CHEMISTRY
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Accepted Article

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Authors: Björn Borgström, Xiaoli Huang, Cecilia Hegardt, Stina Oredsson, and Daniel Strand

To be cited as: *Chem. Eur. J.* 10.1002/chem.201603621

Link to VoR: http://dx.doi.org/10.1002/chem.201603621

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10.1002/chem.201603621

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Structure-activity relationships in salinomycin – cytotoxicity and phenotype selectivity of semi-synthetic derivatives.

Björn Borgström,[a] Xiaoli Huang,[b] Cecilia Hegardt,[c] Stina Oredsson,[b] and Daniel Strand*[a]

Abstract: The ionophore salinomycin has attracted attention for its exceptional ability to selectively reduce the proportion of cells with stem-like properties in cancer cell populations of varying origin. Targeting the tumorigenicity of such cells is of interest as they are implicated in recurrence, metastasis, and drug resistance. Structural derivatives of salinomycin are thus sought after, both as tools for probing the molecular mechanism(s) underlying the observed phenotype effects, and for improving selectivity and activity against cancer stem cells. Here, synthetic strategies for modification of each of the directly accessible functional groups of salinomycin are presented and the resulting library of analogs was investigated to establish structure-activity relationships, both with respect to cytotoxicity and phenotype selectivity in breast cancer cells. 20-*O*-Acylated derivatives stand out by exhibiting both improved selectivity and activity. Mechanistically, the importance of the ionophore properties of salinomycin is highlighted by a significant loss of activity by modifications directly interfering with either of the two primary ion coordinating motifs in salinomycin, the C11 ketone and the C1 carboxylate.

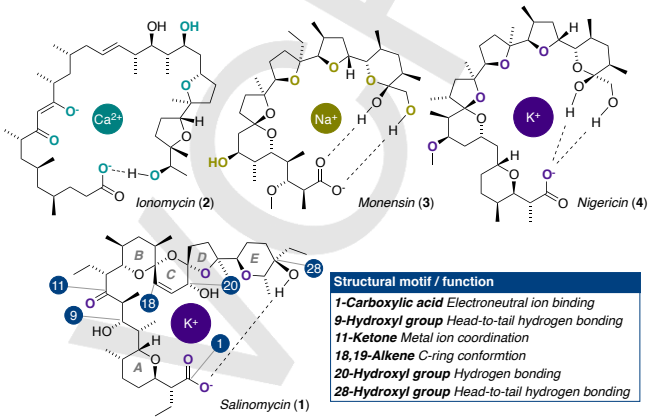


Figure 1. Structure, ion binding preference, and mode of complex formation of diverse polyether antibiotics, the oxygen atoms providing coordination sites for ions (bold) and head-to-tail bonding (dashed lines) are indicated.

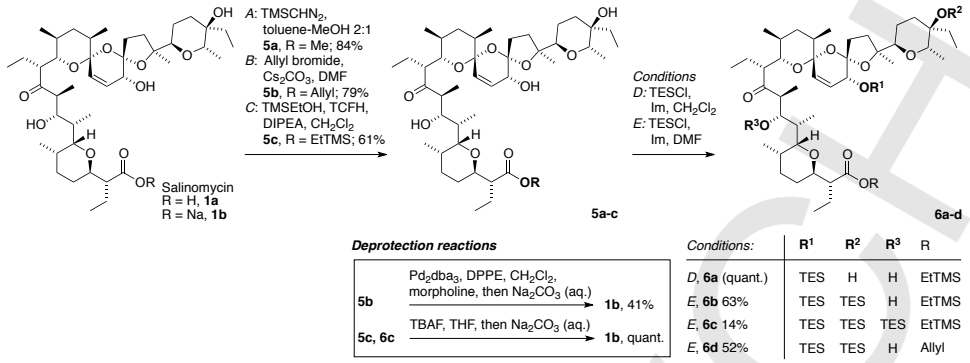
Introduction

Phenotypic heterogeneity within tumors has emerged as a key factor behind many clinical challenges in cancer treatment.[1] Subpopulations of cancer cells sharing traits otherwise associated with stem cells including self-renewal and an ability to spawn differentiated progeny, often referred to as cancer stem cells (CSCs) or tumor initiating cells,[2] are particularly important in this context. Such cells have been implicated in recurrence and metastasis of cancer and are additionally problematic as they exhibit a lower sensitivity to cytotoxic agents compared to regular cancer cells.[3] To moderate the malignant properties of CSCs, small molecules capable of reducing the tumorigenic properties of such cells by inducing phenotypic shifts are much sought after. In 2009, the ionophore salinomycin (Figure 1) was shown by Gupta et al. to exhibit a 100-fold improved selectivity against CSCs compared to clinically used paclitaxel.[4]

In a preceding communication, we described how selective *O*-acylation of the hydroxyl groups of salinomycin could be used to significantly increase its activity against cancer cells.[5] Importantly, this increase was recently shown to also translate to enhanced activity against CSC traits as demonstrated by both marker-based and functional assays;[6] selected 20-*O*-acylated analogs showed significant CSC activity already at 50 nM concentrations where salinomycin itself was inactive. Of mechanistic significance, we also recently showed that conversion of the carboxylate group to hydroxamic acid derivatives that strongly coordinates alkali metal ions but exhibit a decreased ionophore activity also lack activity against CSCs.[7] In addition, a number of studies have been directed at biological and synthetic investigations of salinomycin.[8] Modifications to the carboxylate group of salinomycin have been systematically studied by the Huczyński group to include esters,[9a] amides,[9b] and conjugates with bioactive compounds.[9c] Wu and co-workers have investigated epimerization of the C17 and C21 positions, as well as 20-epi-esters,[10] and Jiang described similar epimeric 20-tetrazole derivatives of salinomycin.[11] In a CSC context however only C1 and C20 derivatives have been extensively explored,[6, 12] and a systematic exploration of structure–activity relationships focusing on both basal toxicity and phenotype selectivity have not been described.

[a] B. Borgström, Prof. D. Strand
Centre for Analysis and Synthesis, Lund University
Box 124, 22100 Lund (Sweden)
E-mail: daniel.strand@chem.lu.se
[b] Dr. X. Huang, Prof. S. Oredsson
Department of Biology, Lund University
Sölvegatan 35/37, 223 62 Lund (Sweden)
[c] Dr. C. Hegardt
Division of Oncology and Pathology, Department of Clinical Sciences, Lund University
Medicon Village, 223 81, Lund (Sweden)

Supporting information for this article is given via a link at the end of the document. It contains experimental procedures and full characterisation data, as well as details of the X-ray analysis of compounds **11** and **12**. CCDC 1496546 (**11**), 1496545 (**12**) contain the supplementary crystallographic data for this paper. These data are provided free of charge by The Cambride Crystallographic Data Centre.



Conditions:	R1	R2	R3	R
D, 6a (quant.)	TES	H	H	EtTMS
E, 6b 63%	TES	TES	H	EtTMS
E, 6c 14%	TES	TES	TES	EtTMS
E, 6d 52%	TES	TES	H	Allyl

Scheme 1. Methods for selective protection and de-protection of salinomycin. Im = imidazole.

Here, we give a full account of our efforts to establish structure–activity relationships of salinomycin with respect to both basal toxicity and phenotype selectivity by modification of each of the directly accessible functional groups highlighted in Figure 1. Synthetic highlights include conditions for stereodivergent diastereoselective reduction of the C11 ketone and selective manipulations of the substituents on the C-ring, including an improved semi-synthesis of the natural product SY-9 (20-oxo-salinomycin). The biological investigations show that 20-*O*-acyl derivatives are consistently more active against CSCs than salinomycin at low nM concentrations. Modifications of positions involved in ion coordination result in significantly decreased antiproliferative activity and a loss of CSC selectivity. Mechanistic implications are discussed.

Results and Discussion

Design aspects. At the outset, we aimed to explore selective and individual modification of the C1 carboxylate, the C9, C20, and C28 hydroxyl groups, the C11 ketone, and the C18 olefin. Each of these were selected as they are directly accessible for synthetic modification and each serve a role in either maintaining the three dimensional structure or is involved in metal ion coordination.[13] The targeted library constitutes a diverse set of analogs suitable for connecting antiproliferative and CSC activity to changes in both structure and ion-coordination.

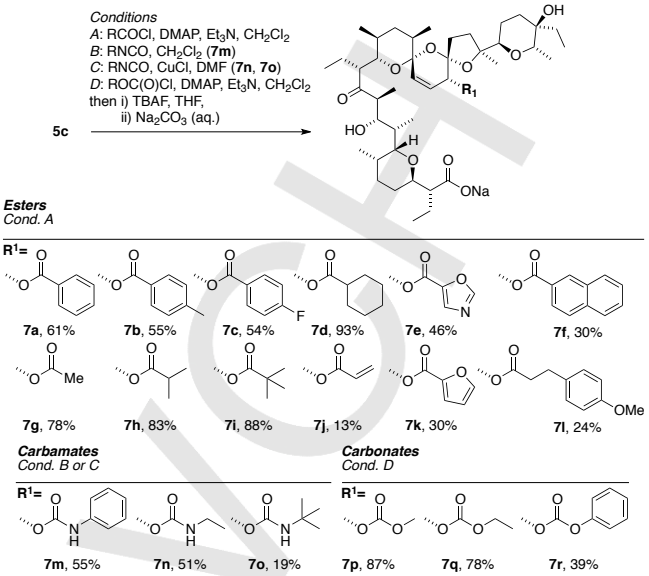
Early work on antibacterial and protozoal properties by Miyazaki showed that salinomycin C20 acetate had an enhanced antibacterial activity suggesting that modifications to the C20 hydroxyl and the C-ring would be beneficial also in the context of cytotoxicity.[14] The C9 and C28 hydroxyls are important as they form a hydrogen-bonding network to the carboxylate moiety that stabilizes the head-to-tail conformation that encapsulates metal cations during membrane crossing. The hydrogen bond between the carboxylate and the C28 hydroxyl is of particular interest as disruption of this interaction is required for catch and release of metal ions. The C11 ketone is the only functional group, other than the cyclic-ethers and the carboxylate that is directly involved in coordination of metal ions. Finally, modification of the C-ring olefin with or without re-hybridization of the C20 carbon in combination with changes to the oxidation state of the C20 carbon, presents opportunities to introduce small but well-defined changes to the C-ring conformation.

Selective protection and deprotection of salinomycin. To enable selective modification of each of the hydroxyl groups of salinomycin, methods for selective protection were first investigated. Esterification of the carboxylate was facile using TMS diazomethane to give the known 1-methyl ester **5a** in good yields (Scheme 1). Unfortunately, this ester proved highly resistant to hydrolysis despite extensive experimentation, which led us to instead investigate protecting groups that could be removed under mild and orthogonal conditions. The allyl ester **5b** was thus readily prepared using allyl bromide. Formation of EtTMS ester **5c** required more careful experimentation to arrive at the uronium type TCFH coupling reagent.[5] The procedures however enabled preparation of both esters **5b** and **5c** on multi-gram scale and the esters could be cleaved using Pd(0) catalysis or TBAF respectively without compromising the integrity of structure. The TMSEt group was particularly convenient as it could typically be removed in essentially quantitative yield without need for subsequent chromatographic purification. Selective protection of the 20-hydroxyl group was then readily achieved with TESCl and imidazole in CH_2Cl_2 to quantitatively give mono-TES ether **6a**. A switch of solvent to DMF increased the reactivity and gave the bis-TES ether **6b** as the major product along with minor amounts of the tris-TES ether **6c**. The persilylated **6c** could be globally deprotected in good yield with TBAF in THF to give salinomycin sodium salt following a wash with Na_2CO_3. This product was indistinguishable from commercial salinomycin, which corroborates the retained structural integrity throughout the protective group manipulations.

Selective acylation of the C20 hydroxyl group. Early synthetic work by Miyazaki revealed that the 20-hydroxyl group could be selectively esterified directly by treatment with aliphatic and non-

bulky anhydrides in pyridine.[14b] In our hands, these protocols were not general and obtaining material of sufficient homogeneity for biological testing was difficult. On the other hand, acid chlorides in the presence of amine bases cleanly afforded the desired esters upon reaction with the 20-hydroxyl group of EtTMS ester **5c**.[15] Similarly, the homologous 20-*O*-carbonates could be obtained by reaction of ester **5c** with chloroformates, and 20-*O*-carbamates by reaction with isocyanates. Addition of catalytic amounts of Cu(I) enabled also reactions with the less reactive ethyl and *tert*-butyl isocyanates. Deprotection with TBAF followed by a wash with aqueous sodium carbonate gave the corresponding sodium complexes of each of the 20-*O*-acylated analog **7a-r**, typically in good yields over three steps (Scheme 2). Acylation of the C20 position increases the overall lipophilicity of the structure and removes the possibility of forming a hydrogen bond from the C20 hydroxyl group to the carboxylate.

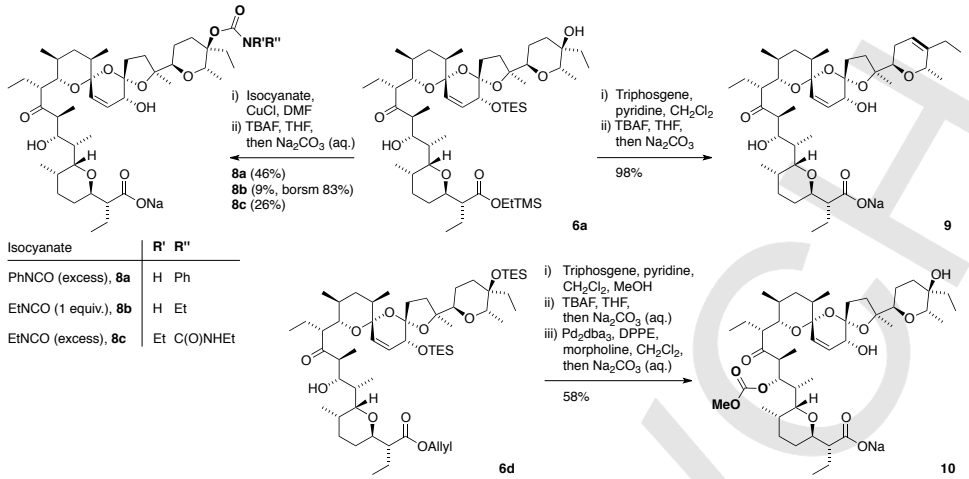
***O*-Acylation beyond the C20 position.** We then turned to selective acylation of the remaining C9 and C28 hydroxyl groups (Scheme 3). The mono-TES or bis-TES protected silyl ethers **6a**, or **6b** failed to react with acyl chlorides. Instead, an excess of the more reactive phenyl isocyanate in presence of catalytic amounts of CuCl was used with a clean and selective acylation of the C28 hydroxyl group **6a** as a result to provide phenyl carbamate **8a**. It is noteworthy that the tertiary C28 hydroxyl is the more reactive of the two hydroxyl groups of **6a**. Employing the same conditions with the smaller ethyl isocyanate resulted primarily in the formation of allophenate **8c** but even so, the ethyl carbamate **8b** could be isolated at low conversion by limiting the amount of isocyanate used. We were unable to isolate any carbamoylated product when exposing the bis-silylated **6b** to isocyanates. The challenge of acylating the C9 alcohol is two-fold; this hydroxyl group is sterically shielded by the C8 and C11 methyl groups and activation of this hydroxyl results in a structure that is highly prone to elimination to the corresponding α,β-unsaturated ketone. To access an acyl derivative at this position, we instead turned to stepwise construction of a carbonate via an intermediate 9-*O*-chloroformate. Interestingly, treatment of TES ether **6a** with phosgene resulted in a clean elimination of the C28 hydroxyl to give olefin **9** as a single observed regioisomer. A favourable anti-periplanar orientation of the axial proton at C27 and the C28 hydroxyl group accounts for the high regio-selectivity of the



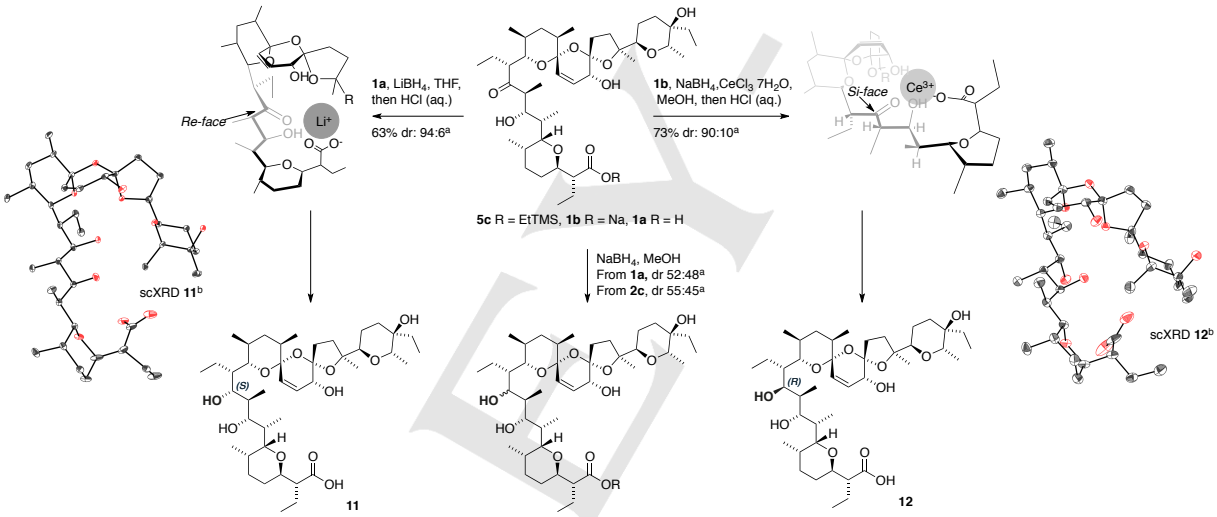
Scheme 2. Selective 20-*O*-acylation of salinomycin.

elimination. While not initially targeted, the elimination product **9** is an interesting analog in its own right, well suited to probe the importance of the C28 hydroxyl group for ionophore activity. A hydrogen bond from this hydroxyl group to the carboxylate has been proposed to stabilize the encapsulation of metal ions during membrane passage and in order to release a bound metal ion, this interaction must be disrupted to allow the necessary rotation around the C24-C25 to open up the structure.

With a protected C28 hydroxyl group, a reaction of the C9 hydroxyl group with phosgene was cleanly achieved and following addition of methanol to the reaction mixture, the desired C9 methyl carbonate could be isolated. As this carbonate is prone to elimination, fluoride mediated deprotection of the EtTMS group could not be accomplished without elimination of the carbonate despite extensive experimentation. Instead, allyl ester **6d** was synthesized following the same strategy and the milder Pd-catalyzed deprotection provided the desired C9 carbonate analog **10** in good yield to complete the targeted library of *O*-acylated analogs.



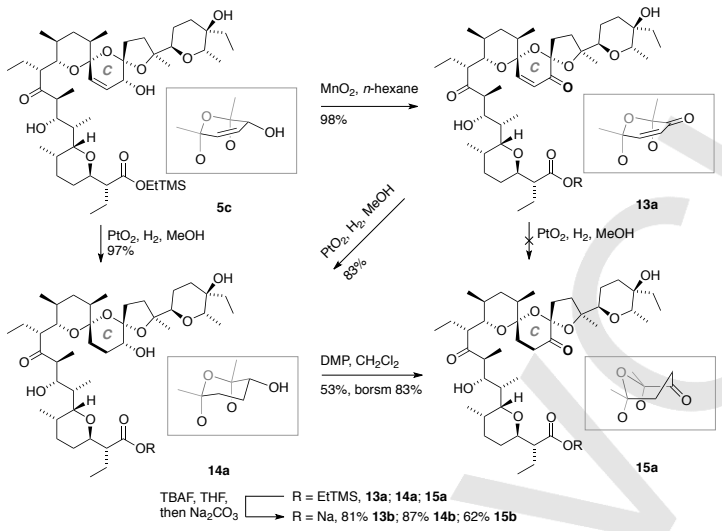
Scheme 3. Selective synthetic modification of the C9 and C28 positions. Borsm = Based on recovered starting material.



Scheme 4. Diastereoselective reductions of the C11 ketone of salinomycin and rationalization of selectivity. [a] The ratio of diastereoisomers was measured by integration of ^{1}H NMR signals in the crude reaction mixture. [b] Thermal ellipsoids shown at 30% probability. Hydrogen atoms and solvate water molecules are omitted for clarity.

Stereodivergent diasteroselective reduction of the C11 carbonyl group. Besides the carboxylate group, the only accessible functionality of salinomycin that is directly involved in ion-coordination is the C11 ketone. Reduction of salinomycin using $NaBH_4$ was early shown to give close to a 50:50 mixture of diastereomers **11** and **12**,[14b] though the relative stereochemistry was not established (Scheme 4). To facilitate preparative access to these compounds in stereochemically pure form, we investigated the possibility of increasing the distereoselectivity of the reduction. We found that under Luche conditions ($CeCl_3{\cdot}7H_2O$, $NaBH_4$), a highly *syn*-selective reduction (with respect to the C10 methyl group) of the sodium complex **1b** occurred whereas exposing the acid **1a** to $LiBH_4$ gave the complimentary *anti*-selectivity (Scheme 4). The minor diastereomer was in each case separable by chromatography and the relative stereochemistry of **11** and **12** was unambiguously established by scXRD after crystallization of each diastereomer from water/MeOH (for **11**) and water/MeCN (for **12**) mixtures.

The reason for observed high selectivity in each case is not obvious in particular as the sodium salt of salinomycin exposes only the *Re*-face to nucleophiles.[16] To gain insight into the factors governing the stereochemical outcome, we performed a small set of complimentary experiments. Exposure of EtTMS ester **5c** to $NaBH_4$ gave a 50:50 mixture of diastereomers whereas $LiBH_4$ under otherwise identical conditions gave a selectivity of ~90:10 in favor of the anti-diastereomer. The C11 ketone of ester **5c** was not reduced under Luche conditions,[17]



Scheme 5. Synthetic manipulation of the C-ring conformation of salinomycin. Conformational changes imposed by C-ring modifications shown as truncated structures. Borsm = Based on recovered starting material.

which suggest that a Ce^{3+} ion is coordinated by the carboxylate during reduction of **1a**.

To account for the selectivity in the $LiBH_4$ reduction, salinomycin lithium salt was modeled using molecular mechanics. The lowest energy conformation found was highly similar to that of the corresponding sodium salt **1b** with the *Re*-face of the C11 ketone exposed. The higher selectivity using $LiBH_4$ compared to $NaBH_4$ is thus likely a reflection of the smaller size of the Li^+ ion which permits rehybridization of the carbonyl group during the reduction. (Scheme 4). The apparently hindered approach to the *Re*-face of the ketone under Luche conditions can be similarly explained by the size of the Ce^{3+} ion. In an analogous conformation as with Li^+, the larger Ce^{3+} ion hinders movement of the carbonyl oxygen in the direction of the metal during reduction. A low-energy conformation of a Ce^{3+} salt of salinomycin that exposes the *Si*-face of the ketone could not be located with an unrestricted model (molecular mechanics, molecular dynamics, DFT, or semi-empirical methods). To account the observed selectivity, we instead suggest that the *Si*-face becomes exposed by rotation around the C11-C12 and C12-C13 bonds that constitutes one of the hinge regions involved in the catch and release mechanism of metal ions.[13] This results in a relocation of the B-C-D ring system away from the ketone and exposes the *Si*-face. In this conformation, the *Re*-side is also shielded by substituents on both sides of the carbonyl group. Access to a similar conformation likely also contributes to the low selectivity observed with $NaBH_4$.

Conformational changes to the C-ring. Synthetic manipulation of the olefin and the C20 position of salinomycin enables introduction of well-defined conformational changes to the C-ring (Scheme 5). Structures **13b** and **14b** have previously been synthesized directly from salinomycin,[14b] but performing these manipulations on protected EtTMS ester **5c** proved superior in our hands, both in terms of reproducibility, yields, and ease of isolation of the final analogs.

Selective oxidation of the C20 hydroxyl group was thus accomplished using MnO_2 to give ketone **13a**. The use of hexane as the reaction medium proved the key to achieve an efficient oxidation. Deprotection of this structure then gave the α,β-unsaturated ketone **13b** which is also a natural product, SY-9.[18] Hydrogenation to the saturated structure **14a** using Adams catalyst followed by deprotection then gave **14b** in good yield. Expanding on these modifications, we were also able to selectively oxidize the C20 hydroxyl group of the C18,C19-dihydro analog **14a** with Dess-Martin periodinane to access dihydroketone **15a**. This analog could not be obtained by hydrogenation of **13a** with Adams catalyst as hydrogenation was followed by a reduction of the C20 ketone under these conditions.[19] We were not able to obtain crystal structures of the analogs **14b** or **15b**, but modeling the sodium salt of each structure with molecular mechanics[20] showed a retained global structure throughout the series, but a spectrum of C-ring conformations from the comparatively flat **13b**[21] to the boat-like conformation of **15a**. The proposed boat-like conformation of **15a** has experimental precedence in related tetrahydropyranone systems.[22]

Biological investigation and identification of SARs. With access to the full library of 28 analogs varying at each of the targeted positions, we turned to investigating differences in biological activity both with respect to antiproliferative activity and CSC selectivity. All analogs were first evaluated by an MTT assay in two breast cancer cell lines, JIMT-1 and MCF-7. The

20-*O*-acylated analogs were consistently more active than the native structure with IC_{50} values down to below 1/5th that of salinomycin. Enhanced biological activity of benzoate **7a** compared to salinomycin was investigated recently also in HT-29, HGC-27, and MDA-MB-231 cell lines by Wu.[10] From a SAR perspective it is interesting that acylation of the 9- and the 28-hydroxyl groups are well tolerated with only small changes to antiproliferative activity. In line with this, olefin **9**, which is deprived of the C28 hydroxyl group, showed a similar activity to salinomycin. The activity of this compound further shows that hydrogen bonding from the C28 hydroxyl to the C1 carboxylate is not vital for activity. The manipulations of the C-ring gave slightly reduced activity across the series with more pronounced conformational changes resulting in less active analogs. As expected, interference with functional groups that are directly involved in ion coordination, the carboxylate and the C11 ketone, resulted in a significant loss of the activity. It is however noteworthy that although more than one order of magnitude less active than salinomycin, these compounds retain IC_{50} values in the double-digit µM range. Combined, the MTT-data for the full library can be condensed into a map of activity domains for basal toxicity shown in Figure 2.

To further clarify the relationship between the basal toxicity and phenotype selectivity, the ability of selected analogs to reduce the proportion of $CD44^+/CD24^-$ cells in the JIMT-1 cell line was investigated. This phenotype displays increased invasive potential and was early identified by Al-Hajj et al. as a marker for putative CSCs in breast cancer.[23] The proportion of $CD44^+/CD24^-$ cells in the JIMT-1 cell line constitute 50-70% of all cells, but less than 1% in the MCF-7 cell line.[6] In JIMT-1 this phenotype can be reduced by ~60% by treatment with salinomycin at 0.5 µM concentration. The more active 20-*O*-acyl derivatives, carbonate **7q**, carbamate **7n**, and acetate **7g**, have also been shown to give a ~75% reduction when used at a 50 nM concentration, which corresponds to ~IC_{25} for these compounds. For comparative purposes, all analogs were screened at a 50 nM concentration.[24] Salinomycin itself does not show a significant reduction in the proportion of $CD44^+/CD24^-$ cells at this concentration, however the series of 20-*O*-acylated derivatives consistently reduced this phenotype by ~75% compared to control, irrespective of the differences in basal toxicity (Figure 2B). Analogs with a similar or lower activity compared to salinomycin in the MTT assay did not show selective activity against $CD44^+/CD24^-$ cells when used at 50 nM concentrations.

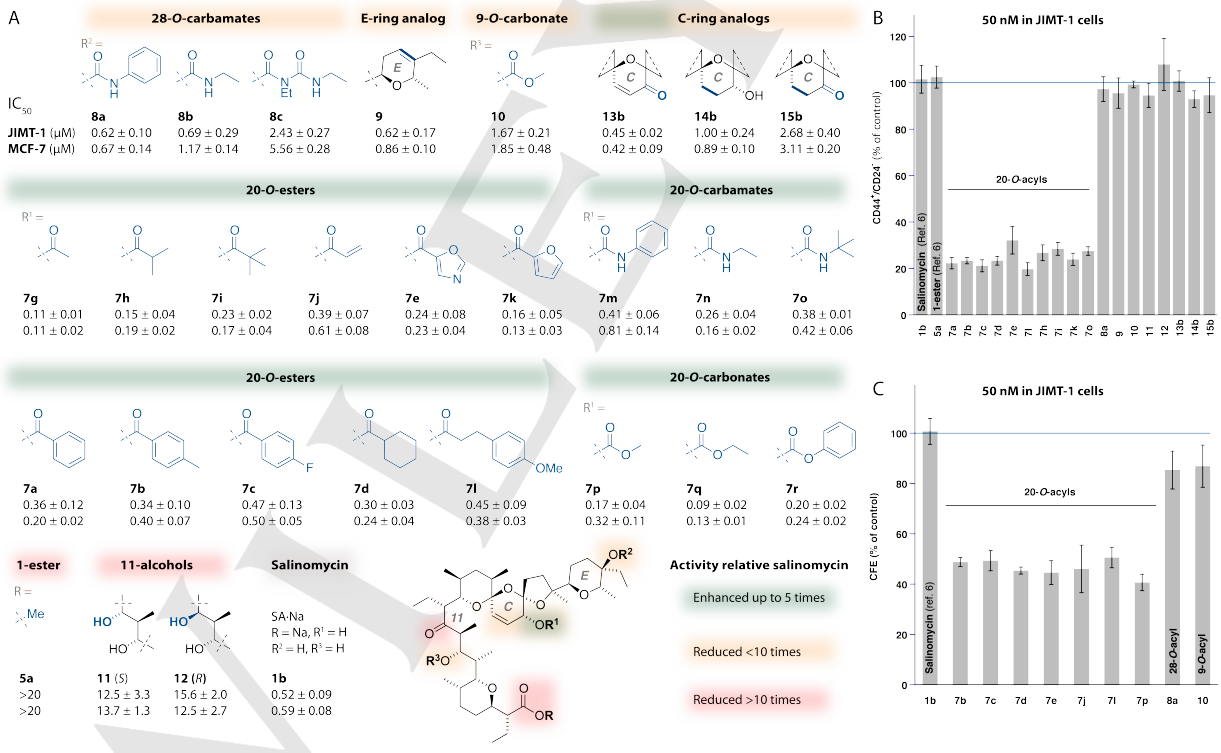


Figure 2. Antiproliferative activity and reduction of putative CSCs by salinomycin analogs at a 50 nM concentration in JIMT-1 cells. A) MTT-based dose–response assay. IC_{50} values are the mean (±SE) for 50% reduction of MTT compared to control. MTT reduction is assumed to be directly proportional to the cell number. For all entries (except **5a**) n = 3. R = Na, R^1 = H, R^2 = H, and R^3 = H unless otherwise stated. B) Reduction of the population of $CD44^+/CD24^-$ cells by salinomycin analog treatment. Bars show the mean of $CD44^+/CD24^-$ cells (±SE) in % of control. For all entries n = 3. C) Reduction in colony forming efficiency (CFE) by salinomycin analog treatment. Bars show the mean CFE (±SE) in % of control. For all entries n = 3.

A selection of analogs with higher or similar activity compared to salinomycin were also investigated in a serum free colony forming efficiency (CFE) assay (Figure 2C). This assay is a functional measure of the ability of individual cancer cells to form colonies in medium lacking serum, a property associated with stem-like cancer cells that have increased tumorigenicity.[25] Salinomycin and the more active analogs **7q**, **7n**, and **7g** were previously shown to give a ~50% reduction of CFE in JIMT-1 cells when used at the respective IC_{50}.[6] When treated with a 50 nM concentration of analogs, the differences in activity in the CFE assay followed the trend seen in the $CD44^+/CD24^-$ assay; the more active 20-*O*-acylated analogs reduced CFE to around 50% whereas salinomycin and the less active analogs **8a** and **10** were inactive.

Conclusions

The design, synthesis, and biological evaluation in breast cancer cells of a library of 28 semi-synthetic structural analogs of salinomycin covering modifications at all directly accessible functional groups are described. The work comprises the first systematic investigation of structure-activity relationships of salinomycin with respect to both basal toxicity and phenotype selectivity. The biological investigation provides a set of activity domains where acylation of the C20 hydroxyl group stands out by giving considerably more active analogs, both with respect to toxicity and selectivity against traits associated with CSCs.

On a structural level, the hydrogen bond motif that stabilizes the head-to-tail conformation of the metal ion complex does not appear to be significant for retaining the biological activity. In light of the impact on biological activity by modification of the C20 hydroxyl group, it is also noteworthy that modulation of the C-ring conformation by modification of the C18-C19 positions results only in slightly reduced activity where larger conformational changes result in less active analogs. Interference with key ion coordinating motifs of the structure results in significantly decreased activity, which supports the notion that the CSC activity of these compounds is intrinsically connected to ionophore properties. Further investigations of the origin of the observed differences in activity within this library of analogs are under way and will be reported in due course.

Acknowledgements

We thank the Swedish Research Council (VR), the Swedish Cancer Society (CF), the Crafoord Foundation, the Royal Physiographical Society, the Mrs. Berta Kamprad Foundation, and the Percy Falk Foundation for funding. Martin Pošta is acknowledged for explorative synthetic work. We thank Anders Sundin for assistance with molecular modeling, Sofia Essén for HRMS measurements, and Eva Dahlberg for seeding cells and preparing medium.

Keywords: Salinomycin • Phenotype selectivity • Structure-activity relationships • Cancer treatment• Ionophores

10.1002/chem.201603621

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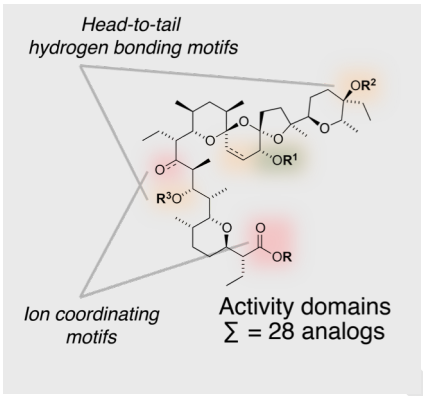
FULL PAPER

The ionophore salinomycin selectively targets cancer stem cells. Synthetic strategies for modification of each of the directly accessible functional groups provide activity domains with respect to cytotoxicity and phenotype selectivity. Acylation of the C20 hydroxyl group gave improved activity and selectivity against putative CSCs, while disruption of ion coordinating motifs was not tolerated.



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