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Preliminary communication

Synthesis and biological activity of salinomycin conjugates with floxuridine

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ABSTRACT

As part of our program to develop anticancer agents, we have synthesized new compounds, which are conjugates between well-known anticancer drug, floxuridine and salinomycin which is able to selectivity kill cancer stem cells. The conjugates were obtained in two ways *i.e.* by copper(I) catalysed click Huisgen cycloaddition reaction performed between 3′-azido-2′,3′-dideoxy-5-fluorouridine and salinomycin propargyl amide, and by the ester synthesis starting from salinomycin and floxuridine under mild condition. The compounds obtained were characterized by spectroscopic methods and evaluated for their *in vitro* cytotoxicity against seven human cancer cell lines as well as antibacterial activity against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). The conjugate obtained by esterification reaction showed a significantly higher antiproliferative activity against the drug-resistant cancer cells and lower toxicity than those of salinomycin and floxuridine towards normal cells, as well as standard anticancer drugs, such as cisplatin and doxorubicin. The conjugate compound revealed also moderate activity against MRSA and MRSE bacterial strains. Very high activity of floxuridine and 5-fluorouracil against MRSA and MRSE has been also observed.

1. Introduction

One of the current concepts in the anticancer drug design and development is synthesis of new hybrid compounds (molecular hybridization/bioconjugation) of improved affinity and efficacy relative to those of the parent drugs [1,2]. Pharmacophore conjugation is believed to be analogous to the conventional combination therapy, but with the two drugs covalently linked and available as a single entity [3].

The development of nucleoside analogues for medicinal use has significant impact on clinical chemotherapy as applied to antiviral and anticancer treatment [4–8]. Since its first discovery in 1957, 5-fluorouracil (**FUra**) has been a well-known anti-tumour agent used in the treatment of several neoplastic diseases, such as colon or breast cancers [9,10]. **FUra** is an anticancer drug with tremendous clinical potential, but has had limited efficacy due to its high toxicity [11,12]. Numerous **FUra** derivatives have been developed up to now. One of the best known **FUra** derivative is 5-fluoro-2′-deoxyuridine (**FdU**, Floxuridine), which is known for its high anti-tumour activity against cancer metastases [13,14]. **FdU** and other nucleoside analogues have been extensively used in the treatment of a variety of cancers over the last 40 years, and their mechanisms of action are well understood. **FdU** has been used to treat human solid tumours. However, **FdU** exhibited various side effects in the clinical treatment and its therapeutic effect was limited by the efficiency of cellular uptake and bioavailability of the drug [15]. In order to overcome these limitations various synthetic approaches have been proposed to improve its physicochemical properties and reduce its toxicity [16].

On the other hand, it is well known that natural products have provided some of the most effective drugs for the treatment of cancer, including paclitaxel, vinblastine, vincristine and doxorubicin [17,18]. Very interesting and time-consuming studies were performed in 2009. In these studies 16,000 natural and commercial chemical compounds have been screened for their anticancer

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activity and only one compound, salinomycin (**SAL**) (Scheme 1), an antibiotic belonging to a large group of natural polyethers isolated from *Streptomyces albus*, has been found to selectively kill breast cancer stem cells (CSCs) with 100-fold greater activity than the known anticancer agent – paclitaxel [19]. Extensive research work has been undertaken to explain the unusual anticancer properties of this compound, because CSCs play crucial role in tumour progression, chemoresistance and recurrence [20]. It has been also shown that **SAL** was able to induce apoptosis by multiple mechanisms in many human cancer cells resistant to cell death, but not in normal cells [21]. **SAL** showed strong inhibition of proliferation, migration and invasion of cancer cells, such as osteosarcoma, hepatocellular carcinoma, cholangiocarcinoma and gastric cancer [22–25]. Until now the synthesis and biological evaluation of various amides, esters and *O*-acylated derivatives of **SAL** have been described. Results of the tests have clearly shown that the obtained **SAL** derivatives exhibited potent anticancer activity against different human cancer cell lines, including drug-resistant cell lines [26–31].

Since 2012, **SAL** has been approved for testing in clinical studies on patients with invasive head, neck, breasts and ovary carcinoma. The results have shown inhibition in progress of the disease over an extended period of time. Acute side effects were rare and the serious long-term adverse side effects were not observed [32].

Recently, it has been proved that combination therapy with **SAL** and **FUra** had a synergistic anticancer effect against liver cancer both *in vitro* and *in vivo* [33]. This interesting observation inspired us to obtain and evaluate biological conjugates of these compounds, in which these compounds will be connected by covalent bond. Based on the idea that cancer cells are killed by **FdU** and **SAL**, we synthesized two different conjugates of **FdU** and **SAL** to enhance the anticancer activity of starting compounds.

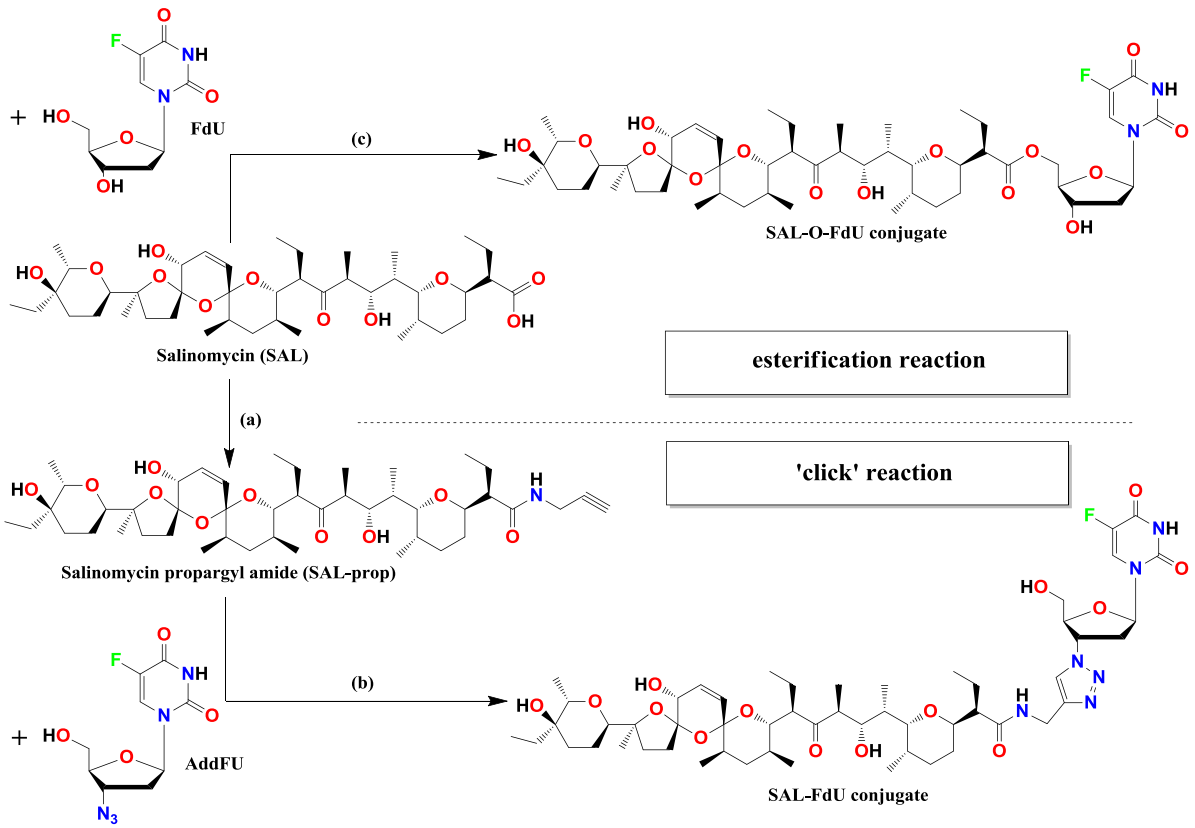
The present paper describes two efficient methods for the synthesis of two new bioconjugates. The first method is based on the 'click' chemistry and involves the copper(I) catalysed 1,3-dipolar Huisgen cycloaddition reaction. The 'Click' chemistry has been previously often applied for the synthesis of new nucleoside bioconjugates and it is one of the powerful tools for the generation of new drug candidates [1]. Many researchers used the 'click' chemistry as a synthetic tool for generation of pharmalogically valuable drugs. In the 'click' reaction, salinomycin propargyl amide (**SAL-prop**) and 2′,3′-dideoxy-3′-azido-5-fluorouridine (**AddFU)** react directly giving the first conjugate (**SAL-FdU**). In the second method two fragments **FdU** and **SAL** are joined by the ester bond in mild reaction conditions, to give **SAL-O-FdU** conjugate.

The structures of the compounds obtained were characterized using elemental analysis, FT-IR and NMR and ESI MS methods. The *in vitro* anticancer activity of these compounds against seven drug-sensitive and drug-resistant human cancer cell lines, as well as their antibacterial activity, especially against MRSA and MRSE hospital strains, were determined and discussed.

2. Results and discussion

2.1. Chemistry

The purity and structures of obtained compounds were determined on the basis of elemental, FT-IR, NMR and ESI MS analysis. The ^{1}H and ^{13}C NMR signals were assigned using one- and two-dimensional ($^{1}H–^{1}H$ COSY, $^{1}H–^{13}C$ HETCOR, $^{1}H–^{13}C$ HMBC) spectra. ^{1}H and ^{13}C as well as 2D NMR spectra of both conjugates are included in the Supplementary material (Figs. S1–S5 and



Scheme 1. *Reagents and conditions*: (a) DCC, HOBt, propargylamine, CH_2Cl_2/THF (3/1), 0 °C, 1 h; then rt,24 h; (b) $CuSO_4$, sodium ascorbate, dioxane/water (3:1); rt, 12 h; (c) DCC, PPy, *p*-TSA, CH_2Cl_2, 0 °C,6 h; then rt, 18 h. Time for completion of the reaction is indicated by TLC.

S8–S12).

Salinomycin sodium salt (**SAL-Na**) was isolated from the commercially available veterinary premix – SACOX® following the procedures described previously [27]. The structure and homogeneity of isolated **SAL-Na** was confirmed using spectroscopic methods. **SAL** was obtained from **SAL-Na** by the extraction with sulphuric acid solution (pH = 1.5) in dichloromethane.

To optimize the preparation of salinomycin conjugate with floxuridine, an easy, versatile and efficient synthetic methodology was required and so the 'click' chemistry approach was taken to construct the conjugates.

The alkyne component for the click reaction *i.e.* salinomycin propargyl amide (**SAL-prop**) was synthesized in the reaction between **SAL** and propargylamine in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC) as a coupling agent and 1-hydroxybenzotriazole (HOBt) as an activator, following our procedure described previously [27].

The structure of **SAL-prop** was confirmed by single crystal X-ray analysis. The crystallographic data and structure refinements, as well as bond lengths and angles characterizing the geometry of the **SAL-prop** amide molecule are summarized in Supplementary data (Tables S1–S3). Furthermore, its molecular structure is presented in Fig. 1. The pseudo-cyclic conformation of this compound is stabilized by the presence of the intramolecular O–H···O and N–H···O hydrogen bonding interactions. It is worth noting that the propargyl group is directed outwards of the molecule. Moreover, the alkyne group is available to perform the 'click' reaction.

The azide component for the 'click' reaction *i.e.* 3′-azido-2′,3′-dideoxy-5-fluorouridine (**AddFU**) was synthesized from 5-fluoro-2′-deoxyuridine (**FdU**) *via* 2,3′-anhydro-5′-*O*-benzoyl-5-fluoro-2′-deoxyuridine intermediate, as has been described recently [34].

The first conjugate (**SAL-FdU**) was prepared using 3′-azido-2′,3′-dideoxy-5-fluorouridine (**AddFU)** as the azide component and salinomycin propargyl amide **(SAL-prop)** as the alkyne component in the presence of copper(I) cations generated *in situ* from copper(II) sulphate and sodium ascorbate in dioxane-water system (Scheme 1). This reaction proceeded giving the desired product (**SAL-FdU**) in 67% yield, following column chromatography.

The main evidence for the formation of the compound **SAL-FdU** is the absence of three characteristic bands in its FT-IR spectrum, which are present in the FT-IR spectra of the azide and alkyne substrates at 2096 cm^{-1}, 3319 cm^{-1} and 2253 cm^{-1}, and are assigned to the $\nu(N_3)$, $\nu(\equiv C-H)$ and $\nu(C\equiv C)$ stretching vibrations, respectively (Supplementary material, Fig. S6). The absence of those bands in the FT-IR spectrum of the product of the 'click' reaction indicates that azide and alkyne had been fully consumed in the reaction.

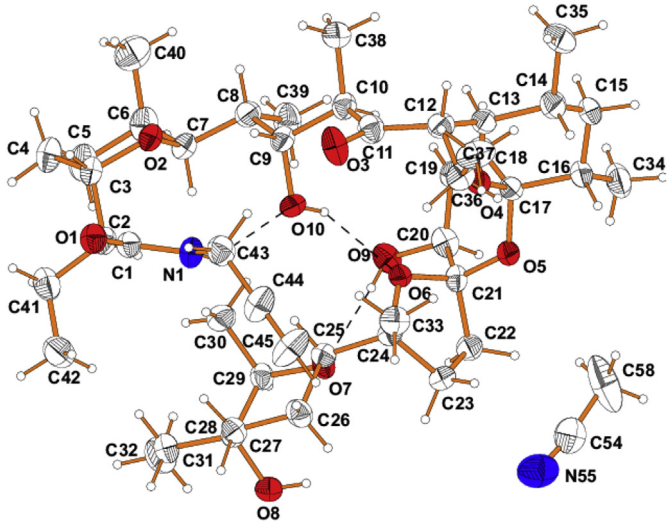


Fig. 1. View of the molecular structures of SAL-prop amide in the crystal.

This observation was also supported by its ^1H NMR spectrum, which showed a singlet for triazole proton at δ 8.01 ppm, thus confirming its formation.

The azide (**AddFU**) and alkyne (**SAL-prop**) components are necessary to perform the 'click' chemistry reaction. It was very interesting to check if a permanent connection between previously unmodified salinomycin (**SAL**) and floxuridine (**FdU**) can be formed in the simple and direct reaction. For this reason, we planned to obtain another conjugate of these compounds using a simpler and less time consuming procedure.

Therefore, on the basis of our experience in the synthesis of ester derivatives of polyether ionophores, including **SAL** [30], we decided to obtain the second conjugate of **SAL** and **FdU** using the esterification reaction. **SAL** is very sensitive to acidic conditions and heating. For these reasons, mild reaction conditions were chosen for **SAL** esterification. Previously, we have developed a valuable method for the synthesis of **SAL** esters, based on the reaction between **SAL** and the appropriate alcohol in the presence of *N,N′*-dicyclohexylcarbodiimide (DCC), 4-pyrrolidinopyridine (PPy) and *p*-toluenesulfonic acid monohydrate (*p*-TSA). This method was used to obtain **SAL-O-FdU** conjugate and gave the desired compound in a moderate 55% yield (Scheme 1).

In the ^{13}C NMR spectra of **SAL-O-FdU conjugate** the most characteristic signal of C(1) atom of ester group was observed at 175.7 ppm, while the signal of C(1) atom of carboxyl group of **SAL** was at 177.8 ppm. The characteristic ^{13}C signal of the $-OCH_2-$ group in the ester substituent was observed at 64.5 ppm.

In the ^1H NMR spectra of **SAL-O-FdU**, the characteristic signals of the $-OCH_2-$ protons from the ester substituent were observed as two separate doublets of doublets at 4.73 ppm and 4.18 ppm.

In the FT-IR spectrum of **SAL-O-FdU conjugate** a broad band arises with a maximum at about 1710 cm^{-1} due to the overlapping of $\nu(C{=}O)$ vibrations of ketone, ester group and 5-fluorouracil moiety as shown in Supplementary material (Fig. S13).

2.2. Anticancer in vitro activity

The most important aim of the study was to determine biological activity of the obtained conjugates, especially their antiproliferative activity. Multidrug resistance (MDR) of cancer cells is actually one of the greatest problems in the application of chemotherapy and in effective fight against neoplastic diseases. For this reason, novel **SAL-FdU** and **SAL-O-FdU** conjugates, as well as their precursors *i.e.* **SAL, FUra**, **FdU, AddFU** were tested against seven human cancer cell lines, including two drug-resistant cell lines. The tests were performed on promyelocytic leukaemia cells and its vincristine-resistant subline (HL-60 and HL-60/vinc, respectively), colon adenocarcinoma cells and its doxorubicin-resistant subline (LoVo and LoVo/DX, respectively), and 3 other colon cancer cell lines (HT-29, LS-180, SW707). Prolonged treatment of cancer patients with chemotherapeutic antitumour agents leads to the development of multidrug resistance toward numerous antitumour drugs. One of the mechanisms responsible for the multidrug resistance (MDR) phenotype of cancer cells is overexpression of ATP-dependent drug efflux protein: the 170-kDa P-glycoprotein (P-gp) encoded by the MDR1 gene [35]. Both multidrug resistant cell lines used in these studies represent this type of MDR [36,37]. On the other hand, the primary colon cancer and especially the one with metastatic potential can be treated usually as palliative rather than curative. Palliative treatment in metastatic

colon cancer consists of systemic chemotherapy with 5-fluorouracil (**FUra**), the only agent available for the treatment for decades, to which leucovorin (LV) was added. However, more recently new agents have been used in concomitant therapy with **FUra** such as oxaliplatin and irinotecan resulting in increased overall clinical benefit [38,39]. Therefore we chose for our studies the colon cancer cell lines with different characteristic: Lovo [40], Ls-180, HT-29 [41], SW-707 [42].

The reference compounds in these studies were two commonly used anticancer agents, cisplatin and doxorubicin. Anticancer drugs may be considered potentially more useful when show higher activity against tumour cells than against to non-tumour cells. Therefore, all tested compounds were also evaluated for their cytotoxicity against normal murine embryonic fibroblasts (BALB/3T3). The concentrations (in μM) of individual compounds at which 50% growth inhibition of cancer cells was observed, are summarized in Table 1. Comparison between the cancer cell lines and the corresponding normal cell line BALB 3T3 was made to define the *in vitro* selectivity index as a measure of the therapeutic potential (Table 2). The *in vitro* selectivity index (SI) of a drug is defined as the ratio of the toxic dose to the therapeutic dose (*in vitro* SI = IC_{50} non-tumour cell line/IC_{50} tumour cell line).

After the IC_{50} values were obtained for each tested cancer cell line for all studied compounds, the resistance index (RI) for a given agent was calculated as the ratio of the IC_{50} value for the resistant cell line to the IC_{50} value for the sensitive one.

According to Harker et al. [43], cells can be classified to one of three categories depending on the RI value: if the RI value is in the range of 0–2 the cells are drug sensitive, in the range of 2–10 the cells are moderately drug resistant, and if the RI value is higher than 10 the cells are markedly drug resistant.

The inhibitory activities of the compounds studied against the used cancer cell lines were different. As follows from the data collected in Tables 1 and 2, almost all the compounds tested are more or less active in the specified concentration range depended on the tested cell line. Among all these compounds, **AddFU** showed the lowest inhibitory activity to all cancer cell lines, whereas **SAL** cytotoxic activity against cancer cells as well as its high SI and low RI indexes clearly show that it has very strong anticancer properties and relatively low toxicity against normal cells and rightly is regarded as one of the most promising new anticancer agent. It is worth noting that the currently used drugs, like **FUra**, **FdU**, cisplatin and doxorubicin, have worse IC_{50}, SI, and RI values than **SAL**.

SAL-O-FdU conjugate obtained by esterification reaction showed emphatically higher anticancer activity than the corresponding **SAL-FdU** obtained by the 'click' reaction. Moreover, **SAL-O-FdU** exhibits also better anticancer activity than its precursors **FUra** and **FdU**, and also than cisplatin and doxorubicin. The anticancer activity of **SAL-O-FdU** is similar to that exhibited by **SAL**. The selectivity index (SI) values are almost always better for **SAL-O-FdU** than observed for **SAL.** The *in vitro* SI for **SAL-O-FdU** is in the range from 11.45 to 87.24, indicating high selective cytotoxic activity and low toxicity against the non-tumour cells. The results revealed that one of conjugates (**SAL-O-FdU)** exhibited better or comparable *in vitro* anticancer activity to **FUra**, **FdU** and **SAL** as well as low toxicity in the normal cells. As shown in Tables 1 and 2, **SAL-O-FdU** exhibited better antitumour activity than the anticancer drugs, cisplatin and doxorubicin. The results demonstrated that the presence of the **SAL** component in the hybrid compound was critical for selectivity and cytotoxicity in human cancer cells as the hybrid is much more potent than doxorubicin or **FUra** in inhibition of cell proliferation and promotion of death of cancer cells (Table 2). **SAL-O-FdU** broke strongly the drug resistance of LoVo/DX cancer cells (RI = 0.64) and moderately that of HL-60/vinc (RI = 7.62).

It is worth noting that we do not observe simple synergy for both conjugates, but the selectivity of one of them **SAL-O-FdU** is better than that of parent compounds and other attested anticancer drugs (Table 2).

All discussed biological properties of the conjugates obtained indicate that the chemical modification of **SAL** can change the anticancer activity of substrates towards two definite directions *i.e.* to obtain weaker (**SAL-FdU)** and stronger (**SAL-O-FdU)** anticancer drug candidates.

2.3. Antibacterial in vitro activity

Actually, the most common single MDR bacterium is MRSA, which is resistant to methicillin and related β-lactam antibiotics, and is now a very serious problem in hospitals worldwide. Therefore, new antibiotics are highly needed in response to continuously emerging resistances. Preliminary tests of the antimicrobial activity of several derivatives of **SAL** have shown that it was active against Gram-positive standard bacterial strains. For these reasons, for the first time the well known cytotoxic compounds like **FUra**, **FdU, AddFU** and **SAL** and their derivatives **SAL-FdU** and **SAL-O-FdU** were subjected to tests of their antimicrobial activity against different strains of Gram-positive and especially against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). Hospital strains of methicillin-resistant Staphylococcus were isolated from different biological materials from patients of the Warsaw Medical University Hospital. Unfortunately, infections caused by this organism are becoming more difficult to treat as further evolution of drug resistance occurs within the pathogen.

Table 1
Antiproliferative activity of tested compounds. Data are given as IC_{50} [μM].

Compound	Cancer cells							Normal cells
	HL-60	HL-60/vinc	LoVo	LoVo/DX	HT-29	LS-180	SW707	BALB/3T3
SAL	0.29 ± 0.05	2.09 ± 0.39	0.36 ± 0.08	0.76 ± 0.25	1.03 ± 0.27	0.61 ± 0.27	1.73 ± 0.25	9.33 ± 3.08
FUra	22.54 ± 7.85	21.15 ± 2.54	4.69 ± 1.54	6.00 ± 1.46	25.08 ± 13.15	38.92 ± 22.62	20.46 ± 12.15	3.23 ± 1.85
FdU	0.24 ± 0.16	17.40 ± 1.34	19.07 ± 5.12	16.67 ± 3.82	115.12 ± 39.43	140.28 ± 51.46	23.86 ± 4.43	23.90 ± 12.97
AddFU	179.74 ± 18.41	293.84 ± 49.00	43%[a]	290.11 ± 61.18	25%[a]	25%[a]	41%[a]	97.31 ± 36.53
SAL-FdU	28.65 ± 2.51	20%[a]	41.25 ± 10.02	70.42 ± 12.29	30.06 ± 0.11	32.81 ± 4.05	34.96 ± 9.40	29.60 ± 4.20
SAL-O-FdU	0.37 ± 0.11	2.82 ± 1.25	1.46 ± 0.57	0.93 ± 0.19	2.02 ± 0.41	2.25 ± 1.11	1.99 ± 0.23	32.28 ± 6.41
doxorubicin	0.04 ± 0.04	0.88 ± 0.26	0.15 ± 0.06	5.46 ± 1.56	0.89 ± 0.09	0.09 ± 0.04	0.18 ± 0.02	0.18 ± 0.07
cisplatin	1.00 ± 0.23	6.87 ± 1.63	3.70 ± 1.20	5.20 ± 1.93	10.70 ± 1.27	6.30 ± 1.33	6.47 ± 1.87	5.30 ± 2.93

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); colorectal adenocarcinoma cell line (HT-29); colon adenocarcinoma cell line (type B according to Dukes scale; LS-180); colorectal cancer cell line (SW707); normal murine embryonic fibroblast cell line (BALB/3T3). Data are expressed as the mean ± SD.

[a] IC_{50} undetectable in the tested concentrations; inhibition of proliferation at the highest concentration does not exceed 50%.

Table 2
The calculated values of the indexes of resistance (RI) and selectivity (SI) of tested compounds.

Compound	HL-60	HL-60/vinc		LoVo	LoVo/DX		HT-29	LS-180	SW707
	SI	SI	RI	SI	SI	RI	SI	SI	SI
SAL	32.17	4.46	7.21	25.92	12.28	2.11	9.06	15.30	5.39
FUra	0.14	0.15	0.94	0.69	0.54	1.28	0.13	0.08	0.16
FdU	99.58	1.37	72.50	1.25	1.43	0.87	0.21	0.17	1.00
AddFU	0.54	0.33	1.63	–	0.34	–	–	–	–
SAL-FdU	1.03	–	–	0.72	0.42	1.71	0.98	0.90	0.85
SAL-O-FdU	87.24	11.45	7.62	22.11	34.71	0.64	15.98	14.35	16.22
doxorubicin	4.50	0.20	22.00	1.20	0.03	36.40	0.20	2.00	1.00
cisplatin	5.30	0.77	6.87	1.43	1.02	1.41	0.50	0.84	0.82

The RI (Resistance Index) indicates how many times a resistant subline is chemoresistant relative to its parental cell line. When RI is 0–2 the cells are sensitive to tested compound; RI of the range 2–10 means that the cell shows moderate sensitivity to a drug; RI 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.

This antibacterial activity was evaluated by the MIC value (Minimum Inhibitory Concentration) in μM (Table 3). The tests performed have clearly shown that **SAL-O-FdU** has higher antibacterial activity in comparison with **SAL-FdU** against the reference strains as well as MRSA and MRSE. Conjugate **SAL-O-FdU** exhibits good potency in inhibiting the growth of MRSA with MICs in the range of 8.17–32.69 μM, and is more active than the reference antibiotic – ciprofloxacin (MIC = 96.68–193.35 μM).

It is interesting to note that both conjugates demonstrate relatively low antibacterial activity, whereas **SAL-FdU** is inactive. Recorded by us very high antibacterial effect of **FUra** and **FdU** is rather connected with cytotoxicity of these compounds than with their antibiotic properties, because these compounds are cytotoxic toward normal cells (the SI is very low, see Table 2).

Table 3
Antibacterial activity against MRSA and MRSE designated as Minimum Inhibitory Concentration – MIC (μM).

Staphylococcus strains	SAL	FUra	FdU	AddFU	SAL-FdU	SAL-O-FdU	Ciprofloxacin
Reference strains							
S. aureus ATCC 4163	2.66	1.92	≤0.016	3.69	30.22	8.17	0.76
S. aureus ATCC 25923	2.66	1.92	≤0.016	3.69	30.22	8.17	1.51
S. aureus ATCC 6538	2.66	3.85	≤0.016	3.69	30.22	4.09	0.76
S. aureus ATCC 29213	2.66	3.85	≤0.016	7.38	60.43	4.09	1.51
S. epidermidis ATCC 12228	2.66	3.85	≤0.016	1.85	30.22	4.09	0.76
S. epidermidis ATCC 35984	2.66	1.92	≤0.016	7.38	30.22	4.09	0.38
Hospital strains of methicillin resistant *Staphylococcus aureus* (MRSA)							
452/11	1.33	3.85	≤0.016	3.69	60.43	32.69	96.68
456/11	1.33	3.85	≤0.016	3.69	60.43	32.69	96.68
462/11	2.66	7.69	≤0.016	7.38	60.43	32.69	193.35
514/11	2.66	3.85	≤0.016	3.69	241.74	8.17	96.68
522/12	2.66	7.69	≤0.016	7.38	241.74	8.17	96.68
537/12	2.66	7.69	≤0.016	7.38	241.74	8.17	193.35
572/12	1.33	3.85	≤0.016	3.69	241.74	8.17	193.35
573/12	1.33	3.85	≤0.016	7.38	60.43	8.17	96.68
585/12	2.66	3.85	≤0.016	3.69	120.87	16.34	193.35
586/12	2.66	3.85	≤0.016	3.69	120.87	8.17	193.35
Hospital strains of methicillin-resistant *Staphylococcus epidermidis* (MRSE)							
459/11	2.66	3.85	≤0.016	0.92	30.22	32.69	48.34
460/11	2.66	1.92	≤0.016	0.92	30.22	32.69	0.38
461/11	2.66	0.96	≤0.016	1.85	30.22	32.69	0.76
466/11	2.66	0.48	≤0.016	0.46	30.22	32.69	6.04
467/11	2.66	0.48	≤0.016	0.92	60.43	32.69	48.34
468/11	2.66	0.48	≤0.016	0.92	60.43	32.69	48.34
469/11	2.66	1.92	≤0.016	3.69	60.43	32.69	24.17
470/11	2.66	0.48	≤0.016	0.92	30.22	8.17	0.38
488/11	2.66	1.92	≤0.016	0.92	120.87	32.69	48.34
489/11	2.66	0.48	≤0.016	3.69	30.22	8.17	0.76

The simple HPLC experiment preformed for both conjugates clearly shows that they are stable in 0.1 M phosphate buffer (pH 7.2) because we observed only one chromatographic peak assigned to a respective conjugate. An exemplary chromatogram of the sample of **SAL-O-FdU** after incubation in 0.1 M phosphate buffer (pH 7.2) after 96 h is presented in Fig. S16 (Supplementary material). A well-shaped peak is observed in the chromatogram at 6.45 min. In this single fraction is recorded ESI MS analysis shows the presence of species having $m/z = 1002$ which is assigned to the signal of **[SAL-O-FdU + Na]**$^+$ cation. Thus, chromatographic ESI MS analysis showed that conjugates do not undergo disintegration in a physiological buffer for up to 96 h.

Besides, it has been shown that carboxylesterases, which are among the best characterised prodrug hydrolysing enzymes involved in the activation of several therapeutic ester prodrugs, are able to cleavage ester bonds in several prodrugs of floxuridine [44]. It has been also demonstrated that several factors including the choice of ester prodrug group, esterification site, and nucleoside structure are the key factors determining carboxylesterase activity [45].

According to the above facts we can explain strong differences in the anticancer activity between obtained conjugates by the ability to cleavage ester bond, present in the structure of **SAL-O-FdU** by cellular carboxylesterases [46].

The amide bond present is the structure of **SAL-FdU** is ostensibly less promising for prodrug synthesis because of the chemical and enzymatic stability of this bond [46,47]: in this context, cellular amidases appear to be less promising than esterases and therefore **SAL-FdU** probably does not hydrolyse to salinomycin. Similarly to amides the triazole products are typical passive linkers which are not able to undergo hydrolysis under physiological conditions as well as by the specific cellular enzymes [46].

SAL-O-FdU in not a typical prodrug because after its probable hydrolysis in cancer cells it gives two active compounds *i.e.* salinomycin which is able to kill multidrug resistant (MDR) cancer cells and cancer stem cells (CSCs), and floxuridine which is well known high antitumour drug. Possible chemotherapy using such compounds should be more precise and more effective because CSCs are responsible for tumour initiation, progression and resurgence.

We previously observed that several salinomycin derivatives such as esters and amides exhibited relatively higher selectivity index in comparison with unmodified salinomycin and much higher in comparison with other anticancer drugs, *i.e.* cispaltin or doxorubicin [26–30].

3. Conclusions

Two examples of conjugates of floxuridine (**FdU**) and salinomycin (**SAL**) were synthesized for the first time using two different procedures. The first conjugate **SAL-FdU was** obtained in Cu(I) catalysed Huisgen azide–alkyne cycloaddition between 2′,3′-dideoxy-3′-azido-5-fluorouridine (**AddFU)** and salinomycin propargyl amide **(SAL-prop)** with 67% yield. The simple one-pot reaction of **SAL** and floxuridine with addition DCC, PPy and *p*-TsOH led to a quick synthesis of a new **SAL-O-FdU** (55% yield). The advantages of the protocol include simple reaction workup, easily available starting materials and convenient isolation.

The results of biological studies of the obtained conjugates indicate that the antiproliferative and antibacterial effects strictly depend on chemical nature of the conjugates. The **SAL-O-FdU** displayed antiproliferative activity in various cell types in the low micromolar range and showed selectivity index from 11.45 up to 87.24 providing an excellent starting point for further drug discovery optimisation. **SAL-O-FdU** also highly broke the drug resistance of LoVo/DX cancer cells.

We have provided evidence that the **SAL-O-FdU** shows antibacterial activity against human pathogenic bacteria, including drug resistant strains of *S. epidermidis* and *S. aureus*. It is interesting to note that the most antiproliferative active conjugate **SAL-O-FdU** is also the one showing the strongest antibacterial activity. These results are important for the development of molecules with dual potential anticancer and antibacterial activity.

Anticancer activity of salinomycin derivatives which have blocked carboxylic group is probably associated with their ability to transport cations through the cellular and mitochondrial membranes by the biomimetic and/or electrogenic mechanism. Therefore the conjugates obtained are still ionophores and they are able to transport metal cations. On the other hand, in the structures of both conjugates the moieties of 5-fluorouracil or floxuridine, which are typical antimetabolites, are also present. For these reasons both conjugates are potentially able to block DNA synthesis. The higher activity of **SAL-O-FdU** conjugate is probably connected with high cleavage of ester group by cellular carboxylesterases which leads to the presence of floxuridine and salinomycin in cancer cells, which consequently can kill those cells by the independent mechanisms.

On the basis of the above results, their structure-activity relationships data (SAR) were still not very clear, because the linker, the positions of substituents in **FdU** and the kind of chemical connection between **SAL** and floxuridine had different impacts on their bioactivities. However, our studies clearly show that compound **SAL-O-FdU** should be worthwhile for further study as the lead compound in the search for strong and broad-spectrum anticancer agents.

4. Experimental

4.1. General

All precursors for the synthesis and solvents were obtained from *Sigma Aldrich* or *Fluka* and were used as received without further purification. CD_2Cl_2 and $CDCl_3$ spectral grade solvents was 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 F254, 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 conjugates was carried out on Vario ELIII (Elementar, Germany).

4.2. Spectroscopic measurements

The ^1H and ^{13}C spectra were recorded on a Bruker Avance DRX 600 spectrometer. ^1H NMR measurements of the compounds obtained (0.07 mol dm^{-3}) in CD_2Cl_2 or $CDCl_3$ were carried out at the operating frequency 600.055 MHz; flip angle, pw = 45°; 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 in the chemical shift value was 0.01 ppm. The ^{13}C NMR spectra were recorded at the operating frequency 150.899 MHz; pw = 60°; 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 in chemical shift value was 0.1 ppm. All spectra were locked to deuterium resonance of CD_2Cl_2 or $CDCl_3$.

The ^1H and ^{13}C NMR signals were assigned using 2D (^1H–^1H COSY, ^1H–^{13}C HETCOR, ^1H–^{13}C HMBC) spectra shown in the Supplementary material (Fig. S3–S5 and S10–S12). 2D spectra were recorded using standard pulse sequences from Varian and Bruker pulse-sequence libraries.

The FT-IR spectra of the obtained compounds in the mid infrared region were recorded in KBr.

The HPLC/ESI-MS analyses were performed using a Waters/Micromass (Manchester, UK) ZQ mass spectrometer. The instrument was coupled to a Waters model 2690 HPLC pump (Milford, MA, USA). Using an autosampler, the sample solutions were injected onto the XBridge C18 column (3.5 μm, 100 mm × 2.1 mm i.d., Waters) run at 20 °C. Chromatographic peaks were identified with a UV detector (Waters). The injection volume was 2 ml. The isocratic acetonitrile with a flow rate of 0.2 ml/min, was applied. The electrospray ionisation source potentials were 3 kV for capillary, 0:5 kV for lens, 4 V for extractor and 30 V for cone voltage. The source and desolvation temperatures were 120 °C and 300 °C; respectively. Nitrogen was used as a nebulising and desolvation gas at flow rates of 100 and 600 l h^{-1}, respectively.

The ESI (Electrospray Ionisation) mass spectra were recorded also on a Waters/Micromass (Manchester, UK) ZQ mass spectrometer equipped with a Harvard Apparatus syringe pump. The samples were 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 ml 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 = 200 to m/z = 1400, as well as from m/z = 300 to m/z = 1300.

4.3. X-ray measurement

X-ray intensity data for the **SAL-prop** crystal was collected using graphite monochromatic MoKα radiation on a four-circle κ geometry KUMA KM-4 diffractometer with a two-dimensional area CCD detector. The ω-scan technique with Δω = 1.0° for each image was used for data collection. 930 images for six different runs covering over 99% of the Ewald sphere were taken. One image was used as a standard after every 50 images for monitoring of the crystal stability and the data collection. No correction for the relative intensity variations was necessary. Data collections were made using the CrysAlis CCD program [48]. Integration, scaling of the reflections, correction for Lorentz and polarisation effects and absorption corrections were performed using the CrysAlis Red program [48]. The

structure was solved by the direct methods using SHELXS-97 and refined using SHELXL-97 program [49]. The hydrogen atoms bonded to the carbon atoms were introduced in their geometrical positions. The hydrogen atoms involved in the hydrogen bonds were located in the difference Fourier maps and were constrained. The final difference Fourier maps showed no peaks of chemical significance. Details of the data collection parameters, crystallographic data and final agreement parameters are collected in Table S1. Visualisations of the structure was made with the Diamond 3.1 program [50]. The information on geometry of hydrogen bonding interactions is given in Table S2 and selected geometrical parameters are listed in Table S3. The structure has been deposited with the Cambridge Crystallographic Data Center in the CIF format, CCDC 1021026. Copies of the data can be obtained free of charge on the application to CCDC, 12 Union Road, Cambridge, CB21EZ, UK, (fax: (+44) 1223-336-033; email: deposit@ccdc.cam.ac.uk).

4.4. Synthesis

4.4.1. Isolation of *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 obtained product was purified by Dry Column Vacuum Chromatography [51] (gradient solvent mixture hexane/dichloromethane) giving 6 g pure **SAL-Na**. The spectroscopic data of **SAL-Na** were in agreement with previously published assignments [27].

4.4.2. Synthesis of *SAL*

SAL-Na was dissolved in dichloromethane and stirred vigorously with a layer of aqueous sulphuric acid (pH = 1.5). The organic layer containing **SAL** was washed with distilled water, and then dichloromethane was evaporated under reduced pressure to dryness giving **SAL**. The spectroscopic data of **SAL** were in agreement with previously published assignments [27].

4.4.3. Synthesis of *SAL-prop*

To a mixture of **SAL** (500 mg, 0.66 mmol) in dichloromethane (15 ml), the following compounds were added: DCC (206 mg, 1.0 mmol), HOBt (45 mg, 0.33 mmol) and propargylamine (110 mg, 2.0 mmol). Firstly, the mixture was 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 propargylamide of **SAL** (460 mg, 88% yield) as a white solid state.

4.4.4. Synthesis of *SAL-FdU*

To a solution of **AddFU** (70 mg, 0.26 mmol) and **SAL-prop** (205 mg, 0.26 mmol) in a 1:3 mixture of water and dioxane (8 ml), sodium ascorbate (17 mg, 0.084 mmol) followed by copper(II) sulphate pentahydrate (5.24 mg, 0.021 mmol, 21 μL of 1 M water solution) were added. The reaction mixture was stirred at room temperature for 12 h, after which time TLC indicated that the reaction was complete. Then the mixture was evaporated under reduced pressure and the residue was purified by silica gel column chromatography using as an eluent the mixture chloroform–methanol (from 20:1 to 10:1, v/v) to afford **SAL-FdU** (185 mg, 67% yield) as white powder. All FT-IR, NMR and ESI MS spectra of **SAL-FdU** are included in Supplementary material (Fig. S1–S7).

4.4.5. Synthesis of *SAL-O-FdU*

To a mixture of **SAL** (500 mg, 0.66 mmol) in dichloromethane (15 ml) the following compounds were added: DCC (206 mg, 1.00 mmol), PPy (50 mg, 0.33 mmol), floxuridine (197 mg, 0.8 mmol) and *p*-TSA (29 mg, 0.15 mmol). Firstly, the mixture was 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 **SAL-O-FdU** (358 mg, 55% yield) as colourless oil. All FT-IR, NMR and ESI MS spectra of **SAL-O-FdU** are included in Supplementary material (Fig. S8–S14).

4.5. Sample preparation for stability tests

Samples of the analysed **SAL-FdU** and **SAL-O-FdU** conjugate were weighed on analytical scales to an accuracy of 0.01 mg. Ten mg of a respective conjugate was placed in a 25 cm^3 round bottom flask, to which 15 cm^3 of 0.1 M phosphate buffer (pH 7.2) was added, and the contents were well mixed subjected to continuous stirring for 96 h at room temperature. 2 mL of this solution were placed in 25 cm^3 volumetric flask and diluted to 25 ml with acetonitrile. The sample was analysed by HPLC/ESI MS chromatography.

4.6. Antiproliferative activity

4.6.1. Cells

Human colon cancer cell line HT-29 was received from the German Cancer Research Center (Deutsches Krebsforschungszentrum, DKFZ) Heidelberg, Germany, the origin of cell line: Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The BALB/3T3 mouse embryonic fibroblasts cell line and human colon cancer cells Ls-180 and SW-707 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 being maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Both leukaemia cell lines were cultured in ISCOVE medium (IITD, Wroclaw, Poland) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 10% foetal bovine serum (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). Ls-180 cells were cultured in Eagle medium (IITD, Wroclaw, Poland) with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose (all from Sigma–Aldrich Chemie GmbH, Steinheim, Germany), and 10% foetal bovine serum (Thermo-Fisher Scientific Oy, Vataa, Finland). LoVo, LoVo/DX, HT-29 and SW-707 cells were cultured in RPMI 1640+Opti-MEM (1:1) (both from IITD, Wroclaw, Poland), and BALB/3T3 in Dulbecco medium (IIET, Wroclaw, Poland) supplemented with 2 mM L-glutamine (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), 5% foetal bovine serum (Thermo-Fisher Scientific Oy, Vataa, Finland). The culture of LoVo/DX and HL-60/vinc cells was supplemented with 10μg/100 ml and 1μg/100 ml of doxorubicin and of HT-29 and LoVo cells with 1.0 mM of sodium pyruvate (both from Sigma–Aldrich Chemie GmbH, Steinheim, Germany). All culture media were supplemented with 100 units/ml penicillin, and 100 μg/ml streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland). All cell lines were grown at 37 °C with 5% CO_2 humidified atmosphere.

4.6.2. An anti-proliferative assay in vitro

24 h before addition of the compounds tested, the cells were plated in 96-well plates (Sarstedt, Germany) at density of 1×10^4 cells per well. An assay was performed after 72 h exposure to varying concentrations of the tested agents. For adherent cells sulforhodamine B (SRB) assay was performed and MTT assay for leukaemia cells.

The results were calculated as an IC_{50} (inhibitory concentration 50) – the concentration of tested agent which inhibits proliferation of 50% of the cancer cell population. IC values were calculated for each experiment separately and mean values ± SD are presented in the tables. Each compound in each concentration was tested in triplicate in a single experiment, which was repeated 3–5 times.

4.6.3. SRB assay

The cells were attached to the bottom of plastic wells by fixing them with cold 50% TCA (trichloroacetic acid, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) on the top of the culture medium in each well. The plates were incubated at 4 °C for 1 h and then washed five times with tap water. The cellular material fixed with TCA was stained with 0.1% sulphorhodamine B (SRB, Sigma–Aldrich Chemie GmbH, Steinheim, Germany) dissolved in 1% acetic acid (POCH, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4×) in 1% acetic acid. The protein-bound dye was extracted with 10 mM unbuffered Tris base (POCH, Gliwice, Poland) for determination of the optical density ($\lambda = 540$ nm) on a computer-interfaced Synergy H4 photometer (BioTek Instruments, USA).

4.6.4. MTT assay

20 μl of MTT solution (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma–Aldrich Chemie GmbH, Steinheim, Germany); stock solution: 5 mg/ml) was added to each well and incubated for 4 h. After the incubation time was complete, 80 μl of the lysing mixture was added to each well (lysing mixture: 225 ml Dimethylformamide, 67.5 g Sodium Dodecyl Sulphate (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) and 275 ml of distilled water). The optical densities of the samples were read after 24 h on a computer-interfaced Synergy H4 photometer (BioTek Instruments, USA). at 570 nm.

4.7. Antimicrobial activity

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. The 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 [52]. Antifungal activities were assessed using Muellere Hinton agar +2% glucose and 0.5 mg/mL Methylene Blue Dye Medium [53].

Sterile filter paper discs (9 mm diameter, Whatman No. 3 chromatography paper) were dripped with tested compound solutions (in EtOH) to load 400 mg 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, GIZ) 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 [54]. The stock solution of a tested agent was prepared in EtOH and diluted in sterile water. Concentrations of tested agents ranged from 0.0625 to 512 μg/ml. The final inoculums 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.2015.01.045. These data include MOL files and InChiKeys of the most important compounds described in this article.

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