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# Semisynthesis of SY-1 for Investigation of Breast Cancer Stem Cell Selectivity of C-Ring-Modified Salinomycin Analogues

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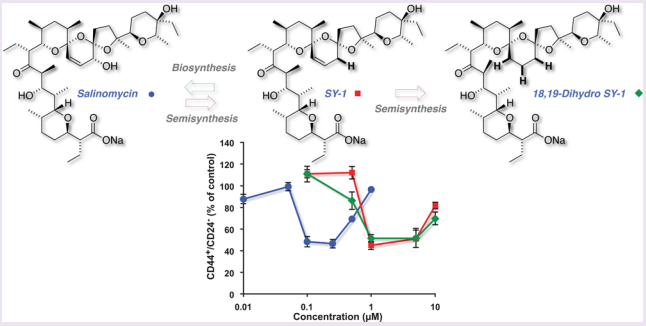
Supporting Information

**ABSTRACT:** Salinomycin, a naturally occurring polyether ionophore was recently found to selectively reduce the proportion of $CD44^+/CD24^-$ cells, a phenotype associated with breast cancer stem cells. Subsequent studies from our group showed that chemical modification of the allylic C20 hydroxyl of salinomycin, located at the C-ring, can enhance the activity of derivatives against breast cancer cells over 5-fold compared to the native structure. Access to C-ring-modified salinomycin analogues is thus of interest from both a mechanistic and a synthetic perspective. Here, we report efficient strategies for gram scale synthesis of the natural product SY-1 (20-deoxy salinomycin), and a saturated analogue, 18,19-dihydro SY-1, for a comparative *in vitro* investigation of the biological profiles of these compounds with that of salinomycin. Across several assays, the deoxygenated structures required higher concentrations to elicit similar cellular responses to that of salinomycin. Similarly to salinomycin, SY-1 or 18,19-dihydro SY-1 treatment was found to reduce the proportion of $CD44^+/CD24^-$ cells with essentially complete selectivity up to ~$IC_{25}$. Importantly, the proportion of $CD44^+/CD24^-$ cells showed a pronounced U-shaped dose response curve for salinomycin and its derivatives, but not for paclitaxel. The concentration for maximum response in this assay followed differences in $IC_{50}$ for salinomycin and its analogues, which emphasizes the importance of taking concentration dependence into account when comparing effects on the $CD44^+/CD24^-$ phenotype. Small differences in the global conformation within the triad of compounds investigated together with differences in activity across assays emphasize the importance of substitution at C20 for the activity of salinomycin and its derivatives.




Semisynthesis of biologically active natural products through selective deoxygenation of more abundantly available structures is an attractive strategy to access material for investigations that would otherwise be prohibited by limited supply. Of equal importance, such strategies can enable access also to unnatural analogues for elucidation of structure−activity relationships and aid in the development of new and potentially superior structures.[1−5] Noteworthy recent examples include the syntheses of prostratin and 12-deoxyphorbol-13-phenylacetate (DPP) via deoxygenation of phorbol[6,7] and strategies for site-selective deoxygenation of erythromycin.[8] Semisynthesis as a strategy is particularly attractive for analogue generation of abundantly available and highly complex structures such as salinomycin (Figure 1, panel a).[9] Salinomycin has received considerable interest recently as a proposed entry to suppress cancer recurrence as it was identified, along with a subset of compounds including the related polyether ionophore nigericin, to efficiently and selectively reduce putative cancer stem cell (CSC) populations.[10−12] Recurrence has been linked to lingering CSCs that remain even after a seemingly efficient treatment and signs of complete remission.[13,14] Such cells are rare in a tumor and have properties that resemble those of normal stem cells including self-renewal and the ability to give rise to all differentiated cellular phenotypes of a tumor.[15,16] Curative cancer treatment has been proposed to require eradication of these cells, which, in turn, has prompted considerable efforts toward investigating the properties of CSCs, as well as identification of small molecules that selectively target them, potentially for use in combination with drugs that target non-CSCs. $CD44^+/CD24^{-/low}$ cells isolated from breast cancer have been shown to exhibit an increased ability to form tumors in immune-compromised mice and have been defined as a putative

Received: March 20, 2014
Accepted: May 19, 2014
Published: May 19, 2014

   dx.doi.org/10.1021/cb5002153 | ACS Chem. Biol. 2014, 9, 1587−1594

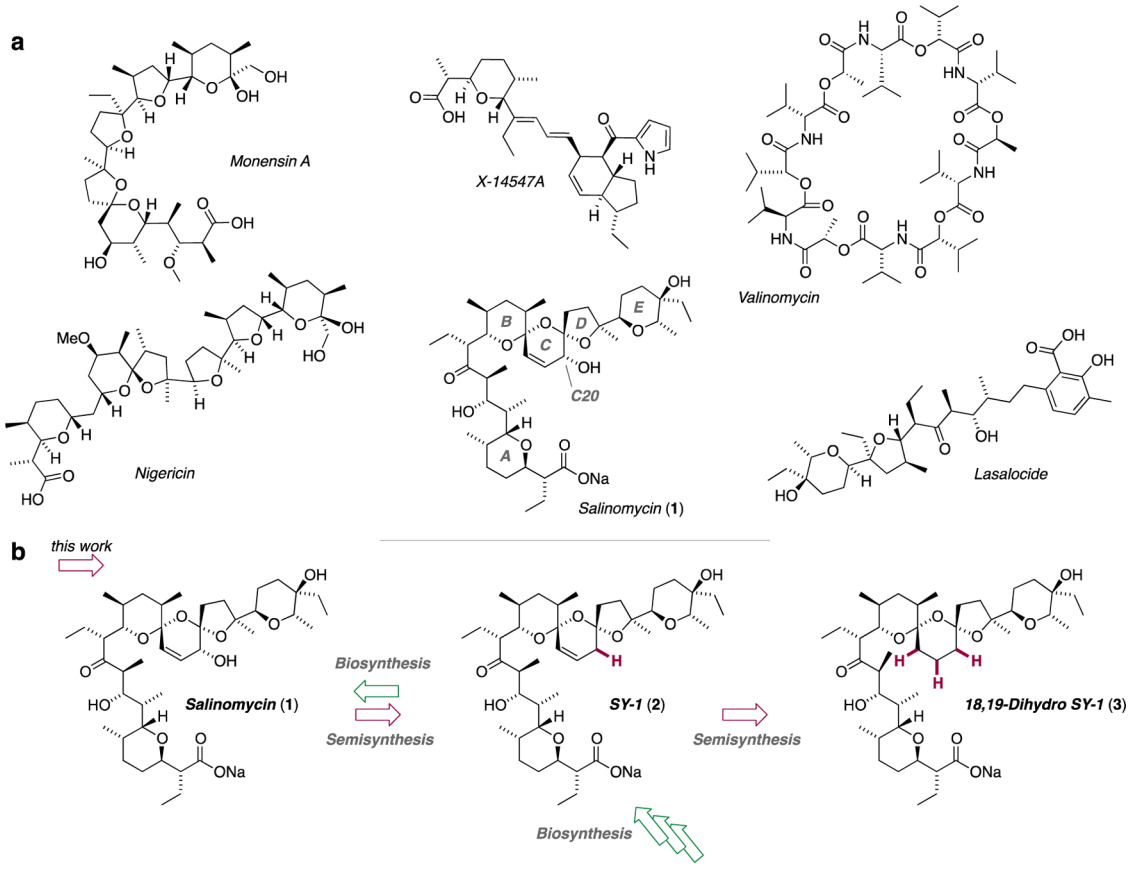
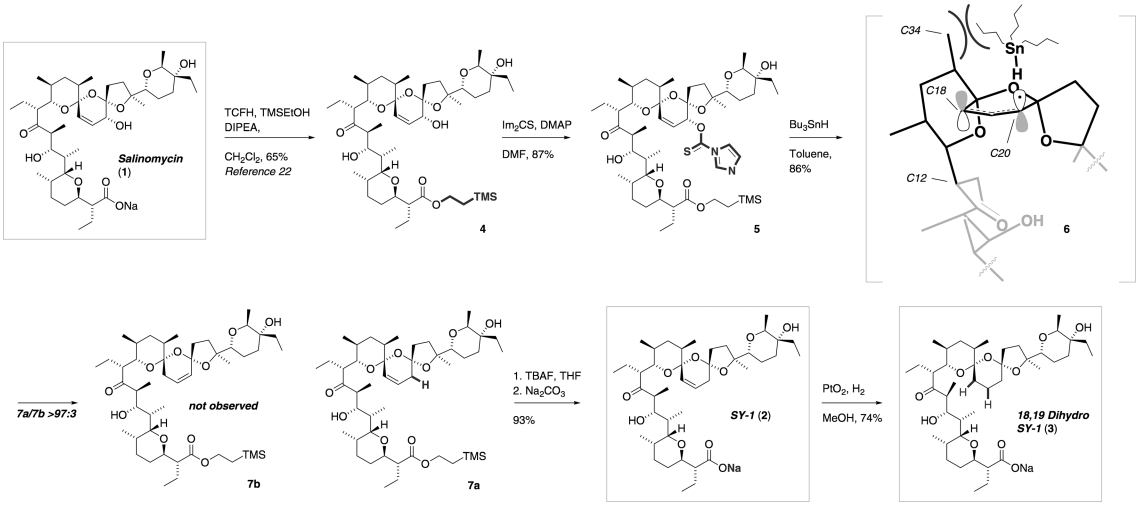



**Figure 1.** (a) Selected ionophore natural products. (b) Reversal of the biosynthesis of salinomycin from SY-1.

**Scheme 1. Synthesis of SY-1 and Its Saturated Analogue 18,19-Dihydro SY-1 Starting from Salinomycin**




CSC population.[17] In this context, salinomycin was identified as the most active out of a library of over 16 000 compounds in reducing the proportion of $CD44^+/CD24^-$ cells as well as in inhibiting mammosphere formation *in vitro*, and mammary tumor growth *in vivo*.[10]

The synthesis and biological activity of salinomycin analogues modified at the carboxylate position have been extensively studied.[18−21] We recently reported synthetic strategies for selective protection and chemical modification of each of the hydroxyl groups in salinomycin.[22] In line with early work on the antibacterial properties of related derivatives,[23] acylation of the allylic C20 hydroxyl, situated on the salinomycin C-ring, was found to enhance the activity of certain analogues over five times compared to the native structure in two breast cancer cell lines.[22] In light of this observation, SY-1 (20-deoxy salinomycin), a natural product structurally related to salinomycin but deprived of the key C20 hydroxyl group, represents an attractive structure for further investigation of structure−activity relationships in a CSC context. SY-1 is an intermediate in the biosynthesis of salinomycin[24] and was isolated and characterized in 1977 as a minor constituent of the fermentation broth of *Streptomyces albus*.[25,26] Unlike salinomycin, however, SY-1 is not readily


dx.doi.org/10.1021/cb5002153 | *ACS Chem. Biol.* 2014, 9, 1587−1594

available and no chemical synthesis of this compound has been reported. While less active against Gram-positive bacteria than salinomycin, SY-1 was shown to exhibit similar activity against mitochondrial function and was moreover reported as 5−10 times more active in inhibiting ADP- or valinomycin-stimulated glutamate oxidation.[27] The effects of SY-1 and related ionophores on multidrug resistant cell lines have also been investigated.[28]

Herein, we report a practical, short, and efficient (four steps, 45% overall yield) semisynthesis of SY-1 starting from readily available salinomycin (Figure 1, panel b). Outgoing from SY-1, a novel 18,19-dihydro analogue of SY-1 was also synthesized, which enabled a comparative *in vitro* investigation of the biological profiles of a triad of compounds varying in substitution at the C-ring: salinomycin, SY-1, and 18,19-dihydro SY-1. The effects of the compounds were investigated in JIMT-1 and HCC1937 breast cancer cell lines with respect to activity as well as selectivity against putative CSCs. At concentrations below $IC_{25}$, both SY-1 and 18,19-dihydro SY-1 selectively reduced the $CD44^+/CD24^-$ phenotype associated with CSC properties. Both compounds also reduced colony forming efficiency and decreased colony size in a serum free soft agar assay. Compared to salinomycin, its C20-deoxygenated congeners SY-1 and 18,19-dihydro SY-1, however, required higher concentrations to give similar responses, which emphasizes the importance of substitution at C20 for the activity of such structures and their derivatives.

## RESULTS AND DISCUSSION

**Efficient Synthetic Conversion of Salinomycin to SY-1 and Its C-Ring-Saturated Analogue 18,19-Dihydro SY-1.** Our strategy for removing the C20 hydroxyl of salinomycin relied on an allylic Barton−McCombie radical deoxygenation reaction as the key transformation.[29] A complicating factor in this process is the intermediacy of an allylic radical, which in principle can give rise to two regioisomeric products **7a** and **7b** (Scheme 1).[30−32] Inspection of an X-ray structure of a nonion coordinated salinomycin derivative[33] was, however, encouraging in this respect, as the southern (C1−C12) part of the structure blocks the approach of a hydride donor to the 18*Re*/20*Si*-faces of the allylic radical **6**. The C34 methyl group on the B-ring further shields against approach to C18 from the *Si*-face (Scheme 1), leaving the desired C20 position as the apparent site for hydrogen abstraction through delivery to the 20*Re*-face.

With respect to purification and isolation of intermediate structures, synthetic modification of salinomycin is greatly facilitated by protection of the carboxylic acid moiety. Salinomycin was thus converted into TMSEt ester **4** on a multigram scale (65% yield), as described previously.[22] Subsequent treatment of alcohol **4** with $Im_2CS$ in DMF using stoichiometric DMAP then gave the desired thiocarbamate **5** in 87% yield with a complete selectivity for the allylic C20 hydroxyl.

Allylic deoxygenation of thiocarbamate **4** was cleanly achieved in 86% yield by a slow addition of $Bu_3SnH$ (excess) in toluene to a refluxing solution of thiocarbamate **5** in the same solvent. It is noteworthy that, in line with the proposed model, the desired 18,19-unsaturated product **7a** was obtained as a single detected regioisomer ($^1H$ NMR spectroscopy of the reaction crude). Addition of radical initiators such as ABCN or $Et_3B$[34] gave significant byproduct formation. Other conditions, including varying the solvent (benzene, dichloroethane, or $CCl_4$), temperature, or running the reaction in neat $Bu_3SnH$,[35] were screened but did not allow for the isolation of appreciable amounts of the desired product. Instead, only complex mixtures were obtained in these experiments.

The synthesis of SY-1 was completed by a fluoride-mediated cleavage of the TMSEt ester of **7a**, which proceeded cleanly with TBAF in THF at ambient temperature. Following chromatographic purification and a $Na_2CO_3$ wash (sat. aq), SY-1·Na was isolated in 93% yield as a single detected isomer, identical to the natural product by optical rotation and IR. The position of the C-ring-olefin at C18/C19 was confirmed by a combination of 1D- and 2D-NMR spectroscopy techniques. A diagnostic NOESY correlation between the C34 methyl group and the olefinic protons corroborated the retained stereochemical integrity at C17 as well as the correct position of the C-ring-olefin at C18/C19. A strong $^3J_{CH}$ coupling from the C16 proton to the C17 carbon in the HMBC spectrum further strengthened this assignment. Spiking a sample of technical grade salinomycin containing trace SY-1 with synthetic SY-1 gave an increase in all signals attributed to trace SY-1 (see Supporting Information for details).

To enable further studies of differences in the activity of C-ring-modified salinomycin derivatives, a novel synthetic analogue of SY-1, 18,19-dihydro SY-1, was also synthesized in 74% yield by hydrogenation of SY-1·Na over Adams catalyst. The attempted hydrogenation of TMSEt ester **4** under the same conditions reduced the C18/C19 unsaturation as evident from the crude $^1H$ NMR spectrum, but resulted in a complex mixture of products.

For a structural comparison, salinomycin·Na, SY-1·Na, and 18,19-dihydro SY-1·Na were modeled using the X-ray structure of SY-1·Na as the initial geometry.[36] Each structure was geometry-optimized using the OPLS2005 force field with GBSA solvation in chloroform (as a model for cell membranes, calculations in gas phase resulted in similar geometries) utilizing the full matrix Newton−Rapson method.[37] In these models, the global conformation is essentially preserved throughout the series; the only significant differences were found in the local conformation of the C-ring (Figure 2).

**Salinomycin, SY-1, and 18,19-Dihydro SY-1 Impair the Proliferation of Breast Cancer Cells in a Dose Dependent Manner.** The biological activities of the sodium salts of salinomycin, SY-1, and 18,19-dihydro SY-1 were investigated in

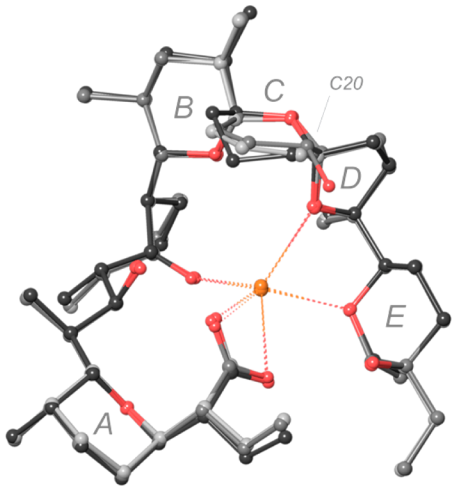
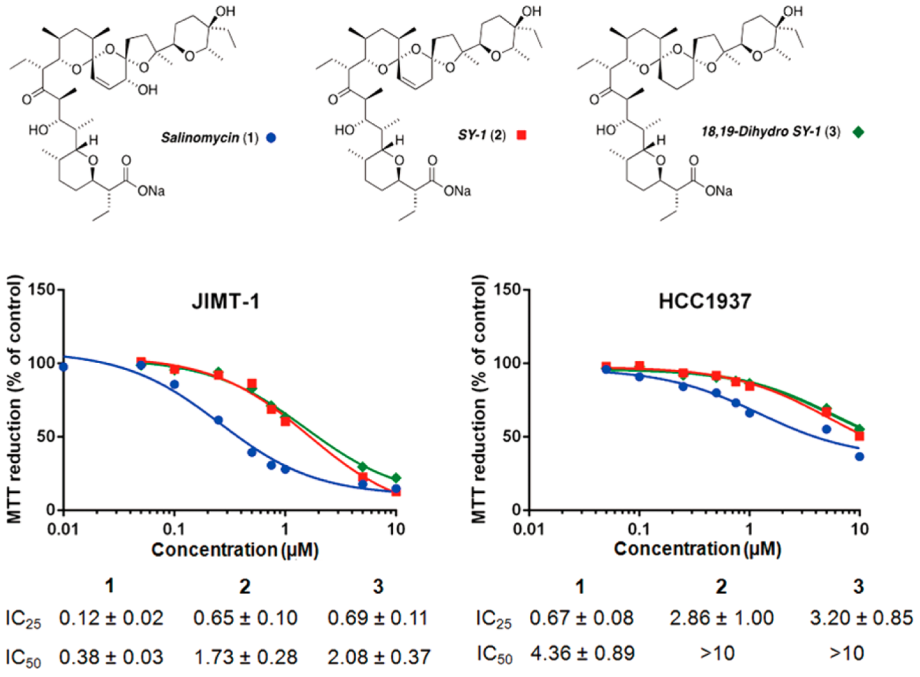



**Figure 2.** Overlay between geometry-optimized models of salinomycin·Na (dark gray), SY-1·Na (light gray), and 18,19-dihydro SY-1·Na (black). Oxygen atoms are shown in red, sodium atoms (pentacoordinate in all structures) in orange. Letters denote rings; see Figure 1.


dx.doi.org/10.1021/cb5002153 | *ACS Chem. Biol.* 2014, 9, 1587−1594




| | JIMT-1 | | | HCC1937 | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| $IC_{25}$ | 0.12 ± 0.02 | 0.65 ± 0.10 | 0.69 ± 0.11 | 0.67 ± 0.08 | 2.86 ± 1.00 | 3.20 ± 0.85 |
| $IC_{50}$ | 0.38 ± 0.03 | 1.73 ± 0.28 | 2.08 ± 0.37 | 4.36 ± 0.89 | >10 | >10 |

**Figure 3.** Dose response curves showing the antiproliferative effect of salinomycin, SY-1, or 18,19-dihydro SY-1 treatments in JIMT-1 and HCC1937 cells. The cells were seeded in the wells of 96-well plates and then incubated for 24 h before addition of compounds to the concentrations shown. The cells were treated for 72 h and the dose response was then evaluated with an MTT assay where MTT reduction is assumed proportional to cell number. The dose response curves shown are the mean of three different dose response experiments with six replicates for each data point in each experiment. $IC_{25}$ and $IC_{50}$ values ($\mu$M) are reported as the mean ± SE ($n$ = 3).

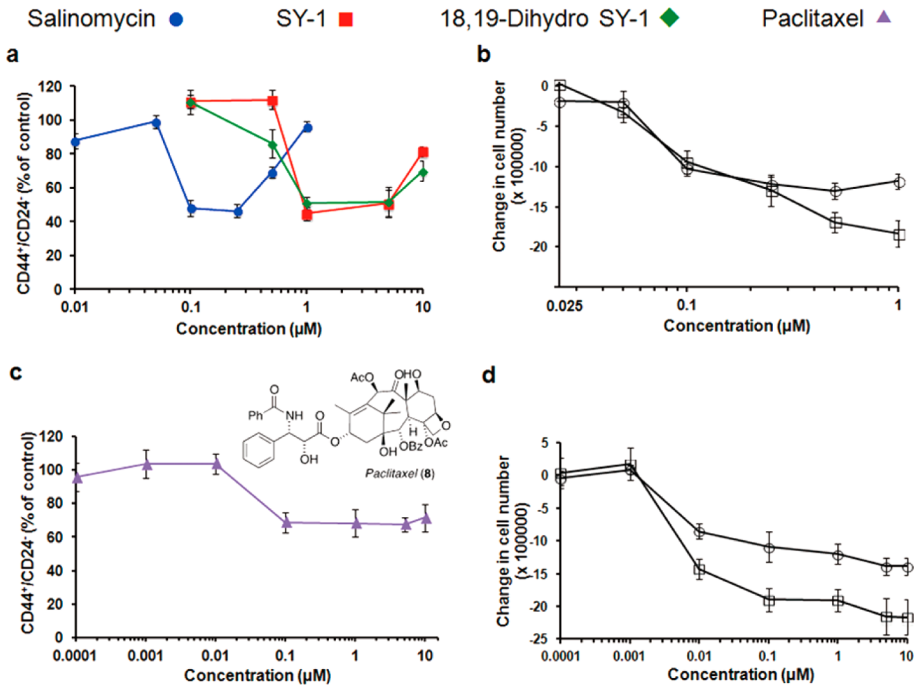



**Figure 4.** Dose response pattern of changes in the $CD44^+/CD24^-$ population in JIMT-1 cells after treatment with (a) salinomycin, SY-1, or 18,19-dihydro SY-1, and (c) paclitaxel. JIMT-1 cells were treated for 72 h at the concentrations indicated. Reduction in the total number of cells (□) and in the number of $CD44^+/CD24^-$ cells (○) after treatment with (b) salinomycin or (d) paclitaxel compared to control. The mean ± SE ($n$ = 4−8) is shown for all entries.

two breast cancer cell lines, JIMT-1 and HCC1937. All compounds gave a dose dependent decrease in MTT reduction for both cell lines indicating a dose dependent decrease in cell numbers (Figure 3). The decrease in cell numbers was caused by an inhibition of cell proliferation and not cell death, as evidenced by growth curve experiments using 0.01−5 $\mu$M salinomycin (not shown).

Salinomycin was more active than SY-1 or 18,19-dihydro SY-1 in both cell lines. As inferred by the $IC_{25}$ and $IC_{50}$ values, JIMT-1 cells were more sensitive to all three compounds than HCC1937


dx.doi.org/10.1021/cb5002153 | ACS Chem. Biol. 2014, 9, 1587−1594

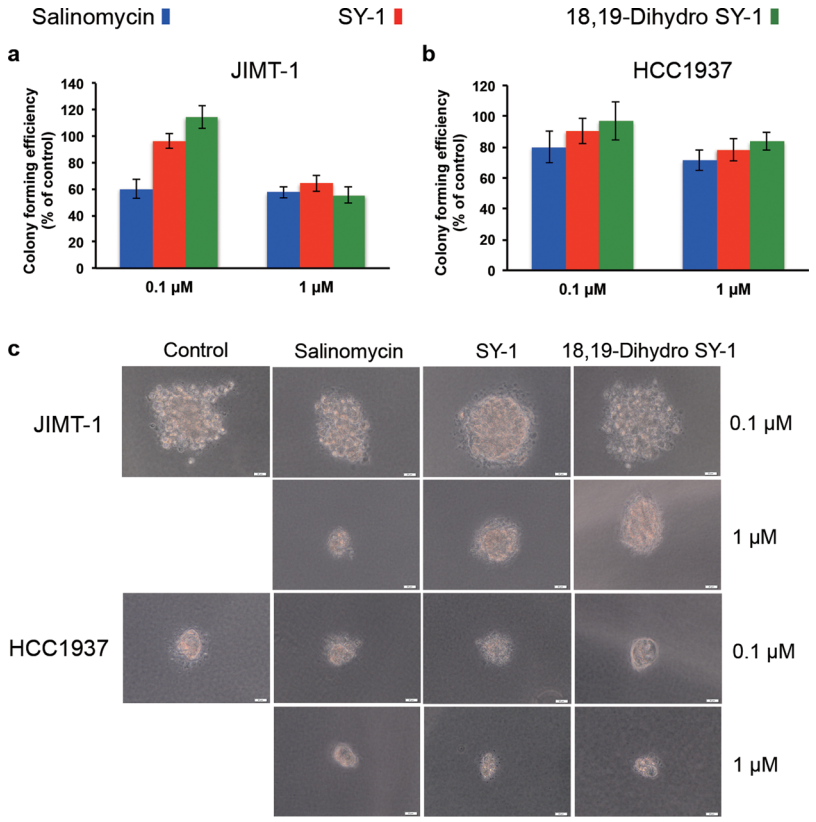



**Figure 5.** Salinomycin, SY-1, or 18,19-dihydro SY-1 treatment reduced colony formation and size of JIMT-1 and HCC1937 cells. The cells were treated for 72 h with the indicated concentrations and then reseeded in serum free medium containing soft agar. Colonies were counted and photographed after 14 days of incubation. Colony forming efficiency of JIMT-1 cells (a) and HCC1937 cells (b) after treatment is expressed as the mean ± SE ($n = 4$). (c) Representative images of JIMT-1 and HCC1937 colonies formed in serum free soft agar after treatment with salinomycin, SY-1, or 18,19-dihydro SY-1. Bar = 20 $\mu$m.

cells. The approximate 10-fold difference in sensitivity between JIMT-1 and HCC1937 cells may in part be a reflection of differences in population doubling time, which is approximately 24 h for JIMT-1 and 35 h for HCC1937 under the culture conditions used. The two cell lines also have different genomic profiles[38] and origins in the breast.[39,40]

**Salinomycin, SY-1, and 18,19-Dihydro SY-1 Selectively Reduce the Proportion of CD44$^+$/CD24$^-$ Cells below $IC_{25}$ in the JIMT-1 Cell Line.** The effect of treatment with salinomycin on the CD44$^+$/CD24$^-$ population in JIMT-1 and HCC1937 cells was investigated using flow cytometry after incubating live cells with fluorescein isothiocyanate (FITC)-conjugated CD44 antibodies and phycoerythrin (PE)-conjugated CD24 antibodies. Treatment with 0.1 $\mu$M salinomycin resulted in a decrease in the CD44$^+$/CD24$^-$ population to 48% of control in the JIMT-1 cells and to 72% of control in HCC1937 cells. In HCC1937 cells, increasing the concentration to 1 $\mu$M gave a similar level of reduction, 70% of control. Surprisingly, when JIMT-1 cells were treated with 1 $\mu$M salinomycin, the proportion of CD44$^+$/CD24$^-$ cells was 96% of control, that is, an increase compared to after treatment with 0.1 $\mu$M. This behavior prompted an investigation with more concentrations in the more sensitive JIMT-1 cell line. The proportion of CD44$^+$/CD24$^-$ cells compared to control was found to go through a minimum when the cells were treated with ∼0.1−0.25 $\mu$M salinomycin (Figure 4, panel a). Both SY-1 and 18,19-dihydro SY-1 showed the same behavior, but being less toxic compared to salinomycin, each displayed minima at a higher concentration range (1−5 $\mu$M) (Figure 4, panel a). To the best of our knowledge, similar observations where the proportion of CD44$^+$/CD24$^-$ cells showed a U-shaped dose response pattern have not been reported. To find a possible explanation for this observation, the decrease in the total cell number following salinomycin treatment was compared to the decrease in the number of CD44$^+$/CD24$^-$ cells (Figure 4, panel b). Up to ∼0.25 $\mu$M, the reduction in cell number resided almost exclusively in a reduction of the number of CD44$^+$/CD24$^-$ cells. At concentrations above ∼0.25 $\mu$M, the number of CD44$^+$/CD24$^-$ cells did not change, while the total cell number continued to decrease, giving an increase in the proportion of CD44$^+$/CD24$^-$ cells as a result (Figure 4, panel a). The change in selectivity appears to occur in the vicinity of the $IC_{25}$ value. The same observation was made for SY-1- or 18,19-dihydro SY-1-treated JIMT-1 cells (not shown). The selectivity of all three compounds against CD44$^+$/CD24$^-$ cells thus seems to be excellent at concentrations below $IC_{25}$ but is decreased at higher doses. The absence of cell death at these concentrations implies that the reduction in the proportion of CD44$^+$/CD24$^-$ cells was a result of inhibition of growth and/or a phenotypical shift of these cells. The decrease in CD44$^+$/CD24$^-$ cells was accompanied by a corresponding increase in the proportion of CD44$^+$/CD24$^+$ cells.

The concentration dependence for salinomycin and its related structures prompted an investigation of clinically used paclitaxel ($IC_{50}$ = 0.005 $\mu$M in JIMT-1 cells) on the CD44$^+$/CD24$^-$ population in JIMT-1 cells. Over a concentration range of 0.0001 $\mu$M to 10 $\mu$M, paclitaxel treatment in JIMT-1 cells did not show a U-shaped dose response pattern such as salinomycin (Figure 4, panel c). Up to 0.01 $\mu$M, the proportion of CD44$^+$/CD24$^-$ cells


dx.doi.org/10.1021/cb5002153 | ACS Chem. Biol. 2014, 9, 1587−1594

remained constant even though the cell number decreased (Figure 4, panel d), which implies no selectivity against the $CD44^+/CD24^-$ population up to this concentration. A decrease to ~70% in the proportion of $CD44^+/CD24^-$ cells compared to control was reached at 0.1 $\mu$M paclitaxel and this level remained constant at the higher concentrations investigated. Previous studies with other cell lines have shown that the $CD44^+/CD24^-$ proportion increased with increasing concentration of paclitaxel.[10,41]

**Salinomycin Is More Efficient in Reducing Colony Forming Ability than SY-1 and 18,19-Dihydro SY-1 at a Low Concentration (0.1 $\mu$M) and Also in Reducing Colony Size at a Higher Concentration (1 $\mu$M).** The colony forming efficiency was investigated in serum free soft agar as a functional assay of survival of cells with stem cell properties.[42] At a 0.1 $\mu$M concentration, salinomycin reduced the colony forming efficiency of JIMT-1 cells by 40% compared to control, while SY-1 or 18,19-dihydro SY-1 treatment did not have a large effect on colony forming efficiency (Figure 5, panel a). At a higher concentration (1 $\mu$M), all three compounds gave a similar decrease in colony forming ability (about 45%) but the colonies were smaller after salinomycin treatment (Figure 5, panel c). Salinomycin was also better than the C20 deoxygenated structures in reducing the colony forming efficiency in HCC1937 cells at 0.1 $\mu$M. Little influence on the colony size was seen for this cell line (Figure 5, panels b/c).

Notably, the colony forming efficiency decreased after treatment with 1 $\mu$M compared to 0.1 $\mu$M even though the $CD44^+/CD24^-$ proportion increased, meaning that a proportion of surviving $CD44^+/CD24^-$ cells were not capable of forming colonies after treatment.

**Conclusions.** An efficient strategy (four steps, 45% overall yield) for the synthesis of the natural product SY-1 (20-deoxy salinomycin) through a selective radical deoxygenation of abundantly available salinomycin is presented. The efficiency of this method reflected in isolation of close to one gram of SY-1·Na during the optimization of the synthesis. Access to SY-1 also enabled the synthesis of an unnatural analogue, 18,19-dihydro SY-1, as well as a comparative biological investigation of the differences in activity and CSC selectivity within a triad of analogous compounds varying at the C-ring: salinomycin, SY-1, and 18,19-dihydro SY-1.

Both SY-1 and 18,19-dihydro SY-1 were found to exhibit similar activities against JIMT-1 and HCC1937 cells as measured by an MTT assay but were less active than salinomycin. The three compounds reduced colony forming efficiency in a serum free soft agar assay with salinomycin again being the more efficient compound at lower concentrations. Salinomycin was found more efficient than SY-1 or 18,19-dihydro SY-1 in reducing the proportion of $CD44^+/CD24^-$ cells, but all compounds target this phenotype with essentially complete selectivity in JIMT-1 cells at concentrations below the respective $IC_{25}$. The similar biological profile of these three compounds suggests that they act through a common mechanism. A U-shaped dose response pattern for $CD44^+/CD24^-$ cells was found, which emphasizes the importance of taking concentration dependence into consideration in assays relying on $CD44^+/CD24^-$ as a marker, in particular, when such assays are used to compare responses between compounds with significant differences in activity. This observation moreover augments the value of also including complementary functional assays as well as other proposed markers for CSCs in breast cancer.[43,44] It is also noteworthy that the performance of each compound in decreasing colony forming efficiency or selectively reducing the $CD44^+/CD24^-$ proportion appears to follow observed differences in activity as measured by the MTT assay.

As the global conformation is largely preserved throughout the series of compounds studied, we interpret differences in activity as primarily related to differences in lipophilicity and ion binding, properties that are expected to reflect in modulated cell uptake and membrane activity. Further studies on these and related compounds as well as the properties of the $CD44^+/CD24^-$ cells that remain after treatment are currently under way.

## METHODS

**Chemical Synthesis.** Salinomycin was isolated from technical grade material (~12%), as described previously.[22] For details on the synthesis and characterization of SY-1 and 18,19-dihydro SY-1, see Supporting Information.

**Cell Lines and Culturing Conditions.** The human breast carcinoma cell lines JIMT-1 and HCC1937 were cultured at 37 °C in a humidified incubator with 5% $CO_2$ in air. The JIMT-1 cell line was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) and was maintained in Dulbecco's modified Eagle's medium/nutrient mixture Ham's F12 medium supplemented with 10% fetal calf serum (FCS), nonessential amino acids (1 mmol $L^{-1}$), insulin (10 $\mu$g $mL^{-1}$), penicillin (100 U $mL^{-1}$), and streptomycin (100 $\mu$g $mL^{-1}$). The HCC1937 cell line was obtained from American Type Culture Collection (ATCC) and was routinely cultured in RPMI 1640 medium supplemented with 10% FCS, nonessential amino acids (1 mmol $L^{-1}$), insulin (10 $\mu$g $mL^{-1}$), epidermal growth factor (20 ng $mL^{-1}$), penicillin (100 U $mL^{-1}$) and streptomycin (100 $\mu$g $mL^{-1}$).

**Dose Response Assay.** An MTT assay was used to evaluate the dose response of the compounds.[45−47] The compounds were dissolved in DMSO and then serially diluted in PBS and used at final concentrations from 0.01 $\mu$M to 10 $\mu$M. The final DMSO concentration in the assays was 0.2% for all concentrations used. Accordingly, control was treated with 0.2% DMSO in PBS. For the assays, cells were seeded in 96-well plates (6000 cells for HCC1937 and 5000 cells for JIMT-1 per well in 180 $\mu$L medium) and the plates were incubated for 24 h before addition of compound. The effect was evaluated by the MTT assay after 72 h of treatment. MTT solution (20 $\mu$L; 5 mg $mL^{-1}$ in PBS) was added to each well and the 96-well plates were returned to the incubator for 1 h. Thereafter, the medium was removed and the blue formazan product was dissolved by the addition of 100 $\mu$L of 100% DMSO per well. The plates were swirled gently for 10 min to dissolve the precipitate and the cells. Absorbance was monitored at 540 nm using a Labsystems iEMS Reader MF (Labsystems Oy) and the software DeltaSoft II v.4.14 (Biometallics Inc.). For each compound, three dose response experiments were performed with six replicates in each experiment. The software program GraphPad Prism was used to plot dose response curves.

**Cell Surface Markers Identified by Flow Cytometry.** Cells were harvested using Accutase and identified based on their expression of the cell surface markers CD44 and CD24 using a BD Accuri C6 Flowcytometer (BD Biosciences). CD44-FITC (clone G44−26), CD24-phycoerythrin (PE) (clone ML5), PE- and FITC-conjugated mouse IgG1 isotype controls (MOPC-21) were purchased from Becton Dickinson. In brief, the cells (300 000 cells per sample) were incubated with the monoclonal antibodies CD44-FITC (1:100), CD24-PE (1:50), isotype-FITC (1:20) and isotype-PE (1:10), respectively, for 15 min on ice. Thereafter, the cells were washed with cold PBS containing 1% FCS before analysis in the flow cytometer. CFlow software was used to evaluate the data. In untreated JIMT-1 cells, the percentage of cells in the $CD44^+/CD24^-$ population varied between 50% and 70% and in untreated HCC1937 between 30% and 50% for different experiments. The data for treated cells are expressed as % of control.

**Colony Formation Assay in Soft Agar.** The colony formation assay was performed in 48-well plates. The wells were coated with poly-HEMA (200 $\mu$L of 5 mg $mL^{-1}$ poly-HEMA in 96% ethanol incubated for 3 days at 37 °C to allow slow evaporation of the ethanol). After 72 h of


dx.doi.org/10.1021/cb5002153 | ACS Chem. Biol. 2014, 9, 1587−1594

treatment with salinomycin, SY-1 or 18,19-dihydro SY-1, the cells were harvested using Accutase for 10 min at 37 °C and then kept on ice. MEBM basal medium containing hydrocortisone, insulin, epidermal growth factor (CC-4136 kit), B27 supplement, basic fibroblast growth factor (20 ng $mL^{-1}$), penicillin (50 U $mL^{-1}$), and streptomycin (50 $\mu$g $mL^{-1}$) was heated to 42 °C and mixed with agarose to a final concentration of 0.4%. Cells were then added to a concentration of 1000 cells $mL^{-1}$, followed by immediate addition of 500 $\mu$L of this mixture to the inner wells of poly-HEMA-coated 48-well plates. To minimize evaporation, outer wells were filled with 1 mL PBS. Plates were wrapped with saran wrap and incubated in 5% $CO_2$ in humidified air at 37 °C for 14 days. The colonies were counted using an inverted phase contrast microscope. In JIMT-1 and HCC1937 cells the colony forming efficiency for control was 73% and 51%, respectively. In untreated JIMT-1 cells, the percentage of cells forming colonies was between 60% and 75% in different experiments. The data for treated cells are expressed as % of control.

**Statistical Analysis.** Data are expressed as the mean ± SE unless otherwise stated.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthetic procedures and copies of the $^{1}H$ and $^{13}C$ NMR spectra for all new compounds and synthetic SY-1. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank the Crafoord Foundation, the Swedish Cancer Society, the Swedish Research Council, the Royal Physiographical Society, the Mrs Berta Kamprad Foundation, the Percy Falk Foundation and the Royal Academy of Sciences for generous financial support. We thank K.-E. Bergquist, S. Essén, and A. Sundin for assistance with NMR spectroscopy, LCMS-MS, and molecular modeling, respectively. We thank E. Dahlberg for seeding cells and preparing medium.

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