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Synthesis, cytotoxicity and antibacterial activity of new esters of polyether antibiotic – salinomycin

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ABSTRACT

A series of 12 novel ester derivatives of naturally occurring polyether antibiotic – salinomycin were synthesized, characterised by spectroscopic method and evaluated for their *in vitro* antibacterial activity and cytotoxicity. The new esters were demonstrated to form complexes with monovalent and divalent metal cation of 1:1 stoichiometry in contrast to the salinomycin which forms only complexes with monovalent cations. All the obtained compounds show potent antiproliferative activity against human cancer cell lines and a good selectivity index for cancer versus mammalian cells. Additionally, 3 compounds showed higher antiproliferative activity against the drug-resistant cancer cells and lower toxicity towards normal cells than those of unmodified salinomycin and standard anticancer drugs such as cisplatin and doxorubicin. Some of the synthesized compounds showed good inhibitory activity against *Staphylococcus* strains and clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). These studies show that salinomycin esters are interesting scaffolds for the development of novel anticancer and Gram-positive antibacterial agents.

1. Introduction

Salinomycin (**1**) (Scheme 1), isolated from *Streptomyces albus*, is an antibiotic belonging to a large group of natural polyether ionophores [1]. Salinomycin and its salts, due to the presence of carboxyl group on one side of the molecule and two hydroxyl groups on the other side, are able to form "head to tail" type of intramolecular hydrogen bonds resulting in formation of a pseudo-cyclic structure. The polyether skeleton of this pseudo-cyclic structure is able to form complexes with metal cations and transport them across lipid cell membranes [2].

The mechanism of salinomycin activity is based on the transport of metal cations (especially Na^+ and K^+) from the extracellular environment through the biological membranes into a cell, where they are exchanged for protons. This leads to disturbance of intracellular pH and to increasing osmotic pressure inside the cell, leading finally to apoptosis [1]. For this reason, salinomycin shows antimicrobial activity against Gram-positive bacteria, including *Staphylococcus aureus* and mycobacteria, *Plasmodium falciparum* or *Eimeria* spp, parasites, and protozoa, which are responsible for coccidiosis in poultry [3]. Salinomycin sodium salt is commercially used in the veterinary medicine as a coccidiostatic and non-hormonal growth-promoting agent.

In addition to the well recognised antibacterial activity of **1**, in 2009 this compound was shown to be nearly 100-fold more effective against breast cancer stem cells than commonly used cytostatic drug – Taxol (Paclitaxel) [4].

Recent studies have proved that **1** is able to induce mass programmed death of human tumour cells of various tissues showing multidrug resistance (MDR), for example leukemic stem cells, by the expression of ATP-binding cassette (ABC) transporters [5]. Additionally, **1** inhibits Wnt signalling pathway and induces apoptosis of tumour cells in patients with chronic lymphocytic leukemia [6]. The ability of **1** to reduce the subpopulations of breast cancer stem cells, as well as colon carcinoma stem cells, has been also described [7]. Moreover, **1** blocks the growth and migration of chemoresistant prostate cancer cells [8]. *In vitro* tests have

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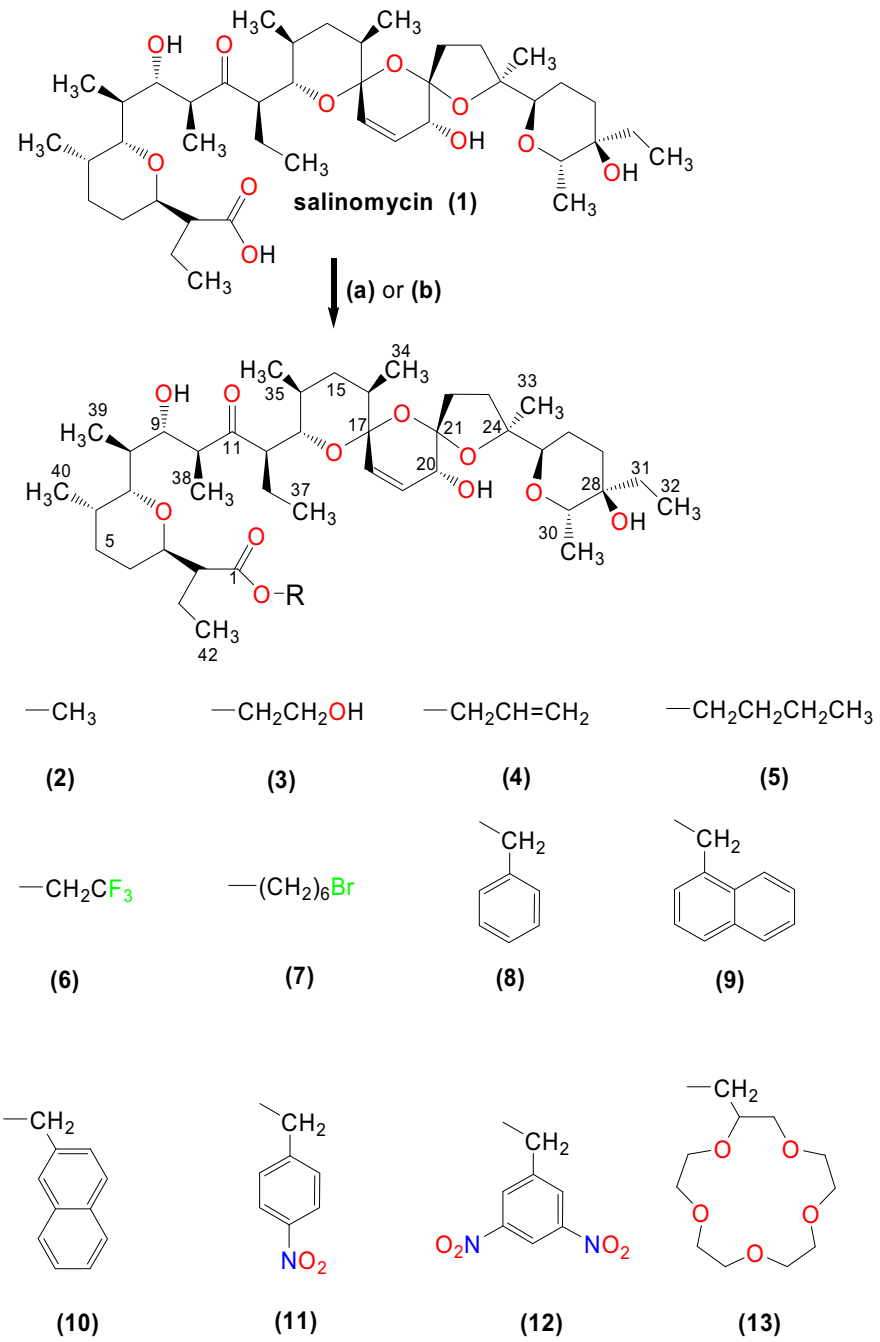

Scheme 1. Synthetic access to salinomycin ester derivatives. *Reagents and conditions*: (a) **1** (1 eq), R–OH (7.5 eq), DCC (1.5 eq), PPy (0.5 eq), *p*-TSA (0.23 eq), CH_2Cl_2, 0 °C to rt, 24 h; (b) **1** (1 eq), R-*X* (bromides or chlorides) (2.2 eq), DBU (1.75 eq), toluene, 90–100 °C, 5 h.

confirmed the strong antitumor activity of this compound against the lung cancer cell lines [9]. Furthermore, sensitizing effects of salinomycin during irradiation and treatment with cytostatic agents suggest that the sensitizing mechanism of action of the antibiotic is preserved both in the application of radiotherapy and chemotherapy [10,11]. Additionally, it has been showed that the sodium salt of **1** is able to selectively deplete breast cancer stem cells with efficiency comparable to that of **1** [12].

Chemical modifications of **1** yielded its various derivatives characterised by significantly lower toxicity and better biological activity than the unmodified antibiotic, and this discovery has opened an interesting direction of research. Until now the synthesis, structure and biological activity of the series of amides [13,16], *O*-acyl derivatives [15], as well as one ester of salinomycin with 1-hydroxybenzotriazole [14,16] have been described. Among the tested compounds, **1** and its several amide derivatives showed high activity against Gram-positive bacteria [13]. The antiproliferative activity tests of **1** and its amide derivatives have clearly shown that some of the amides possess high antiproliferative effect against normal and drug resistant cancer cells, and these compounds were less toxic against normal cells than commonly used cytostatic agents – cisplatin and doxorubicin [16].

Furthermore, the antiproliferative activity tests of *O*-acyl salinomycin derivatives have shown that some of these compounds can be used to improve IC_{50} values up to one fifth in comparison to salinomycin against breast cancer cells [15]. Up to now the most interesting results of biological activity have been obtained for only one ester of salinomycin with 1-hydroxybenzotriazole. This compound exhibited relatively high activity against strains of hospital isolates methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) [14]. In addition, it showed high antiproliferative activity against different human cancer cells with relatively low toxicity [16].

It is postulated that derivatives of polyether antibiotics with modified carboxylic groups (amides, esters) can transport cations *via* an electrogenic or biomimetic mechanism while unmodified antibiotics transport cations always by an electroneutral mechanism, by virtue of their negatively charged carboxylate groups [2,17]. Therefore, the modification of carboxylic group of salinomycin can change ionophoretic properties of its derivatives and the mechanism of cation transport through the cell lipid membrane.

The main purpose of our research was to design and synthesise derivatives of salinomycin showing considerable activity against Gram-positive bacteria and cytotoxic activity against human cancer cell lines. In this paper we present 12 completely new salinomycin esters synthesized using two different methodologies. The structures of new salinomycin esters were evaluated using X-ray and spectroscopic methods. The antimicrobial activity of salinomycin derivatives, especially against a series of clinical isolates of methicillin-resistant *Staphylococcus*, as well as antiproliferative activity against the drug-resistant human cancer cell lines were studied and are discussed here.

2. Results and discussion

2.1. Chemistry

For better structure–activity relationship analysis (SAR) twelve new salinomycin esters (**2**–**13**) were synthesised such as: saturated alkyl chain (methyl **2**, butyl **5**), alkyl chain containing OH group (2-hydroxyethyl **3**), unsaturated alkyl chain (allyl **4**), alkyl chain containing halogen atoms (2,2,2-trifluoroethyl **6** and 6-bromohexyl **7**) aromatic ring (benzyl **8**, α-naphthylmethyl **9**, β-naphthylmethyl **10**), polar benzyl group (*p*-nitrobenzyl **11**, di-*o*-nitrobenzyl **12**), crown ether (methyl-15-crown-5 **13**).

Salinomycin sodium salt (SAL-Na) was isolated from veterinary premix – SACOX®, following the procedures described previously [13]. The structure and homogeneity of isolated salinomycin sodium salt was confirmed using spectroscopic methods [13]. Salinomycin acid (**1**) was obtained from salinomycin sodium salt by the extraction with sulphuric acid solution (pH = 1.5) in CH_2Cl_2 [13].

Most procedures of esterification require rather harsh conditions such as the presence of strong acids, bases or other catalysts and proceed at high temperatures. Salinomycin is very sensitive to acidic conditions and heating [18]. Therefore, mild reaction conditions for the salinomycin esterification were chosen. Initially, the reaction between salinomycin, respective alcohol and DCC (*N,N′*-dicyclohexylcarbodiimide) as a coupling agent was tested. Unfortunately, this procedure was characterized by low to moderate chemical yields *e.g.* allyl ester (**4**) was obtained only with 12% yield. Thus, modification of the synthetic method was necessary. We developed a valuable method of synthesis of salinomycin esters based on the reaction between **1** and the appropriate alcohol in the presence of DCC, PPy (4-pyrrolidinopyridine) and *p*-TSA (*p*-toluenesulfonic acid monohydrate). This method was quite efficient and gave the esters (**2**–**3**, **6**, and **13**) in relatively high yields (40–70%) (Method A, Table 1). However, under the same reaction conditions, the salinomycin benzyl ester (**8**) was obtained only with a yield of 25%.

Much more reliable strategy was the esterification of (**1**) based on direct alkylation of the carboxylate ion. In this method, the appropriate alkyl halide (chlorides or bromides) with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were used as effective nucleophilic catalysts (Method B, Table 1). Under this reaction conditions the yield increased, for example compound **8** was obtained with 85% yield. This method was used also for preparation of

Table 1
The yields of the synthesis and characteristic NMR signals of new salinomycin esters.

Compound	Yield (%)		Analytical NMR signals (ppm) w CD_3CN		
			^{13}C NMR	^{1}H NMR	
	Method A	Method B	C(1) = O	$C(43)H_2$	$C(43)H_2$
1	–	–	177.8	–	–
2	59.4	–	176.9	52.8	3.78 (s) (OCH_3)
3	40.7	–	176.5	67.1	4.27 (ddd, *J* = 10.02, 5.25, 4.32 Hz, 1H) 4.27 (td, *J* = 9.96, 4.71 Hz, 1H)
4	42.1	79.3	175.9	66.1	4.84 (tdd, *J* = 13.4, 5.9, 1.4 Hz, 1H), 4.70 (tdd, *J* = 13.4, 5.8, 1.4 Hz, 1H)
5	–	67.0	176.3	65.3	4.33 (td, *J* = 10.9, 6.7 Hz, 1H), 4.13 (td, *J* = 10.9, 6.5 Hz, 1H)
6	71.4	–	184.9	76.8	4.00 m
7		49.0	176.4	65.5	4.34 (td, *J* = 10.9, 6.8 Hz, 1H), 4.11 (td, *J* = 10.9, 6.4 Hz, 1H)
8	25.4	85.3	176.0	67.2	5.46 (d, *J* = 12.5 Hz, 1H), 5.18 (d, *J* = 12.5 Hz, 1H)
9	–	75.0	175.6	65.4	5.90 (d, *J* = 13.0 Hz, 1H), 5.75 (d, *J* = 12.9 Hz, 1H)
10	–	28.6	176.0	67.3	5.64 (d, *J* = 12.6 Hz, 1H), 5.35 (d, *J* = 12.6 Hz, 1H)
11	–	86.7	175.2	65.5	5.64 (d, *J* = 13.8 Hz, 1H), 5.35 (d, *J* = 13.8 Hz, 1H)
12	–	47.9	175.5	65.0	5.82 (dd, *J* = 13.7, 0.5 Hz, 1H), 5.38 (dd, *J* = 13.8, 0.4 Hz, 1H)
13	49.1	–	176.2	≈68.2	≈3.96

Method A: synthesis from respective alcohols and with addition of DCC, PPy i TsOH.
Method B: synthesis from respective alkyl halides with addition of DBU (see experimental section).

esters when the alcohol needed for Method A was expensive or difficult to access, whereas the corresponding alkyl halides were commercially available, like in the synthesis of compounds **4**–**5** and **7**–**12**. It should be noted that the use of alkyl chlorides instead of alkyl bromides decreases the yields of respective esters. The esterification methods show also a remarkable solvent dependence. Dichloromethane was the best of all solvents tested for Method A and toluene for Method B, because all reactants and products were soluble in these solvents.

All esters can be easily isolated in pure form following the purification by Dry Column Vacuum Chromatography. This method was efficient and gave the esters in high yields of up to 28–86% (Table 1). The purity and structures of compounds **1**–**13** 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 Supplementary Material.

In the ^{13}C NMR (all in CD_3CN) spectra of salinomycin esters, the most characteristic signal of C(1) atom of the ester group was observed in a narrow range 175.5–179.6 ppm, while the signal of C(1) atom assigned to the carboxylic group in salinomycin acid (**1**) was at 177.8 ppm. Only for trifluoroethyl ester (**6**) the signal of C(1) atom of the ester group was at 184.9 ppm due to high electron-withdrawing inductive effect in the ester substituent. The characteristic ^{13}C signal of the $-OCH_2-$ group in the ester substituent was observed in the range 65.3–68.2 ppm with two exceptions: methyl ester **2** (52.8 ppm) and trifluoroethyl ester **6** (76.8 ppm).

In the ^{1}H NMR spectra of any salinomycin ester, the characteristic signals of the $-OCH_2-$ protons from the ester substituent were observed in the range 3.78–5.90 ppm (Table 1).

Structural data of the salinomycin derivatives are very important to understand their anticancer and antimicrobial properties, as well as to make the structure–activity relationship analysis (SAR) and related investigation. We have therefore characterized compound **3** by single crystal X-ray diffraction method. The single crystals of **3** were grown by crystallisation in acetonitrile solution and the X-ray crystallographic analysis confirmed the structural assignment (Fig. 1). The crystallographic data and structure refinement of compound **3** are summarized in Table 2. The bond lengths and angles characterizing the geometry of the molecule are presented as supplementary material (Table S1). The absolute configuration of salinomycin ester **3** is *2R*, *3R*, *6S*, *7R*, *8S*, *9S*, *12R*, *13S*, *14S*, *16R*, *17R*, *20R*, *21S*, *24S*, *25R*, *28R*, *29S* and is analogous to that found for salinomycin and its other derivatives [13,14,16,19–22]. The salinomycin skeleton of **3** contains four six-membered rings and one five-membered ring. Two six-membered and one five membered rings form tricyclic spiroketal rings system, in which the central ring is unsaturated and six-membered. The arrangement of the atoms around the spiro C17 and C21 heads are tetrahedral, so that the junctions between the five- and six-membered and between both six-membered rings are of the spiro type. The C18–C19 bond of the length 1.315(6) Å indicates a double bond character. The unsaturated six-membered ring with one double bond (C18 = C19) has envelope conformation where C21 is 0.644(5) Å out of the plane formed by the other atoms of the ring. Both saturated six-membered rings have chair conformation and the five-membered ring is twisted, the C21 is 0.226(5) Å out of the plane of the other atoms. The conformation of the rings as well as the C–C and C–O bond lengths in salinomycin derivative 3 are similar to that found in free salinomycin and other structurally characterised salinomycin derivatives [13,14,16,19–22]. However, the conformation of the whole skeleton of molecule **3** is different than in the other derivatives [13,14,16,19–22]. The conformation of the whole molecule of **3** is not pseudo-cyclic, but more linear with both ends of the molecule folded (Fig. 1) for which the intramolecular O–H···O hydrogen bonds are responsible. The hydroxyl group at the C9 atom is a donor of hydrogen bond with O1 atom (acceptor). In addition, the proximity of the hydroxyl group at C20 to both etheric O9 and O10 atoms is responsible for the formation of a bifurcated hydrogen bond (O8–H···O9 and O8–H···O10). In contrast to the pseudo-cyclic conformation of salinomycin skeleton, the exterior surface of a more linear conformation of salinomycin skeleton of **3** shows both hydrophilic and hydrophobic regions, and leads to the interaction between the molecules of **3** in the crystal with formation of intermolecular O11–H···O12^{i} and O12–H···O8^{ii} hydrogen bonds (Fig. 2).

The FT-IR spectra of salinomycin 2-hydroxyethyl ester (**3**) in acetonitrile (dashed line) and in the crystal (solid line) are compared and clear differences between these structures are revealed, especially in the formation of hydrogen bonds.

The most characteristic, in the FT-IR spectrum of crystalline **3** (Fig. 3b), are the bands assigned to the ν(OH) vibrations of the four OH groups. According to the hydrogen bonds parameters collected in Table 3 the band at 3537 cm^{-1} and 3456 cm^{-1} should be assigned to stretching vibrations O(8)H and O(11)H, respectively, because these groups are engaged in weak hydrogen bonds.

The broad bands with maxima at 3374 cm^{-1} and 3331 cm^{-1} are assigned to stretching vibrations of the O(12)H and O(4)H groups, respectively, because they participate in relatively stronger

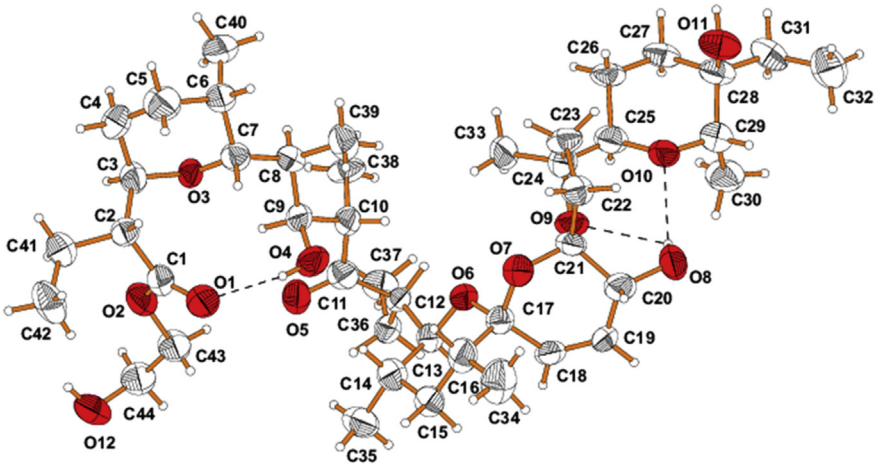

Fig. 1. View of the molecular structure of **3** with the atoms labelling. Dashed lines represent the hydrogen bonds.

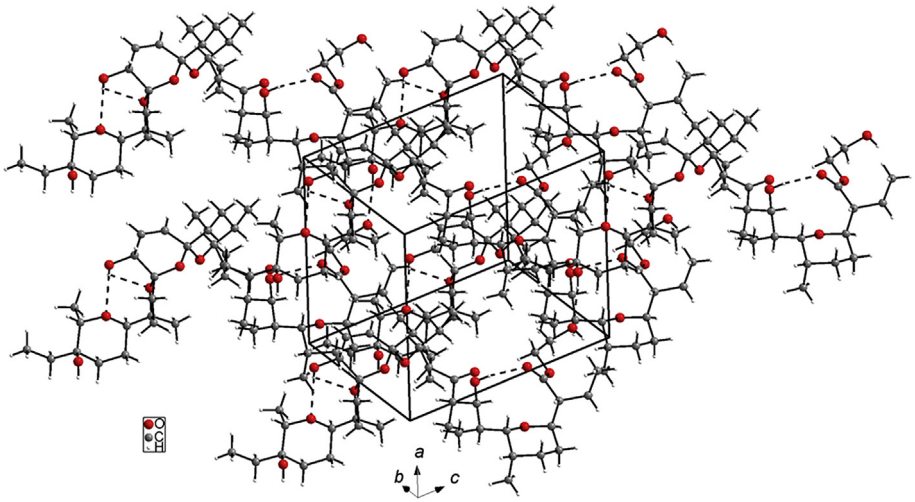

Fig. 2. Arrangement of **3** in the unit cell. Dashed lines represent the intra- and intermoleuclar O–H···O hydrogen bonds.

hydrogen bonds than the first ones. In the spectrum of **3** in acetonitrile (Fig. 3b, dashed line) the bands at 3331 cm^{-1} and 3457 cm^{-1} vanish, and additionally the intensity of the band at 3525 cm^{-1} increases indicating that in the acetonitrile solution the O11–H···O12^{i} and O12–H···O8^{ii} intermolecular hydrogen bonds are broken (Table 3).

In the FT-IR spectrum of crystalline **3** (Fig. 3c) a broad band arises with a maximum at about 1710 cm^{-1} due to the overlapping of ν(C=O) vibrations of both ketone and ester groups. In the spectrum of **3** in solution, instead of one band at about 1710 cm^{-1} two bands at 1713 cm^{-1} and 1728 cm^{-1} are observed. The position

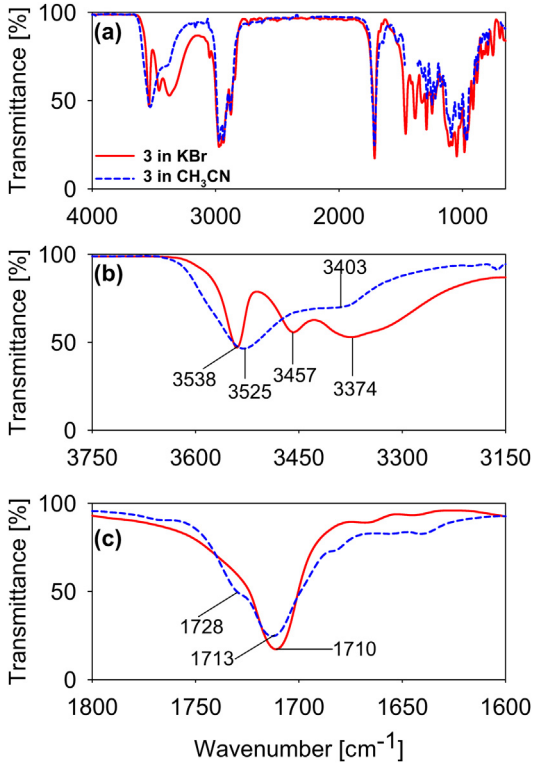

Fig. 3. FT-IR spectra of **3** crystals: (–) in KBr, (- - -) in acetonitrile solution: (a) 4000–650 cm^{-1}; (b) 3750–3150 cm^{-1}; (c) 1800–1600 cm^{-1}.

of the second band is typical of ν(C=O) vibrations of non hydrogen-bonded ester group, demonstrating that the intramolecular O(4)–H···O(1) = C(1) bond existing it the solid state is broken in the acetonitrile solution. This fact is also confirmed by the shift of the band assigned to the ν(OH) vibrations of the O(4)–H group 3331 cm^{-1} observed in the FT-IR spectrum of crystalline **3** to higher frequency range in the spectrum of **3** in solution.

Polyether antibiotics exhibit interesting biological effects because they are able to form lipophilic complexes with metal cations and transport them through cell membranes disturbing their natural Na^+/K^+ ion balance. The formation of such complexes with monovalent (Li^+, Na^+, K^+, Rb^+, Cs^+) and divalent (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}) cations was studied by Electrospray Ionization Mass Spectrometry (ESI MS) method. The ESI MS spectra of the respective mixtures of the salinomycin esters with mono- and di-valent metal perchlorates (Figs. S8–S11) demonstrate that the esters (**2**–**13**) form exclusively 1:1 complexes with both types of metal cations. Only the ester (**13**) with the crown moiety is able to form different complexes with divalent cations (*M*) *i.e.* the (**13** + $M)^{2+}$ and (13 + $MClO_4$). The last type of complexes has been previously observed for diffident monensin derivatives [23,24].

It is important to note that unmodified salinomycin (**1**) is a typical monovalent ionophore, because it is able to form complexes only with monovalent cations, especially with Na^+ and K^+. The ESI MS studies performed in this study demonstrate clearly that salinomycin ester derivatives form complexes with both monovalent and divalent metal cations and derivative **13** can also form complexes of different composition.

3. Biological activity

3.1. In vitro determination of drug-induced inhibition of human cancer cell line growth

The newly synthesized esters of salinomycin (**2**–**13**) were evaluated for their *in vitro* antiproliferative effect on normal and cancer cells. 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), and 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

Table 2
Crystal data and structure refinement details of compound **3**.

$C_{44}H_{74}O_{12}$	$V = 1138.0\ (2)\ Å^3$
$Mr = 795.03$	$Z = 1$
Triclinic, $P1$	Mo$K\alpha$ radiation, $\lambda = 0.71073$ Å
$a = 9.5948\ (9)$ Å	$\mu = 0.08\ mm^{-1}$
$b = 10.9190\ (9)$ Å	$T = 295$ K
$c = 12.5397\ (12)$ Å	$0.33 \times 0.22 \times 0.21$ mm
$\alpha = 84.298\ (7)°$	$R = 0.0651\ (>2\sigma)$
$\beta = 70.837\ (9)°$	$wR = 0.1295$ (all refls)
$\gamma = 66.581\ (9)°$	$S = 0.998$

Table 3
Hydrogen-bond geometry (Å, °) in the crystal structure of **3**.

D—H···*A*	*D*—H	H···*A*	*D*···*A*	*D*—H···*A*
O4—H···O1	0.82	2.04	2.853 (5)	172
O8—H···O10	0.82	2.19	2.857 (5)	138
O8—H···O9	0.82	2.29	2.701 (5)	111
O11—H···O12^{i}	0.82	2.08	2.896 (6)	171
O12—H···O8^{ii}	0.82	2.00	2.787 (6)	161

Symmetry codes: (i) $x - 1, y + 1, z - 1$; (ii) $x, y - 1, z + 1$.

activity of the compounds studied. The mean $IC_{50} \pm SD$ of the tested compounds are collected in Table 4. To evaluate agents activity against the cells with MDR (multidrug resistance) phenotype two drug resistant cancer cell lines *i.e.* HL-60/vinc and LoVo/dx were tested and the indexes of resistance (IR) were calculated (Table 4). The IR value indicates how many times more resistant is the subline in comparison to its parental cell line.

This study shows that the majority of the synthesized compounds exerted antitumor activities against the four human cancer cell lines to various extents and low toxicity to normal murine embryonic fibroblast cell line.

The results obtained indicate that salinomycin (**1**) and its derivatives **5**, **9** and especially **6** exhibited the highest ability to inhibit the proliferation of different cancer cell lines (Table 4) relative to that of the other obtained salinomycin derivatives. The results imply the following conclusions. Firstly, a different ester substituent attached to the salinomycin might contribute to the activity of the compound synthesized. Secondly, the presence of three trifluoroethyl groups is preferable over the presence of aliphatic or aromatic groups (compound **6** has superior anticancer activity against different cancer cell lines over all tested compounds including unmodified salinomycin as well as cisplatin). The data given in Table 4 show that all compounds tested were active against cell lines expressing drug-resistant phenotype (IR below 10).

Almost all the compounds studied showed moderate to high cytotoxic activity against LoVo/dx cancer line, which was better than the activity of anticancer drugs such as doxorubicin and cisplatin. Compounds **1** and **6** showed maximum inhibitory activity against LoVo/dx cell line with IC_{50} 0.99 and 0.80 μM, respectively. Some tested compounds were found to exert low to moderate inhibitory potential against HL-60/vinc cell line; for instance compounds **7**, **10**–**12** showed relatively low inhibitory activity.

Many chemotherapeutics are toxic against malignant cells as well as normal ones. Therefore, a very serious problem is the degree of toxicity of drugs depending on the doses required to achieve response in clinical trials. 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 respective cancerous cell line. The bioactivity of each compound was evaluated on the basis of a combination of its IC_{50} value and corresponding SI. Higher values of SI indicate greater anticancer specificity and SI greater than 3 was considered as highly selective. In our experiments salinomycin and its ester derivatives appeared to be less toxic against normal embryonic fibroblasts than cisplatin and doxorubicin. It is worth noting that SI for some tested compounds *i.e.* **1**, **6**, **9**, **11** is very high from 8 to 50, indicating that these compounds will rather selectively kill cancer cells than normal cells (Table 5).

Extremely high anticancer activity was revealed by compound **6**. It exhibits high cytotoxic effect towards cancer cells ($IC_{50} < 1$ μM) and low toxicity ($IC_{50} = 23.8$ μM obtained for BALB/3T3 cell line). Furthermore, this compound was very selective towards almost all types of cancer cell lines HL-60, LoVo and LoVo/dx (SI ranging from 30 to 50) and exhibited more than moderate selectivity towards HL-60/vinc cancer cells with selectivity ratio 7.81.

Table 4
Antiproliferative activity of salinomycin (**1**) and its esters (**2**–**13**) compared with antiproliferative activity of standard anticancer drugs doxorubicin and cisplatin.

Compound	HL-60	HL-60/vinc		LoVo	LoVo/dx		BALB 3T3
	IC_{50} (μM)	IC_{50} (μM)	*IR*	IC_{50} (μM)	IC_{50} (μM)	*IR*	IC_{50} (μM)
1	1.17 ± 0.44	4.74 ± 1.73	*4.05*	1.53 ± 0.41	0.99 ± 0.012	*0.65*	25.82 ± 8.96
2	5.50 ± 1.75	13.91 ± 5.62	*2.53*	7.10 ± 3.92	4.35 ± 0.31	*0.61*	25.42 ± 3.76
3	36.45 ± 3.53	35.13 ± 2.91	*0.96*	17.17 ± 9.51	10.01 ± 6.29	*0.58*	41.52 ± 3.72
4	34.22 ± 4.15	18.05 ± 8.37	*0.53*	15.80 ± 11.49	10.08 ± 8.89	*0.64*	38.41 ± 10.11
5	3.58 ± 0.45	4.15 ± 1.50	*1.16*	4.04 ± 0.09	3.99 ± 0.06	*0.99*	24.32 ± 7.27
6	0.47 ± 0.22	3.05 ± 0.38	*6.49*	0.78 ± 0.24	0.80 ± 0.07	*1.03*	23.82 ± 6.49
7	4.00 ± 2.00	uv[a]	–	4.99 ± 1.29	20.86 ± 4.37	*4.18*	14.69 ± 7.74
8	4.78 ± 2.52	26.73 ± 4.49	*5.59*	6.05 ± 3.72	6.48 ± 3.70	*1.07*	28.42 ± 3.04
9	3.73 ± 0.21	9.33 ± 1.47	*2.50*	7.34 ± 0.35	4.70 ± 0.28	*0.64*	35.80 ± 1.66
10	13.39 ± 8.05	uv[a]	–	45.79 ± 10.09	uv[a]	–	88.25 ± 0.43
11	8.83 ± 4.09	uv[a]	–	7.65 ± 0.07	3.66 ± 0.18	*0.48*	73.83 ± 17.31
12	11.86 ± 4.43	uv[a]	–	7.96 ± 1.33	7.97 ± 4.62	*1.00*	uv[a]
13	19.16 ± 6.80	15.56 ± 4.04	*0.81*	8.07 ± 2.21	5.40 ± 1.39	*0.67*	39.66 ± 6.49
Doxorubicin	0.04 ± 0.02	1.78 ± 0.33	*44.50*	0.17 ± 0.06	6.45 ± 1.82	*37.94*	0.48 ± 0.33
Cisplatin	3.95 ± 3.22	10.53 ± 2.12	*2.67*	5.18 ± 1.06	8.84 ± 0.76	*1.71*	9.47 ± 0.29

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.
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 the cell show moderate sensitivity to a drug and IR above 10 indicates strong drug-resistance.
[a] uv – unavailable in range of concentrations used, IC_{50} could not be calculated due to weak antiproliferative activity of compound across the tested concentrations.

Table 5
The calculated selectivity index (SI) which represents IC_{50} for normal cell line/IC_{50} for cancerous cell line.

Compound	Calculated selectivity index SI			
	HL-60	HL-60/vinc	LoVo	LoVo/*dx*
1	22.07	5.45	16.88	26.88
2	4.62	1.83	3.58	5.84
3	1.14	1.18	2.42	4.15
4	1.12	2.13	2.43	3.81
5	6.79	5.86	6.02	6.10
6	50.68	7.81	30.53	29.78
7	3.67	–	2.94	0.70
8	5.95	1.06	4.70	4.39
9	9.60	3.84	4.88	7.62
10	6.59	–	1.93	–
11	8.36	–	9.65	20.17
12	–	–	–	–
13	2.07	2.55	4.91	7.34
Doxorubicin	12.00	0.27	2.82	0.07
Cisplatin	2.32	0.87	1.77	1.04

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 tumor cells greater than toxicity against normal cells.

3.2. Antimicrobial screening

Antimicrobial activities of the newly synthesized compounds were tested *in vitro* against typical Gram-positive cocci, Gram-negative rods and yeast-like organisms, as well as against a series of clinical isolates of *Staphylococcus*: methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE). Further evaluation was then carried out on compounds showing reasonable growth inhibition zones (Giz) to determine their minimal inhibitory concentration (MIC) values using the twofold serial dilution method.

Hospital strains of MRSA and MRSE were isolated from different biological materials of patients of the Warsaw Medical University Hospital. The other micro-organisms used here were provided by the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

The data concerning the antimicrobial activity of the compounds are summarized in Tables 6 and 7.

Among the compounds tested, only salinomycin (**1**) and three ester derivatives (**4**, **6** and **12**) showed high activity against Gram-positive bacteria (MIC = 1–64 μg/cm^3) (Table 6). The other compounds exhibited very low activity against Gram-positive bacteria (MIC ≥ 256 μg/cm^3). Only one of the derivatives (**6**) showed antifungal activity (*Candida albicans* ATCC 10231 Giz = 17 mm; *C. albicans* ATCC 90028 Giz = 18 mm; *Candida parapsilosis* ATCC 22019 Giz = 20 mm; MIC values were not determined). Unmodified salinomycin was inactive against the tested strains of *Candida*. Moreover, as expected, the cell walls of Gram-negative bacteria do not permit the penetration of hydrophobic molecules of high weights and thus the micro-organisms are not susceptible to the action of salinomycin (**1**). The same applies to its ester derivatives (**2**–**13**). Promising results of microbiological tests performed on drug-susceptible strain of reference Gram-positive bacteria (Table 6) made us check, whether **1** and its three ester derivatives (**4**, **6** and **12**) are also effective against the methicillin-resistant *Staphylococci* strain (MRSA and MRSE). This type of staphylococcal strain is the cause of many life threatening infections, because these bacteria are very troublesome pathogens in hospitals and other healthcare settings. They represent a group of invasive, biofilm-forming microorganisms responsible for many nosocomial infections. Methicillin-resistant strains of *Staphylococcus* are resistant to all β-lactams and they are often resistant to some other widely used antibiotics and belong to the so-called multi-drug resistant strains. One of the few drugs effective in combating infections caused by MRSA is ciprofloxacin (CPX). Therefore, CPX is used by us as a reference drug (Table 7).

Methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) strains are the most important pathogens responsible for a range of illnesses, from minor skin infections to life-threatening diseases, such meningitis, pneumonia and sepsis. *Staphylococcus* has the ability to adhere to the surface of implanted medical devices (bones implants, vascular catheters, artificial heart valves) and produce the biofilm on surfaces of biomaterials. The treatment of nosocomial infections, such as post-operative wound and bloodstream infections is a major medical problem. Under clinical research there are several compounds that are potentially active against multi-drug resistance bacteria. It is necessary to search for new drugs with antimicrobial activity, for example in the group of natural antibiotics such as salinomycin [25,26].

All of the compounds showing antibacterial activity (**1**, **4**, **6** and **12**) in general also have potent antibacterial activity against the twenty tested strains of MRSE and MRSA (Table 7). Esters **4** and **12** exhibit good potency in inhibiting the growth of MRSE, with MICs

Table 6
Antibacterial activity of salinomycin (**1**) and its the most active ester derivatives designated as diameter of Giz (mm) and MIC (μg/cm^3).

Reference strains	Compounds				
	1	**4**	**6**	**12**	Ciprofloxacin
	Giz MIC	Giz MIC	Giz MIC	Giz MIC	Giz MIC
S. aureus NCTC 4163	30 2	12 32	31 1	20 4	26 0.25
S. aureus ATCC 25923	29 2	13 32	29 1	22 4	26 0.5
S. aureus ATCC 6538	34 2	11 32	26 1	24 4	28 0.25
S. aureus ATCC 29213	28 4	12 64	28 2	22 8	22 0.5
S. epidermidis ATCC 12228	34 2	13 64	37 1	25 8	30 0.25
S. epidermidis ATCC 35984	33 2	–64	27 2	23 4	32 0.125

Compounds **2**, **3**, **5**, **7**–**11** and **13** were practically inactive towards all micro-organisms tested (Giz 10–12 mm and MIC ≥ 256 μg/cm^3).

Table 7
Antibacterial activity of salinomycin (**1**), its selected esters (**4**, **6**, **12**) against hospital strains methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus aureus* (MIC (μg/cm^3)).

Staphylococcus strains	Compounds				
	1	**4**	**6**	**12**	Ciprofloxacin
Hospital strains of methicillin-resistant *Staphylococcus epidermidis* (MRSE)					
459/11	16	64	2	8	16
460/11	16	128	2	8	0.125
461/11	16	256	2	4	0.25
466/11	8	64	2	8	2
467/11	16	64	2	4	16
468/11	16	64	2	4	16
469/11	16	128	4	8	8
470/11	16	256	4	8	0.125
488/11	16	128	2	4	16
489/11	16	128	2	4	0.25
Hospital strains of methicillin-resistant *Staphylococcus aureus* (MRSA)					
452/11	16	64	0.5	16	32
456/11	16	32	0.5	8	32
462/11	16	128	0.5	16	64
514/11	8	32	1	16	32
522/12	16	64	0.5	16	32
537/12	16	32	1	16	64
572/12	16	32	0.5	8	64
573/12	16	32	0.5	16	32
585/12	16	32	0.5	8	64
586/12	16	32	0.5	8	64

in the range of 4–256 μg/cm^3, which means that they were slightly less active than unmodified salinomycin (**1**) (MIC = 8–16 μg/cm^3). The compound **6** (trifluoroethyl ester), with the best activity against MRSE strains (MIC = 2–4 μg/cm^3), is found to be more potent than **1**. This compound possess also the antibacterial activity against hospital isolated MRSA (Table 7). Among the derivatives tested, compound **6** exhibited the highest anti-MRSA activity (MIC = 0.5–1 μg/cm^3), which is higher than the activity of unmodified **1** (MIC = 8–16 μg/cm^3), and, what is worth noting, higher than that of the standard drug – ciprofloxacin (MIC = 23–64 μg/cm^3).

Antibacterial and cytotoxic activity evaluation largely depended on various substitutions in the ester group of salinomycin derivatives. Attempts were made to correlate antibacterial activity of these compounds with changing substitution at ester moiety. The study performed clearly demonstrated that trifluoroethyl ester group showed the highest antitumor and antibacterial bioactivities.

This preliminary study of the antitumour and antibacterial activity of the compounds **1**, **4**, **5**, **9**, **12** and especially **6** represents a novel strategy for the discovery of promising lead anticancer and antibacterial compounds which requires further investigation. As follows from the results obtained, the conversion of carboxylic group to the ester derivatives which contain a bulky group in the ester position, resulted in inactivation of these compounds against the test bacteria and had little impact on anticancer activity. Thus, it has been clearly shown that chemical modifications of the carboxylic group of **1** influence the biological properties of salinomycin.

4. Conclusions

Twelve new esters (**2**–**13**) of salinomycin were synthesized using two different synthetic methods. The advantages of the protocol include simple reaction workup, easily available starting materials and convenient isolation. These methods of synthesis of esters can be also used to obtain other acid sensitive natural compounds.

According to the data analyzed above, preliminary structure–activity relationship (SAR) could be concluded. Firstly, trifuloroethyl ester (**6**) appeared to reveal higher antiproliferative and antibacterial activity than unmodified salinomycin, its other esters, as well as cytotoxic drugs like cisplatin or doxorubicin, and antibacterial drug as ciprofloxacin. Secondly, different ester moiety could produce to some extent different bioactivity. Thirdly, esterification of salinomycin could not only increase cytotoxic and antibacterial activity but also reduce toxicity. In general, our preliminary SAR studies demonstrated that the most potent anticancer and antibacterial compounds in this category would contain trifluoroethyl ester group (**6**) or be accompanied by short aliphatic chain (**2** and **5**) or α-naphthylmethyl (**9**) or polar di-*o*-nitrobenzyl (**12**) ester substituents. Further optimizations of salinomycin derivatives should be carried on to discover compounds having potent antiproliferative activity against human cancer cell lines.

5. Experimental

5.1. General

All precursors for the syntheses (alcohols, alkyl halides) and solvents were obtained from Aldrich or Fluka and were used as received without further purification. CD_3CN and CH_3CN spectral-grade solvents were stored over 3 Å molecular sieves for several days. All manipulations with the substances were performed in a carefully dried and CO_2-free glove box. TLC was carried out on precoated plates (TLC silica gel 60 F_{254}, Aluminium Plates Merck) and spots were detected by illumination with an UV lamp and visualized with iodine. All the solvent used in flash chromatography were of HPLC grade (CHROMASOLV from Sigma–Aldrich) and were used as received. The elemental analysis of **1**–**13** were carried out on Vario ELIII (Elementar, Germany).

5.2. Spectroscopic measurements

The ^{1}H, ^{13}C spectra were recorded on a Bruker Avance DRX 600 spectrometer. ^{1}H NMR measurements of **1**–**13** (0.07 mol dm^{-3}) in CD_2Cl_2 were carried out at the operating frequency 600.055 MHz; flip angle, pw = 45^0; spectral width, sw = 4500 Hz; acquisition time, at = 2.0 s; relaxation delay, d_1 = 1.0 s; T = 293.0 K and using TMS as the internal standard. No window function or zero filling was used. Digital resolution was 0.2 Hz per point. The error of the chemical shift value was 0.01 ppm. The ^{13}C NMR spectra were recorded at the operating frequency 150.899 MHz; pw = 60^0; sw = 19,000 Hz; at = 1.8 s; d_1 = 1.0 s; T = 293.0 K and TMS as the internal standard. Line broadening parameters were 0.5 or 1 Hz. The error of chemical shift value was 0.1 ppm. All spectra were locked to deuterium resonance of CD_3CN.

The ^{1}H and ^{13}C NMR signals were assigned using 2-D (COSY, HETCOR, NOESY, HMBC) spectra shown in the Supplementary Materials. 2-D spectra were recorded using standard pulse sequences from Varian and Bruker pulse-sequence libraries.

The FT-IR spectra of **1**–**13** in the mid infrared region were recorded in KBr or CH_3CN solution. A cell with Si windows and wedge-shaped layers was used to avoid interferences (mean layer thickness 170 μm). The spectra were taken on an IFS 113v FT-IR spectrophotometer (Bruker, Karlsruhe) equipped with a DTGS detector; resolution 2 cm^{-1}, NSS = 64. The Happ–Genzel apodization function was used.

The ESI (Electrospray Ionisation) mass spectra were recorded on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. The samples ware prepared in dry acetonitrile (5×10^{-5} mol dm^{-3}). The sample was infused into the ESI source using a Harvard pump at a flow rate of 20 μl min^{-1}. The ESI source potentials were: capillary 3 kV, lens 0.5 kV, extractor 4 V. The standard ESI mass spectra were recorded at the cone voltages: 10 and 30 V. The source temperature was 120 °C and the desolvation temperature was 300 °C. Nitrogen was used as the nebulizing and desolvation gas at flow-rates of 100 dm^3 h^{-1}. Mass spectra were acquired in the positive ion detection mode with unit mass resolution at a step of 1 m/z unit. The mass range for ESI experiments was from m/z = 300 to m/z = 1200.

5.3. X-ray measurement

A colourless single crystal of **3** was used for data collection on a four circle KUMA KM-4 diffractometer equipped with a two dimensional CCD detector. The graphite monochromatized Mo$K\alpha$ radiation (λ = 0.71073 Å) and the ω-scan technique ($\Delta\omega$ = 1.0°) were used for data collection. Data collection and reduction along with the absorption correction were performed using CrysAliss software package [27]. The structure was solved by direct methods using SHELXS-97 program [28] revealing positions of almost all non-hydrogen atoms. The remaining atoms were located from difference Fourier maps. The H atoms of CH, CH_2 and CH_3 groups were constrained with a distance of 0.97 Å and U_{iso} = 1.5 U_{eq} of C joined H. The H atom of OH groups were also constrained with a distance of 0.82 Å. Visualisation of the structure was made with the Diamond 3.0 program [29]. Details on the crystal data, data collection parameters and final refinement parameters are collected in Table 2. Selected geometrical parameters are listed in Table 3.

5.4. Synthesis

5.4.1. Isolation of salinomycin sodium salt (SAL-Na)

SAL-Na was isolated from Sacox®120 microGranulate an anticoccidial feed additive distributed by Huvepharma Polska. 100 g of permix was dissolved in dichloromethane. The solvent was evaporated under reduced pressure and the crude product obtained was purified by Dry Column Vacuum Chromatography (gradient solvent mixture hexane/dichloromethane) giving 6 g pure SAL-Na. The spectroscopic data of SAL-Na data were in agreement with previously published assignments [13].

5.4.2. Synthesis of salinomycin acid (**1**)

SAL-Na was dissolved in dichloromethane and stirred vigorously with a layer of aqueous sulphuric acid (pH = 1.5). The organic layer containing **1** was washed with distilled water, and then dichloromethane was evaporated under reduced pressure to dryness giving **1**. The spectroscopic data of **1** data were in agreement with previously published assignments [13].

5.4.3. General procedure for the synthesis of salinomycin esters (**2**–**13**)

5.4.3.1. Method A. To a mixture of **1** (500 mg, 0.66 mmol) in dichloromethane (15 ml) the following compounds were added: DCC (206 mg, 1.0 mmol), PPy (50 mg, 0.33 mmol), corresponding alcohol (5 mmol) and *p*-TSA (28.5 mg, 0.15 mmol). The mixture was first stirred at a temperature below 0 °C for 6 h and then for further 18 h at room temperature. The solvent was subsequently evaporated under reduced pressure to dryness. The residue was suspended in hexane and filtered off. The filtrate was evaporated under reduced pressure and the residue was purified chromatographically on silica gel (Fluka type 60) to give (**2**–**4**, **6**, **8** and **13**) (yield from 25.4% to 71.4%, Table 1) as a colourless oil showing a tendency to form the glass state. The exemplary spectra of compound **4** obtained in the method A are included in the Supplementary material.

5.4.3.2. Method B

A mixture of alkyl bromide or chloride (1.45 mmol), **1** (500 mg, 0.66 mmol), DBU (175 mg, 1.15 mmol) and 40 ml toluene was heated at 90 °C for 5 h. After cooling, the precipitate DBU-hydrohalide (DBU·HX) was filtered and washed with hexane. The filtrate and the washing were combined and evaporated under reduced pressure. The residue was purified by chromatography on silica gel (Fluka type 60) to give corresponding ester (**4**–**5**, **7**–**12**) (yield from 28.6% to 86.7%, Table 1) as a colourless oil showing a tendency to form the glass state. The exemplary spectra compound **8** obtained in the method B are included in the Supplementary Material.

5.5. Antiproliferative activity of salinomycin and its derivatives

Four human cancer cell lines and one murine normal cell line were used to evaluate antiproliferative activity of salinomycin and its derivatives: human acute promyelocytic leukemia (HL-60) and its vincristine resistant subline – (HL-60/vinc), human colon adenocarcinoma cell lines sensitive and resistant to doxorubicin (LoVo) and (LoVo/dx) respectively, and also normal murine embryonic fibroblast cell line (BALB/3T3). The BALB/3T3 cell line was purchased from the American Type Culture Collection (ATCC Rockville, Maryland, USA), HL-60 cell line – from European Type Culture Collection by courtesy of Professor Spik and Dr. Mazurier (Laboratory of Biological Chemistry USTL, Lille, France) and HL-60/vinc, LoVo and LoVo/dx by courtesy of Prof. E. Borowski (Technical University of Gdańsk, Poland). All the cell lines are maintained in the Institute of Immunology and Experimental Therapy (IIET), Wroclaw, Poland.

Human leukaemia cells were cultured in RPMI 1640 medium (IIET, Wroclaw) containing 10% fetal bovine serum, glucose (5.5 g/l), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma–Aldrich, Germany) and 1 μg/100 ml doxorubicin for HL-60/vinc (Sigma–Aldrich, Germany). Human colon adenocarcinoma cell lines were cultured in mixture of OptiMEM and RPMI 1640 (1:1) medium (Optimem from Gibco, RPMI from PAA, Austria), supplemented with 5% fetal bovine serum (PAA, Austria), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma–Aldrich, Germany) and 10 μg/100 ml doxorubicin for LoVo/dx (Sigma–Aldrich, Germany). Murine embryonic fibroblast cells were cultured in Dulbecco medium (IIET, Wroclaw), supplemented with 10% fetal bovine serum (PAA, Austria), 4 mM glutamine and glucose 4.5 g/l (Sigma–Aldrich, Germany). All culture media contained antibiotics: 100U/ml penicillin and 100 μg/ml streptomycin (Polfa-Tarchomin, Poland). All cell lines were cultured during entire experiment in humid atmosphere at 37 °C and 5% CO_2.

5.5.1. The antiproliferative assays in vitro

Twenty four hours before adding of the tested compounds all cell lines were seeded in 96-well plates (Sarstedt, Germany) in appropriate media with 10^4 cells per well. All cell lines were exposed to each tested agent at four different concentrations of the range 100 to 0.1 μg/ml for 72 h. Cells were also exposed to the reference drug cisplatin (Ebewe, Austria) and doxorubicin (IBA, Poland). Additionally, all cell lines were exposed to DMSO (solvent used for tested compounds) (Sigma–Aldrich, Germany) at concentrations corresponding to these present in tested agents' dilutions. For adherent cells sulphorodamine B assay was performed and MTT assay for leukaemia cells.

5.5.2. SRB

After 72 h of incubation with tested compounds cells were fixed *in situ* by gently adding of 50 μl per well of cold 50% trichloroacetic acid TCA (POCh, Poland) and were incubated at 4 °C for 1 h. Following, wells were washed four times with water and air dried. Next, 50 μl of 0.4% solution of sulphorodamine B (Sigma–Aldrich, Germany) in 1% acetic acid (POCh, Poland) were added to each well and plates were incubated at room temperature for 0.5 h. After incubation time, unbound dye was removed by washing plates four times with 1% acetic acid whereas stain bound to cells was solubilized with 10 mM Tris base (Sigma–Aldrich, Germany). Absorbance of each solution was read at Multiskan RC photometer (Labsystems, Finland) at 540 nm wavelength.

5.5.3. MTT

Proliferation inhibition of leukaemia cells by tested compounds was measured by means of MTT assay. Thus, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (Sigma–Aldrich, Germany) were added to each well and plates were left in cell incubator for 4 h to allow the cells to metabolize yellow MTT to blue formazan. Then, lysing mixture consisting of 225 ml dimethylformamide, 67.5 g sodium dodecyl sulphate (both from Sigma–Aldrich, Germany) and 275 ml of distilled water, was added in 80 μl volume per well. Plates were incubated for 24 h for the formazan crystals to be released from cells and dissolved and than absorbance of each well was read at Multiskan RC photometer (Labsystems, Finland) at 570 nm wavelength.

Results are presented as mean IC_{50} (concentration of the tested compound, that inhibits cell proliferation by 50%) ± standard deviation. IC_{50} values were calculated in Cheburator 0.4, Dmitry Nevozhay software for each experiment. Compounds at each concentration were tested in triplicates in single experiment and each

experiment was repeated at least three times independently. Results are summarized in Tables 4 and 5. The IR was defined as the ratio of IC_{50} for a given compound calculated for resistant cell line to that measured for its parental drug sensitive cell line (Table 4).

5.7. Antimicrobial activity of salinomycin and its derivatives

Microorganisms used in this study were as follows: Gram-positive cocci: *S. aureus* NCTC 4163, *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, Gram-negative rods: *Escherichia coli* ATCC 10538, *E. coli* ATCC 25922, *E. coli* NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* ATCC 15442, *P. aeruginosa* NCTC 6749, *P. aeruginosa* ATCC 27863, *Bordetella bronchiseptica* ATCC 4617 and yeasts: *C. albicans* ATCC 10231, *C. albicans* ATCC 90028, *C. parapsilosis* ATCC 22019.

Other microorganisms used were obtained from the collection of the Department of Pharmaceutical Microbiology, Medical University of Warsaw, Poland.

Antibacterial activity was examined by the disc-diffusion method under standard conditions using Mueller-Hinton II agar medium (Becton Dickinson) according to CLSI (previously NCCLS) guidelines [30]. Antifungal activities were assessed using Mueller–Hinton agar +2% glucose and 0.5 μg/mL Methylene Blue Dye Medium [31].

Sterile filter paper discs (9 mm diameter, Whatman No 3 chromatography paper) were dripped with tested compound solutions (in EtOH) to load 400 μg of a given compound per disc. Dry discs were placed on the surface of appropriate agar medium. The results (diameter of the growth inhibition zone) were read after 18 h of incubation at 35 °C.

Minimal Inhibitory Concentration (MIC) was tested by the twofold serial microdilution method (in 96-well microtiter plates) using Mueller-Hinton Broth medium (Beckton Dickinson) according to CLSI guidelines [32]. The stock solution of tested agent was prepared in EtOH and diluted in sterile water. Concentrations of tested agents ranged from 0.0625 to 512 $\mu g/cm^3$. The final inoculum of all studied microorganisms were 10^5 CFU/mL^{-1} (colony forming units per ml). Minimal inhibitory concentrations (the lowest concentration of a tested agent that prevents visible growth of a microorganism) were read after 18 h of incubation at 35 °C.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2014.02.031. These data include MOL files and InChiKeys of the most important compounds described in this article.

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