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Synthesis, antiproliferative and antibacterial activity of new amides of salinomycin

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ABSTRACT

A series of 11 novel amides of salinomycin were synthesized for the first time. All the obtained compounds were found to show potent antiproliferative activity against human cancer cell lines including the drug-resistant cancer cells. Four new salinomycin derivatives revealed good antibacterial activity against clinical isolates of methicillin-resistant *Staphylococcus epidermidis* (MRSE).

In last decades natural substances have been in a sense rediscovered as important ingredients in pharmaceutical industry either in their chemically unchanged or synthetically modified forms.[1,2]

Salinomycin (**SAL**) isolated from *Streptomyces albus* is an antibiotic belonging to natural polyether ionophores,[3] exhibiting a large spectrum of antimicrobial activity against Gram-positive bacteria, including *Staphylococcus aureus*, mycobacteria, *Plasmodium falciparum* or *Eimeria* spp, parasites, and protozoa.[4] For this reason, sodium salt of salinomycin (Bio-cox™, Sacox™) found commercial application as a coccidiostatic and non-hormonal growth promoting agent in livestock and poultry breeding.[5] Screening of about 16.000 chemical compounds performed in 2009 showed that **SAL** was the most effective against breast cancer stem cells, nearly 100-fold more active than the commonly used anticancer drug–*Paclitaxel* (*Taxol*).[6] Since this discovery, extensive research has been carried out all over the world to elucidate the unusual properties of **SAL**. Further studies proved that **SAL** shows antiproliferative activity against various types of human tumour cells, for example leukemic stem cells, including lymphocytic leukemia, colon carcinoma stem cells, prostate cancer stem cells, as well as lung cancer cell lines.[7] Additionally, it has been shown that the application of **SAL** enhances the anticancer effect of radio- and chemotherapy.[8] Moreover, the sodium salt of **SAL** is able to selectively deplete the breast cancer stem cells with efficiency comparable to that of **SAL**.[9]

The preliminary clinical study of salinomycin has been performed by Cord Naujokat et al. on a small group of patients with metastatic breast cancer or metastatic head and neck cancers. The patients treated with 200–300 μg/kg of **SAL**, every second day for three weeks, have shown partial tumour regression and only transient acute side effects, including tachycardia and mild tremor, with neither severe nor long-term side effects that can be observed to accompany the use of conventional chemotherapeutic drugs. Thus, the preliminary results have permitted determination of a drug dosage that yields clinically significant benefits without excessive toxicity.[10]

The simplest method for preparing biologically effective compounds is chemical modification of substances with proven high biological activity. The synthesis, structure, as well as biological activity of a series of *O*-acyl derivatives,[11] amides[12,13] and one ester[14] of **SAL** have been already described. The **SAL** derivatives showed antimicrobial activity, among others against methicillin-resistant hospital strains of *Staphylococcus* and anticancer activity

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in various cell phenotypes in the low micromolar range, providing an excellent starting point for further drug discovery optimisation. In addition, tests of **SAL** and its derivatives have clearly proven that some of these compounds have a high antiproliferative effect against normal and drug resistant cancer cells. These results indicate that the biological effects of **SAL** derivatives are diverse on different bacterial or cancer cell lines and are strongly dependent on chemical nature of *O*-acyl, amide or ester substituent.[11–14]

The main aim of this Letter is evaluation of anticancer and antimicrobial activity of new derivatives of **SAL**. Therefore, a series of new **SAL** amides was synthesized, characterized by X-ray and spectroscopic methods and tested against their antiproliferative and antibacterial activity. Moreover, as the biological activity of the new **SAL** derivatives is closely related to their ability to make complexes with monovalent and divalent metals, it was tested using the electrospray ionisation mass spectrometry (ESI MS).

In the present study, the antiproliferative effect of eleven **SAL** amides (**1**–**11**) was tested in vitro using human promyelocytic leukemia (HL-60) and its vincristine-resistant subline (HL-60/vinc), human colon adenocarcinoma cell line (LoVo) and doxorubicin resistant subline (LoVo/DX), and normal murine embryonic fibroblast cell line (Balb 3T3). Multi-drug chemoresistance (MDR) remains one of the most common reasons for failure of chemotherapy. The membrane transporter protein belonging to the ABC transporters family has been shown in vitro to effectively reduce the intracellular concentration of several anticancer chemotherapeutic agents such as doxorubicin. On the other hand, it is known that cancer stem cells may act as master regulators during the process of chemoresistance acquisition and are characterized by MDR phenotype.[15,16]

Taking into account this phenomenon, we decided to study the antiproliferative activity of salinomycin derivatives on drug resistant cells, expressing various transporters (e.g., *p*-glycoprotein) and their parent cell lines to observe not only the antiproliferative activity against cancer cells, but also the possibility to break the barrier of chemoresistance.

Antimicrobial activity of compounds **1**–**11** was also tested in vitro on Gram-positive and Gram-negative bacteria and fungi, as well as against a series of clinical isolates of *Staphylococcus*. To investigate the effect of different substitutions of the carboxylic group of **SAL** on its bioactivity, eight new amide derivatives (**1**–**8**) and three dimers (**9**–**11**) of **SAL** were synthesized using the procedure developed previously by our group.[12] To facilitate the structural activity relationship analysis (SAR) we chose salinomycin amides with different substituents such as: unsaturated alkyl chain (propargylamine, **1**), alkyl chains containing oxygen atoms (2-(2-aminoethoxy)ethanol, **3**) biogenic amines like cysteamine, (**2**), putrescine (**4**), histamine (**7**), dopamine (**8**), containing fluorinated aromatic ring (4-fluorobenzylamine, **6**) and crown ether (2-aminomethyl-15-crown-5, **5**). It is generally believed that dimers of biologically active compounds, such as antibiotics, can show enhanced biological activity relative to that of the single ligand. Thus, the symmetrical dimeric **SAL** ligands, in which two **SAL** molecules are linked by different spacer units (1,4-butanediamine **9**; *p*-phenylenediamine **10**; 4,4′-diaminobiphenyl, **11**) were also prepared to check the effects of linker length and its flexibility on the biological activity of **SAL**.

Salinomycin sodium salt was isolated from veterinary premix–SACOX®. Amide derivatives of salinomycin (**1**–**11**) were obtained in the reaction between salinomycin acid (**SAL)** and amines with addition of DCC (*N,N′*-dicyclohexylcarbodiimide) and HOBt (1-hydroxybenzotriazole) following the procedures described previously.[12]

All **SAL** amides can be easily isolated in pure form following the purification by dry vacuum column chromatography.[17] This method was efficient and gave amides **1**–**8** in high yields of up to 43–88% (Scheme 1). The **SAL** dimers (**9**–**11**) were obtained in the moderate yields of about 30% (Scheme 1). The purity and structures of compounds **1**–**11** were determined on the basis of elemental analysis, FT-IR and NMR methods. The ^{1}H and ^{13}C NMR signals were assigned using one- and two-dimensional (COSY, HETCOR, HMBC and NOESY) spectra. The exemplary NMR spectra are included in the Supplementary material (Figs. S1–S4). The analytical signals in the ^{1}H and ^{13}C NMR spectra and the position of the amide I band in the FT-IR spectra of compounds **1**–**11**, are collected in Table S1.

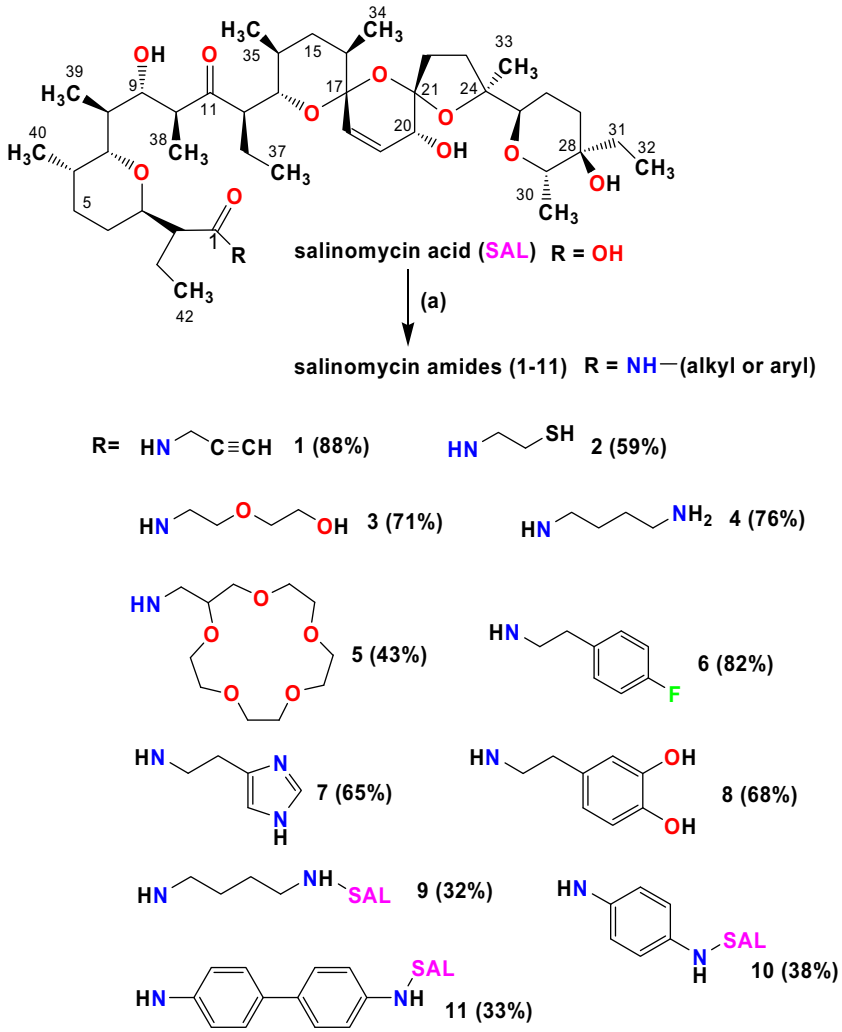
Detailed structural analysis of the biologically active compounds is very important for better understanding of their anticancer and antimicrobial properties for structure–activity relationship analysis (SAR), and related investigation. Therefore, one exemplary compound of the studied series of derivatives that is *p*-fluorobenzylamide (**6**) was characterized by single crystal X-ray diffraction method. The single crystals of **6** were grown by crystallisation in acetonitrile and their structure was determined using X-ray crystallographic technique (Fig. 1). The crystallographic data and structure refinement of compound **6** are summarized in Table S2 (Supplementary material).

The pseudo-cyclic conformation of **6** is stabilised by four N1–H···O10, O10–H···O6, O9–H···O7 and O8–H···O7 weak intramolecular hydrogen bonds showed in Figure 1, and the parameters of this compound are collected in Table S3. The six-membered rings of **6** exhibit the typical chair conformation. The intermolecular O8–H···O1^{i} hydrogen bond between the terminal hydroxyl group (O8–H) of one molecule and the carbonyl atom of amide group of the neighbouring molecule, together with the van der Waals forces, stabilise the arrangement of **6** molecules in the crystal (Fig. S5, Supplementary data). The bond lengths and angles characterizing the geometry of the molecule are presented as Supplementary material (Table S4). The absolute configuration of **6** is unchanged and is the same as determined previously for salinomycin, its amides and benzotriazole ester.[12–14,18,19]

The presence of the pseudo-cyclic structure of salinomycin amides confirmed by X-ray is facilitates the formation of the lipid-soluble pseudo-cyclic complexes of these compounds with the metal cations. Since the biological activity of the polyether antibiotics and its derivatives strongly depends on their ionophoretic properties, the ability of the new **SAL** derivatives to form complexes with monovalent cations such as Li^{+}, Na^{+}, K^{+}, Rb^{+}, Cs^{+} and divalent cations such as Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+} was studied by us using ESI MS technique. The ESI MS spectra of the mixtures of respective **SAL** amides with the monovalent and divalent metal perchlorates (Fig. S6-S10) demonstrate that the amides (**1**–**4**, **6**–**8**) form exclusively 1:1 complexes with both types of metal cations. Only the amide with the crown moiety (**5**) is able to form different complexes with divalent cations (M) that is the $(\mathbf{5}+M)^{2+}$ and $(\mathbf{5}+MClO_4)^{+}$ (Fig. S9). The last type of complexes has been previously observed for different monensin derivatives.[12,13] Additionally, dimers of **SAL** (**9**–**11**) are able to form complexes with monovalent cations in 1:1 and 1:2 stoichiometry (Fig. S10).

In contrast to unmodified salinomycin (**SAL**), which forms complexes of 1:1 stoichiometry only with monovalent cations, especially Na^{+} and K^{+}, salinomycin amides are able to form complexes with both monovalent and divalent metal cations and of different stoichiometries.

The reason why **SAL** and its derivatives exhibit biological effects is their ability to form lipid-soluble pseudo-cyclic complexes with metal cations and transport them trough cell and mitochondrial membranes. Derivatives of **SAL** with modified carboxylic groups like amides can transport cations via an electrogenic mechanism. The ESI MS measurement show the ability of SAL amides to form complexes with monovalent and divalent cation, therefore the biological activity of these compounds is confirmed also by their ionophoretic properties.



Scheme 1. Reaction and conditions: (a) DCC, HOBt, R-NH_2–appropriate amine, CH_2Cl_2/THF (3/1), 0 °C–1 h, then rt–24 h. Time for completion of the reaction at rt as indicated by TLC. Yield of isolated and purified products in brackets. For the experimental procedure, see Ref. 12.

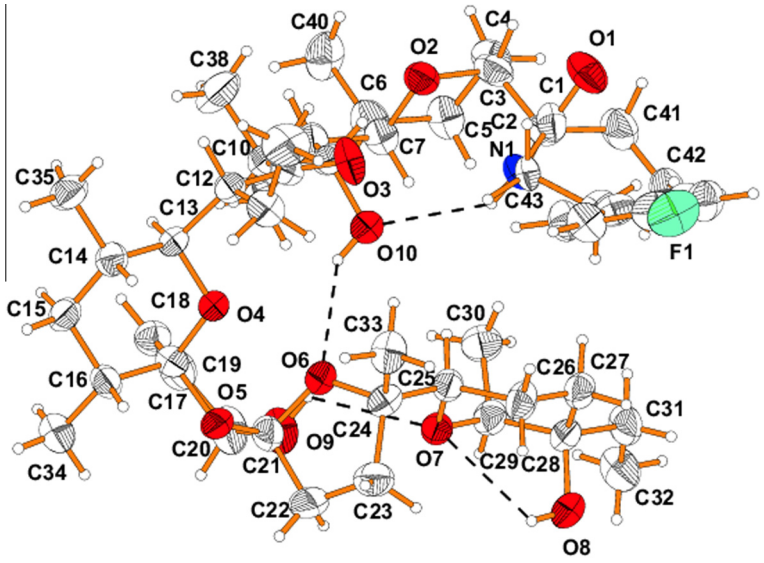


Figure 1. View of the molecular structure of **6** with the atoms labelling. Dashed lines represent the hydrogen bonds.

The new salinomycin amides (**1**–**11**) were evaluated for their in vitro antiproliferative effect on four cancer and one normal cell lines following the previously published procedures.[13] Each compound was tested on two human cancer cell lines displaying various levels of drug resistance, such as human promyelocytic leukemia (HL-60) and its vincristine-resistant subline (HL-60/vinc), human colon adenocarcinoma cell line (LoVo), and doxorubicin resistant subline (LoVo/DX). The antiproliferative effect was also studied on normal murine embryonic fibroblast cell line (BALB/3T3) for better description of cytotoxic activity of studied compounds. The mean IC_{50} ± SD of the tested compounds are collected in Table 1. To evaluate the activity of compounds **1**–**11** against the cells with MDR (multidrug resistance) phenotype, two drug resistant cancer cell lines that is HL-60/vinc and LoVo/DX were tested and their indexes of resistance (IR) were calculated (Table 2). The IR value indicates how many times more resistant is the subline in comparison to its parental cell line.

As shown in Table 1, **SAL** was active at low μM ranges (1.0–4.7 μM) in the entire cancer cell lines tested, being the most active against the LoVo/DX and HL-60 cell lines. This study shows that the majority of the synthesized compounds exerted antiproliferative activities at micromolar concentrations (IC_{50} from 2.0 to 36.7 μM) against the four human cancer cell lines and relatively low toxicity to normal murine embryonic fibroblast cell line. Derivatives **6** and **8** were active in the low micromolar concentration range (IC_{50} 2.3–6.9 μM), whereas compounds **2**, **3**, **5** and **7** showed IC_{50} values between 8.5 and 36.7 μM for HL-60/vinc, LoVo and LoVo/DX cancer line. The most sensitive cell line was HL-60, because almost all **SAL** derivatives are active under low micromolar concentrations. It is interesting to note that **SAL** and its most active derivatives revealed antiproliferative activity against both chemoresistant cell lines similar or higher than that of typical anticancer drugs as cisplatin and doxorubicin against BALB/3T3 fibroblasts and were less toxic than these drugs.

The results obtained indicate that **SAL** and its derivatives **8** and especially **6** exhibited the highest ability to inhibit the proliferation of different cancer cell lines (Table 1) in comparison to those of the other **SAL** derivatives obtained. It clearly shows that different amide substituents attached to the **SAL** moiety might affect the activity and also that the presence of aromatic ring with the polar groups, such as F or OH, is preferable over the presence of aliphatic groups. The data given in Table 1 show that all compounds tested were active against cell lines expressing drug-resistant phenotype (IR below 10), while for doxorubicin IR is over 44. Even six compounds (**1**, **6**, **8**–**11**) showed moderate to high cytotoxic activity against LoVo/DX cancer cell line, which was higher than that of the anticancer drugs such as doxorubicin and cisplatin (Table 1).

Table 2
The calculated values of the indexes of resistance (IR) and selectivity (SI) for salinomycin (**SAL**) and its new amides (**1**–**11**)

Compound	HL-60	HL-60/vinc		LoVo	LoVo/DX	
	SI	SI	IR	SI	SI	IR
SAL	22.07	5.45	4.05	16.88	26.08	0.65
1	6.47	3.29	1.97	5.40	9.60	0.56
2	5.53	0.50	10.99	0.65	0.60	1.07
3	2.22	1.28	1.74	1.08	2.76	0.39
4	9.47	1.52	6.22	1.60	2.78	0.58
5	1.35	1.16	1.16	1.30	3.69	0.35
6	20.27	6.80	2.98	11.20	19.57	0.57
7	7.79	1.04	7.46	1.06	0.99	1.07
8	2.05	0.83	2.48	1.47	1.34	1.10
9	7.28	1.47	4.95	1.85	5.13	0.36
10	10.07	1.26	7.99	2.49	7.20	0.35
11	—	—	—	—	—	0.38
Doxorubicin	12.00	0.27	44.50	2.82	0.07	37.94
Cisplatin	2.33	0.87	2.66	1.77	1.04	1.71

The IR (Index of Resistance) indicates how many times a resistant subline is chemoresistant relative to its parental cell line. When IR is 0–2 the cells are sensitive to tested compound; IR of the range 2–10 means that the cell shows moderate sensitivity to a drug; IR above 10 indicates strong drug-resistance.
The SI (Selectivity Index) was calculated for each compounds using formula: SI = IC_{50} for normal cell line (BALB 3T3)/IC_{50} for respective cancerous cell line. A beneficial SI >1.0 indicates a drug with efficacy against tumour cells greater than toxicity against normal cells.

The selectivity index (SI), an important pharmaceutical parameter that facilitates the estimation of possible future clinical development, was determined as the ratio of IC_{50} value for normal cell line (BALB 3T3) to IC_{50} value for a respective cancerous cell line. The bioactivity of each compound was evaluated by a combination of its IC_{50} value and the corresponding SI. Higher values of SI indicate greater anticancer specificity and SI greater than 3 was considered as highly selective. The results collected in Table 2 indicate that **SAL** and its amides appeared to be less toxic against normal embryonic fibroblasts than cisplatin and doxorubicin. **SAL** and its derivative **6** have high SI from 5.45 to 26.08, indicating that these compounds will rather selectively kill cancer cells than normal ones (Table 2).

The antimicrobial activity of **SAL** and its amides (**1**–**11**) was studied in vitro against the typical Gram-positive cocci, Gram-negative

Table 1
Antiproliferative activity of salinomycin (**SAL**) and its amides (**1**–**11**). Data are given as IC_{50} [μM]

Compound	Cancer cells				Normal cells
	HL-60	HL-60/vinc	LoVo	LoVo/DX	BALB 3T3
SAL	1.2 ± 0.5	4.7 ± 1.7	1.5 ± 0.4	1.0 ± 0.1	25.8 ± 9.0
1	5.7 ± 2.2	11.3 ± 1.3	6.9 ± 1.0	3.9 ± 1.0	37.1 ± 6.4
2	3.1 ± 0.6	34.0 ± 7.4	26.5 ± 6.2	28.5 ± 5.0	17.1 ± 1.0
3	17.8 ± 2.1	30.9 ± 5.4	36.7 ± 2.4	14.3 ± 5.2	39.4 ± 2.1
4	3.7 ± 0.8	23.1 ± 6.2	21.9 ± 3.5	12.6 ± 5.8	35.1 ± 4.7
5	23.2 ± 3.7	27.0 ± 4.7	24.1 ± 2.2	8.5 ± 4.8	31.3 ± 5.2
6	2.3 ± 0.2	6.7 ± 1.2	4.1 ± 0.1	2.3 ± 0.5	45.8 ± 20.9
7	4.4 ± 0.8	33.1 ± 4.6	32.5 ± 5.3	34.8 ± 3.8	34.5 ± 6.6
8	2.8 ± 1.1	6.9 ± 1.1	3.9 ± 0.2	4.3 ± 0.7	5.7 ± 2.2
9	2.7 ± 0.1	13.5 ± 2.1	10.7 ± 3.0	3.9 ± 1.3	19.8 ± 2.9
10	2.0 ± 0.7	16.1 ± 5.1	8.1 ± 2.6	2.8 ± 0.6	20.2 ± 1.9
11	4.4 ± 1.4	NA	8.4 ± 2.8	3.1 ± 0.8	NA
Doxorubicin	0.04 ± 0.02	1.8 ± 0.3	0.17 ± 0.06	6.5 ± 1.8	0.5 ± 0.3
Cisplatin	4.00 ± 3.3	10.6 ± 2.2	5.2 ± 1.1	8.9 ± 0.8	9.2 ± 2.3

The IC_{50} value is defined as the concentration of a compound that corresponds to a 50% growth inhibition. Human promyelocytic leukemia (HL-60) and its vincristine-resistant subline (HL-60/vinc); human colon adenocarcinoma cell line (LoVo) and doxorubicin resistant subline (LoVo/DX); normal murine embryonic fibroblast cell line (BALB 3T3). Data are expressed as the mean ± SD; NA–not active in concentrations used (up to 100 μg/ml).

rods and yeast-like organisms (Table 3), and against a series of clinical isolates of methicillin-resistant *Staphylococcus epidermidis* (MRSE) according to the procedure published by us previously.[12] Hospital strains of methicillin-resistant *Staphylococcus* were isolated from different biological materials of patients of the Warsaw Medical University Hospital. MRSE is a species of antibiotic-resistant bacterium commonly found both on the skin and in the noses of healthy people.

The antimicrobial properties of salinomycin and its derivatives against typical bacteria strains are expressed by the minimum inhibitory concentration (MIC) (Table 3). Antibacterial activity evaluation largely depends on various substituents in the amide group of salinomycin derivatives. It is worth noticing that the compounds which expressed the higher anticancer activity, were not the ones showing the strongest activity against bacteria. Attempts were made to correlate antibacterial activity of these compounds with changing substituents at amide moiety.

Among the compounds tested, only **SAL** and four amide derivatives (**2**–**4** and **11**) showed activity against Gram-positive bacteria. Compounds (**1**, **5**–**10**) were practically inactive towards all microorganisms tested (MIC $\geqslant$256 μg/ml). The amides containing alkyl chains with sulfur, oxygen or nitrogen atoms (**2**–**4**) or dimer of **SAL** with the 4,4′-diaminobiphenyl linker (**11**) show a considerably stronger activity against Gram-positive bacteria than the other salinomycin amides. Salinomycin and its amides are inactive against the strains of *Candida* and Gram-negative bacteria.

Amides **2**–**4** and **11** exhibit good potency in inhibiting the growth of MRSE, with MICs in the range of 16–64 μg/ml, which were slightly less active than unmodified **SAL** (MIC = 8–16 μg/ml).

To summarize, a simple and efficient one-pot methodology for the synthesis of eleven new amides of salinomycin (**1**–**11**) is described. The simplicity of this method, high yields, easy work-up and purification of the products by flash chromatography and crystallization are its key advantages. The molecular structure of one amide (**6**) has been characterised by X-ray method proving the pseudo-cyclic structure of this compound. The ionophoretic properties of obtained compounds were studied by ESI MS showing that amides of **SAL** are able to form complexes with monovalent and divalent cations, while unmodified **SAL** is able to form complexes only with monovalent cations. The activity tests of compounds **1**–**11** clearly show that some of them, especially the amides with dopamine (**8**) and 4-fluorobenzyl substituent (**6**), indicate relatively high antiproliferative effect (e.g., against multidrug-resistant and their parental cancer cell lines). These compounds are less toxic for normal murine fibroblasts cells than the currently used anticancer drugs such as cisplatin and doxorubicin. We have provided evidence that the four new amides containing saturated alkyl chain with additional heteroatom such as sulfur (**2**), oxygen (**3**) and nitrogen (**4**) or **SAL** dimer linked by 4,4′-diaminobiphenyl spacer (**11**) show antibacterial activity against human pathogenic bacteria, including drug resistant strains of *Staphylococcus epidermidis*. It is interesting to note that the most antiproliferative active **SAL** derivatives are not the ones showing the strongest antibacterial activity and vice versa.

These results indicate that the antiproliferative and antibacterial effects of salinomycin amides strictly depend on chemical nature of amide substituent (**1**–**11**). The **SAL** derivatives displayed antiproliferative activity in various cell types in the low micromolar range, providing an excellent starting point for further drug discovery optimisation. These results are important for the development of molecules with dual potential anticancer and antibacterial activity.

Table 3
Antibacterial activity of salinomycin (**SAL**) and its amides (**2**–**4**, **11**) against standard bacteria and methycyllin-resistant hospital strains of *Staphylococcus epidermidis* (MRSE). Data are given as MIC [μg/ml]

Reference strains	SAL	2	3	4	11	Ciprofloxacin
Standard bacteria						
S. aureus NCTC 4163	2	16	32	32	16	0.25
S. aureus ATCC 25923	2	16	32	32	32	0.50
S. aureus ATCC 6538	2	16	32	32	32	0.25
S. aureus ATCC 29213	4	32	64	64	32	0.50
S. epidermidis ATCC 12228	2	32	64	64	32	0.25
S. epidermidis ATCC 35984	2	32	64	32	32	0.125
Methycyllin-resistant Staphylococcus epidermidis						
459/11	16	32	32	32	32	16
460/11	16	32	64	64	64	0.125
461/11	16	32	64	64	32	0.25
466/11	8	32	64	64	32	2
467/11	16	32	32	32	16	16
468/11	8	64	64	32	32	16
469/11	16	64	64	64	64	8
470/11	16	64	64	64	64	0.125
488/11	16	32	64	64	32	16
489/11	16	32	64	32	32	0.25

Amides **1** and **5–10** were practically inactive towards all tested microorganisms (GIZ 10–12 [mm] and MIC $\geqslant$256 [μg/ml]). Ciprofloxacin (control compound) is a synthetic antibiotic of the fluoroquinolone drug class. Salinomycin amides were inactive against tested strains of *Candida* and Gram-negative rods.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.02.042. These data include MOL files and InChiKeys of the most important compounds described in this article.

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