction with diphenylphosphoryl azide (DPPA) as a nucleophile. Azide **3** was obtained with a 73% yield and no C9- or C28-substituted side product was isolated. Unmasking the carboxylic group with tetrabutylammonium fluoride (TBAF) provided the key intermediate **4** on a multi-gram scale with a 68% yield. To generate a diverse library of salinomycin analogs using the CuAAC reaction, a panel of 26 alkynes were selected, including 9 phenyl-containing, 6 nitrogen-containing, 7 hydroxyl-containing, and 4 ether-containing alkynes. As expected, each alkyne was conveniently coupled with azide **4**, respectively, in the presence of $CuSO_4$ and sodium ascorbate under mild conditions to give triazoles **5a–z** with good yields.

To illustrate the structures of triazoles **5a–z**, single-crystal X-ray structures of **5f**-Na^+ and **5f**-K^+ were obtained (Fig. 2). It was found that **5f** can adopt a suitable conformation to chelate ions, Na^+ or K^+, with its oxygen atoms. As expected, the Mitsunobu reaction took place exclusively at C20 with the inversion of the configuration, and, consequently, the CuAAC reaction constructed a triazole sidearm which stretched out of the ion chelation pocket. In this way, both the steric effect and chelation perturbation of ion chelation were successfully avoided.

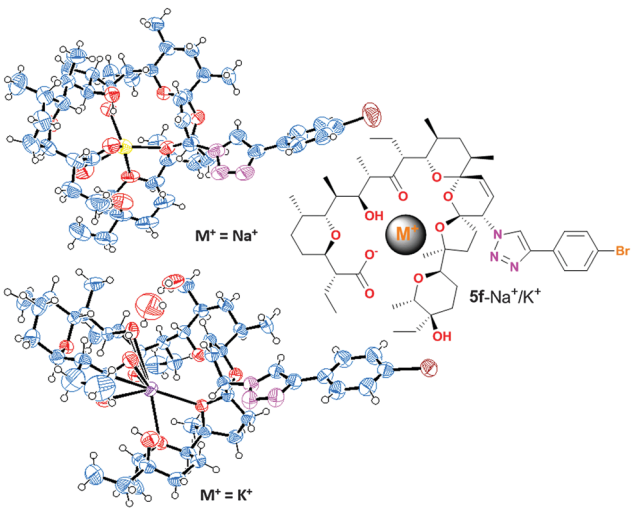


Fig. 2 Single-crystal X-ray structures of **5f**-Na^+ and **5f**-K^+.

The cytotoxicity of these salinomycin analogs **5a–z** together with salinomycin **1** and azide **4** was then evaluated in 4T1 murine breast cancer cells using a MTT assay (Table 1). Surprisingly, azide **4** showed a 27-fold loss of potency as compared to salinomycin. Out of the library, all 9 phenyl-containing triazoles **5a–i** displayed higher or comparable potency to salinomycin. Their EC_{50} values were very favourable to bulky substituents without a heteroatom,

Table 1 EC_{50} of salinomycin **1**, azide **4** and triazole **5a–z** on murine 4T1 cells

Compd no.	EC_{50} (μM)	Compd no.	EC_{50} (μM)	Compd no.	EC_{50} (μM)
Core compounds		N-containing		Ether-containing	
1	3.42	**5j**	13.37	**5w**	1.52
4	95.20	**5k**	2.91	**5x**	33.42
Ph-containing		**5l**	7.02	**5y**	3.95
5a	4.00	**5m**	23.14	**5z**	≫1000
5b	2.46	**5n**	9.97		
5c	2.31	**5o**	≫1000		
5d	2.29	OH-containing			
5e	3.33	**5p**	≫1000		
5f	3.69	**5q**	20.44		
5g	3.07	**5r**	≫1000		
5h	5.08	**5s**	8.80		
5i	3.83	**5t**	≫1000		
		5u	≫1000		
		5v	≫1000		

which display EC_{50} of 2.29–2.46 μM for **5b–d**. With the exception of 3-pyridine substituted **5k**, the EC_{50} of 5 nitrogen-containing triazoles **5j–o** were much higher than that of salinomycin. In these cases, the strong chelation ability of nitrogen may pose a perturbation on ion chelation.[4f] The same trend was observed in the 7 hydroxyl-containing triazoles **5p–v**. It is noteworthy that no significant cytotoxicity was observed for triazoles **5t–v** with ethylene glycol units which are known to chelate ions. Among the 4 ether-containing triazoles **5w–z**, perfluoro-*tert*-butyl ether **5w** turned out to be the most potent one with an EC_{50} of 1.52 μM, which is over 2-fold more potent than salinomycin. Notably, perfluoro-*tert*-butyl is a bulky group ideal for ^{19}F magnetic resonance imaging (^{19}F MRI).[9] Based on these observations, it is clear that bulky substituents on the C20 triazole is preferred for achieving high potency. In contrast, C20 hydroxyl acyl analogs with the least bulky substituent exhibited the highest potency.[4f] Therefore, inversion of the C20 configuration indeed improves the potency.

Based on the initial assay, triazoles **5b**, **5d**, **5k**, and **5w** and salinomycin were selected for further assay on human cells (Table 2). Besides human hepatic cells (L02), a panel of cancer cells were selected, including human glioblastoma cells (U87), cervical cancer cells (Hela), epithelial colorectal adenocarcinoma cells (Caco2), and breast cancer cells (MCF-7). Compared to salinomycin, these triazoles displayed dramatically lower cytotoxicity against normal L02 cells and significantly higher cytotoxicity towards cancer cells. For example, **5d**, **5k** and **5w** displayed over 2-fold higher cytotoxicity towards U87, and **5b** exhibited 2.9-fold higher cytotoxicity towards MCF-7 than that of salinomycin. It is noteworthy that, compared to salinomycin, **5w** showed 29.5-fold higher cytotoxicity towards Caco2 with an IC_{50} of 0.44 μM.

To evaluate the clinical potential of triazoles **5b**, **5d**, **5k**, and **5w**, the selectivity index (SI) was calculated with salinomycin as a comparison (Table 3). It is an indication of a drug with efficacy against cancer cells when SI > 1.0. For selected cancer cells, salinomycin showed a low SI except for in Hela cells. In contrast, these triazoles had a significantly higher SI than salinomycin. For example, **5b** displayed the highest SI of 8.85 for Hela and **5d** displayed the highest SI of 5.89 for Caco2. Among these triazoles, **5w** is very promising because it had high potency and SI for Hela and Caco2 cells.

The SI was calculated using the formula: SI = IC_{50}(L02)/IC_{50} (cancer cell).

With 9 symmetrical fluorine atoms, fluorinated triazole **5w** is also a valuable ^{19}F NMR/MRI probe for better understanding the mechanism of selective cytotoxicity of salinomycin on CSCs. In recent years, ^{19}F MRI has been widely used in tracking targets of interest[10] and monitoring biological reactions.[11] Compared to other imaging technologies, ^{19}F MRI is able to provide high-contrast *in vivo* images without an endogenous background and ionizing radiation. Triazole **5w** gives a strong singlet ^{19}F NMR peak at −70.50 ppm from its nine symmetrical fluorines. Regardless of the size and the chelation pattern of the ions, little chemical shift change was observed when **5w** was chelated with a panel of ions (Fig. 3a), which can dramatically simplify the downstream ^{19}F MRI study. ^{19}F MRI relaxation experiments indicated that **5w** has appropriate relaxation times for ^{19}F MRI with a longitudinal relaxation time T_1 of 843 ms and a transverse relaxation time T_2 of 445 ms. Then, a ^{19}F MRI phantom experiment of **5w** was carried out on an array of its solutions in DMSO. With a short scan time of 128 seconds, **5w** was imaged by ^{19}F MRI at a concentration as low as 1.1 mM (or 10 mM in ^{19}F concentration, Fig. 3b). Because of its high cytotoxicity, selectivity, and ^{19}F MRI sensitivity, **5w** is a promising ^{19}F MRI traceable mechanism probe for CSCs and a drug candidate for imaging-guided cancer therapy.

In summary, a fluorinated salinomycin analog with high potency and selectivity against cancer cells and high ^{19}F MRI sensitivity was discovered. The Mitsunobu reaction enables the site-specific modification of salinomycin with high efficacy, while the CuAAC reaction provides a convenient way to generate a salinomycin analog library under mild conditions. Importantly, inversion of the C20 configuration of salinomycin can relieve steric hindrance, enhance ion chelation, improve potency, and provide

Table 2 IC_{50} of salinomycin **1**, analogs **5b**, **5d**, **5k** and **5w** on a panel of cells

	IC_{50} (μM)				
Compd no.	L02	U87	Hela	Caco2	MCF-7
1	0.69	38.70	0.32	12.99	7.95
5b	2.96	50.02	0.33	1.29	2.75
5d	10.72	18.89	2.01	1.82	27.44
5k	3.52	15.74	0.61	3.31	8.87
5w	1.12	16.64	0.29	0.44	6.48

Table 3 Selectivity index (SI) of salinomycin **1**, triazoles **5b**, **5d**, **5k** and **5w** on a panel of cancer cells over normal cell L02

	Selectivity index SI			
Compd no.	U87	Hela	Caco2	MCF-7
1	0.02	2.16	0.05	0.09
5b	0.06	8.85	2.29	1.07
5d	0.57	5.33	5.89	0.39
5k	0.22	5.76	1.07	0.40
5w	0.07	3.82	2.57	0.17

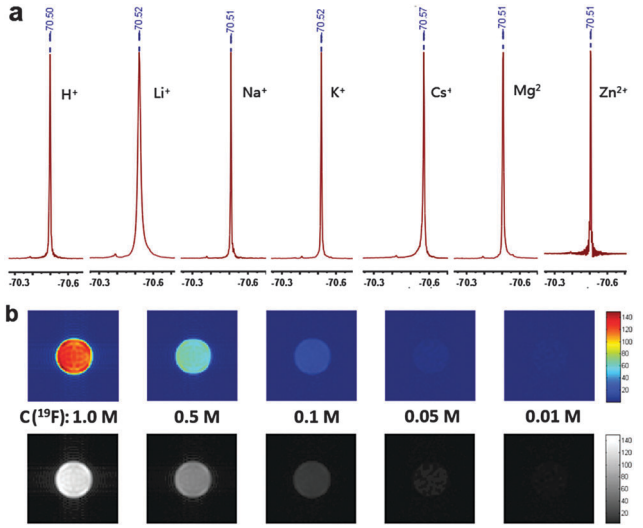


Fig. 3 (a) ^{19}F NMR of **5w** and its ion complexes. (b)^{19}F MRI phantom images of **5w** (upper: coloured; lower: black-white).

a valuable conjugation site for targeted delivery. The results presented here could serve as a starting point for the discovery of clinically useful salinomycin-based anti-cancer agents, ^{19}F NMR/MRI-guided mechanism study of effects of salinomycin on CSCs, and ^{19}F MRI-guided cancer therapy. Using the fluorinated salinomycin analog as a mechanism probe to study its modes of action on CSCs is currently in progress and will be published in due course.

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