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Synthesis and antitumor activities investigation of a C-nucleoside analogue of Ribavirin

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ABSTRACT

SRO-91 is a non-natural ribofuranosyl-1,2,3-triazole C-nucleoside obtained by a synthetic sequence involving a C-alkynyl glycosylation mediated by metallic indium and a Huisgen cycloaddition for the construction of the triazole. Its structure is close to the one of ribavirin, a drug presenting a broad-spectrum against viral infections. SRO-91 antitumor activities were investigated on 9 strains of tumor cells and IC₅₀ of the order of 1 μM were obtained on A431 epidermoid carcinoma cells and B16F10 skin melanoma cells. In addition, studies of ovarian tumor cell inhibitions show an interesting activity in regard to the need for new drugs for this pathology. Finally, cytotoxicity and mouse toxicity studies reveal a favorable therapeutic index for SRO-91.

KEYWORDS

C-nucleoside, antitumor activity, ovarian cancer, Ribavirin

1. Introduction

Nucleosides are the elementary bricks of nucleic acids, carriers of genetic information. As a result, nucleosides constitute a molecular family of choice for the development of active molecules against processes involving the synthesis of genetic material *i.e.* cellular proliferation (cancers, bacterial, viral and parasitic infections). Nucleosides, nucleotides and derived prodrugs are thus a cornerstone of the chemotherapeutic arsenal against cancer and, nucleoside derivatives received a constant interest, as illustrated by FDA approvals in recent decades. These compounds have recently been referenced by the Schinazi team [1] as well as other compounds whose clinical development has been stopped. As an example, we can mention sofosbuvir [2] (Fig. 1) a prodrug of a modified uridine that has become the treatment of choice for hepatitis C. Sofosbuvir has demonstrated its ability to engender complete remission in follicular lymphoma [3]. Likewise other purine nucleoside analogues (PNAs) such clofarabine (CAFdA), nelarabine and forodesine (BCX-1777, immucillin H) are currently in clinical development for acute lymphoblastic leukemia (ALL) [4].

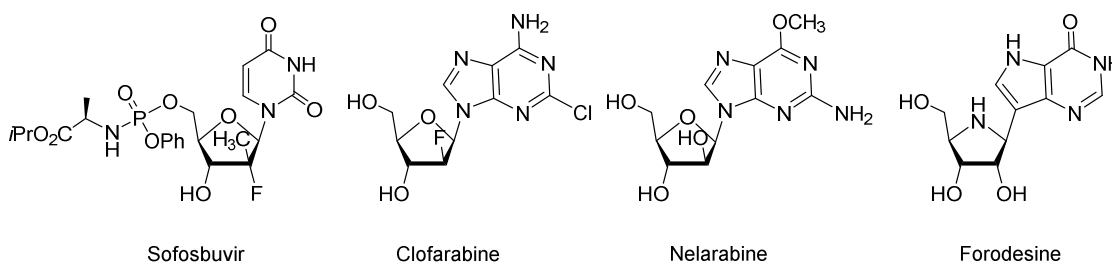


Fig. 1. Sofosbuvir nucleotide and purine nucleoside analogues

Although this class of compounds has been extensively studied for 40 years, particularly for their antiviral activities, their potential in antitumor chemotherapy is underexplored but important, both by the large number of compounds and by their structural variability. Many and various modifications have been introduced at different positions, whether on ribose (deoxy, quaternization, fluoridation ...) or on the nucleobase. Ribose mimetic motifs (carbasugars, iminosugars, acyclic moieties) as well as non natural anomeric bonds such as carbon-carbon links (C-nucleosides) are also found. As far as we are concerned, we are interested in C-

nucleosides, especially in those presenting a triazole as natural base mimetic. This choice was guided by the need of broad-spectrum antivirals, such as ribavirin RBV (Fig. 2). RBV is often prescribed as first-line in hemorrhagic fevers, for example. Ribavirin is a non-naturally occurring nucleoside, described in the 1970s [5,6] and essentially developed in bi-therapy with interferon for the treatment of Hepatitis C [7]. In recent years, like other nucleosides, Ribavirin showed a renewed interest in its antitumor activities as antitumor repositioned drug for multi-target drug development. If the mechanism of action of RBV is multiple, its inhibitor activity on the translation factor eIF4E has been reported [8], as well as its epigenetic action on the expression of targets involved in cancers such as non-Hodgkin lymphoma has been postulated. Ribavirin has also been reported to be active in other forms of cancer (breast [9], thyroid [10], renal cell carcinoma (RCC) [11] in dual therapy with interferon) acute myeloid leukemia (AML) [12] in combination or not with cytarabine. Ribavirin seems particularly relevant as repurposed antiviral agents for anticancer therapy [13]. Moreover RBV is in clinical development for the later pathology; five other clinical trials are currently ongoing [14].

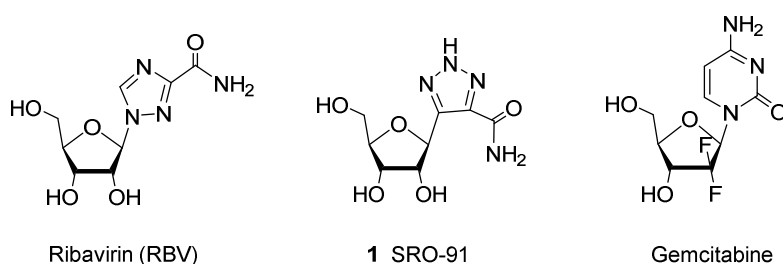


Fig. 2. RBV and its C-nucleoside analogue SRO-91

For all these reasons, we have been interested in the development of a new family of nucleosides structurally closed to RBV such as C-ribosyl triazole carboxamides [15,16]. These compounds present a non-natural C-anomeric linkage providing a greater chemical and enzymatic stability. The potential of C-nucleosides was recently underlined and reported by De Clerq [17]. Moreover, we decided to introduce a 1,2,3-triazole instead of the 1,2,4-triazole heterocycle present in RBV, due to its easy accessibility by the Huisgen cycloaddition and its

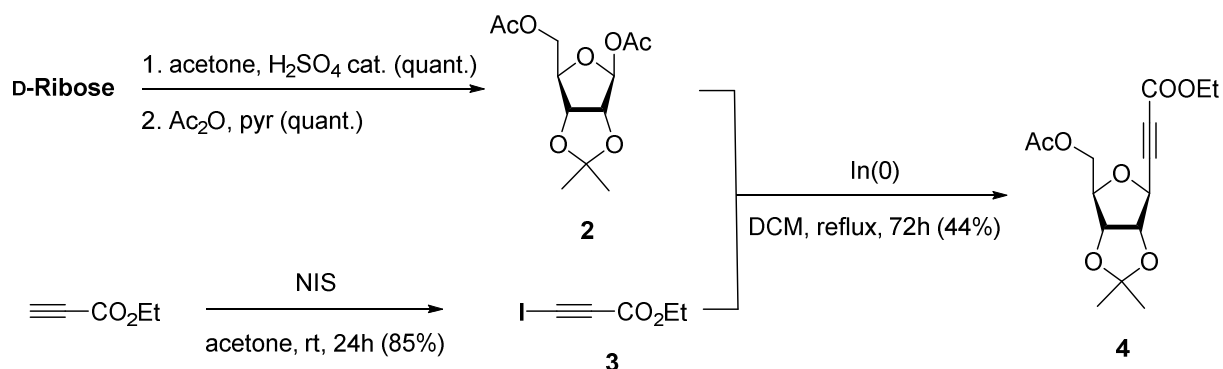
well-known pharmacophore potential [18]. We anticipated that the pharmacological importance of this heterocyclic nucleus will be reinforced by the presence of the carboxamide group [19].

We herein propose to revisit the synthesis of the 5- β -D-ribofuranosyl-1,2,3-triazole-4-carboxamide **1** named SRO-91 (Fig. 2) and to investigate its potential antitumor activities, particularly against the ovarian cancer. For this later, the first-line treatment involves cisplatin chemotherapy, eventually combined with taxoids. But the combination with nucleosides like gemcitabine (Fig. 2) appears relevant since initial resistances to cisplatin therapy disappear [20]. In addition, ovarian cancer cells have been shown to overexpress nucleoside transporters [21]. This observation may allow an efficient intracellular concentration of SRO-91, promoting antitumor activity if the uptake is transporter-dependent as it has been reported for RBV [22]. For all these reasons, we think that the 5-ribosyltriazole-4-carboxamide SRO-91 could present interesting activities.

2. Results and discussion

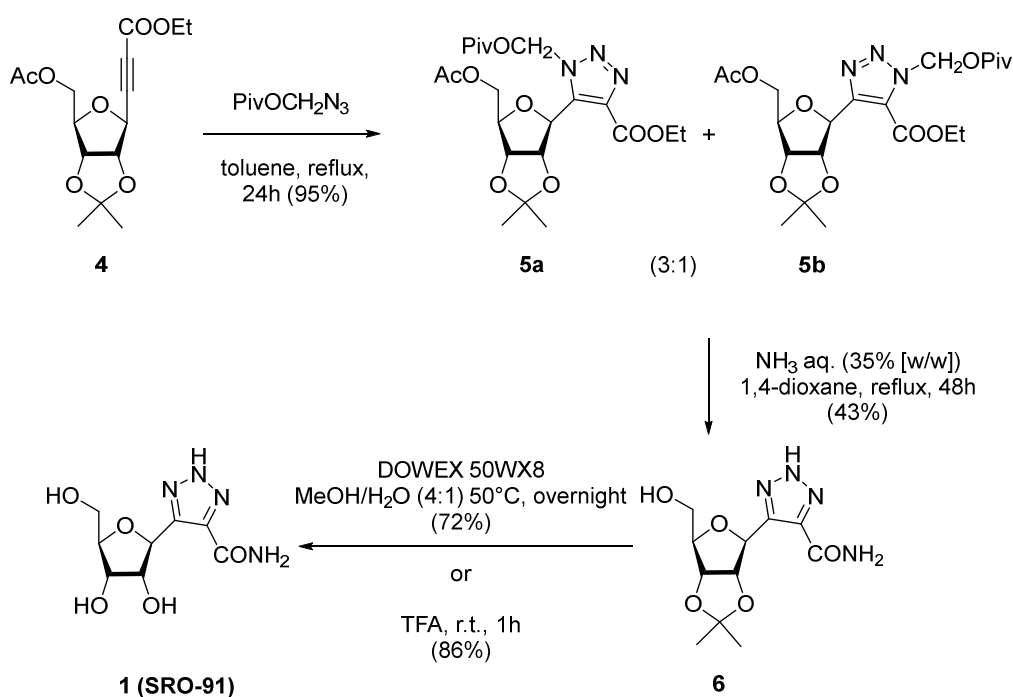
2.1. Chemistry

The synthesis of **1** (SRO-91) is based on two key reactions, namely an indium mediated alkynylation on ribose anomeric position and a Huisgen cycloaddition leading to the triazole moiety. During this work we revisited the synthesis developed in the laboratory in order to simplify the deprotection steps and avoid the palladium catalyzed hydrogenolysis. Alkynyl derivative **4** [25,26] was obtained in Barbier conditions from fully protected ribose **2**, ethyl iodopropiolate (obtained by iodination of ethyl propiolate with NIS) and metallic indium in refluxing DCM for 3 days as illustrated in Scheme 1.



Scheme 1. Preparation of *C*-alkynyl glycoside **4** [25,26]

Ethyl 3-ribose-2,3,5-triacetate **4** was further engaged in Huisgen cycloaddition with azidomethyl 2,2-dimethylpropanoate [27], obtained from chloromethyl 2,2-dimethylpropanoate, resulting in a 3:1 mixture of regioisomers **5a** and **5b**. Next, the mixture of pivaloyloxymethyl-protected triazole nucleosides **5a,b** was transformed by aminolysis to the single nucleoside derivative **6**. In the process of this reaction, pivaloyloxymethyl-protecting group was cleaved, acetyl moiety was aminolyzed and the ester function was amidated in one pot. Finally, the acetonide group removal resulted in target nucleoside **1** (SRO-91) as illustrated in Scheme 2.



Scheme 2. Preparation of nucleoside **1** (SRO-91).

SRO-91 was thus synthesized in 6 steps with 15% overall yield starting from *D*-ribose using this approach. This yield is identical to that obtained according to the benzyl approach previously reported [25]. However the strategy herein disclosed presents the advantage of both avoiding the step of deprotection by hydrogenolysis under pressure (several days under 5 bars with high loading of Pd) and the potential palladium contamination of the final product.

2.2. Biology

2.2.1. Screening on a panel of cancer cell lines

Ribavirine (RBV) and SRO-91 were screened on a panel of nine cancer cell lines. The panel comprised human breast adenocarcinoma (pleural metastasis) cell line (MDAMB231), human melanoma cell lines (MDAMB435) and mouse melanoma cell lines (B16F10), human colorectal carcinoma cell line (Caco2), human hepatocarcinoma cell line (HuH7), prostatic carcinoma cell line (DU145) human (SNB75) and rat (C6) Glioblastoma cell lines and human epidermoid cell line (A431). To evaluate the antiproliferative activity of nucleoside analogues, the MTT test was performed. Interestingly, among the nine cancer lines tested, SRO-91 and RBV respond to all cell lines but the response of RBV is less efficient against MDAMB 231 and DU145 cell lines (Table 1). SRO-91 was the most active compound. Seven cell lines proliferation inhibition reached at least 80% inhibition at the highest concentration tested. HuH7 and DU145 cell lines proliferations were only inhibited by 60% and 70% respectively. For all cell lines, the SRO-91 IC_{50} values are better than the RBV ones. It has to be noticed that for a given cell line the maximum inhibition of proliferation was almost the same range for SRO-91 but with different IC_{50} values. SRO-91 exhibited IC_{50} in the 0.5- 9.5 μ M range while RBV exhibited IC_{50} in the 3- >100 μ M range.

Table 1

Antiproliferative Values (IC₅₀, μM) of the nucleoside analogues on MDAMB231, MDAMB435 (Cell Line Obtained from Breast Cancer Tissue), B16F10 (Cell Lines Obtained from Skin Cancer Tissues), Caco2 (Cell Line Obtained from Colon Cancer Tissue), HuH7 (Cell Line Obtained from Intestine Cancer Tissue), DU145 (Cell Line Obtained from Prostate Cancer Tissue), SNB-75 (human glioblastoma cell line) (SNB75), C6 (rat glioblastoma cell line), A431 (human epidermoid cell line).

	IC ₅₀ values (μM)								
	MDAMB231 ^(a)	MDAMB435 ^(b)	B16F10 ^(c)	Caco2 ^(d)	HuH7 ^(e)	DU145 ^(f)	SNB-75 ^(g)	C6 ^(h)	A431 ⁽ⁱ⁾
RBV	>100	38.3 (±0.2)	3.50 (±0.06)	34.5 (±0.06)	53.7 (±0.1)	>100	18.53 (±0.05)	13.51 (±0.01)	3.18 (±0.03)
SRO-91	9.59 (±0.05)	1.90 (±0.07)	1.52 (±0.05)	8.05 (±0.04)	22.2 (±0.1)	2.14 (±0.07)	9.38 (±0.08)	2.15 (±0.06)	0.58 (±0.12)

^(a) The maximum inhibition of cell proliferation was 40 % at 100 μM for RBV and 80 % at 100 μM for SRO-91

^(b) The maximum inhibition of cell proliferation was 50% at 100 μM for RBV and 80 % at 100 μM for SRO-91

^(c) The maximum inhibition of cell proliferation was 80% at 100 μM for RBV and 80 % at 100 μM for SRO-91

^(d) The maximum inhibition of cell proliferation was 60% at 100 μM for RBV and 90 % at 100 μM for SRO-91

^(e) The maximum inhibition of cell proliferation was 60% at 100 μM for RBV and 60 % at 100 μM for SRO-91

^(f) The maximum inhibition of cell proliferation was 50% at 100 μM for RBV and 85 % at 100 μM for SRO-91

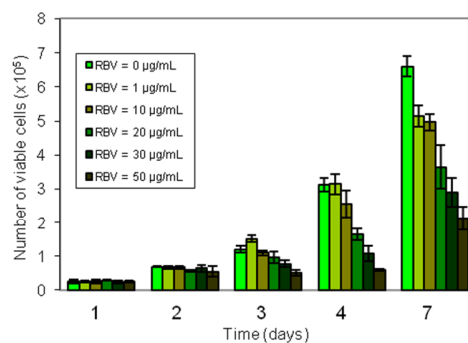
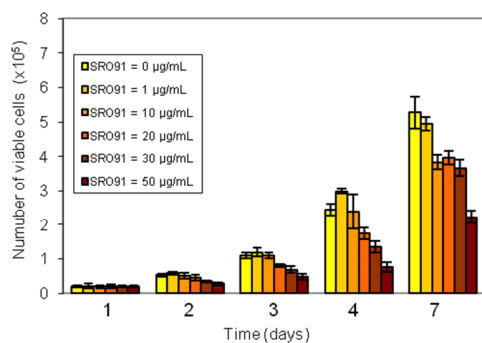
^(g) The maximum inhibition of cell proliferation was 70% at 100 μM for RBV and 70 % at 100 μM for SRO-91

^(h) The maximum inhibition of cell proliferation was 75% at 100 μM for RBV and 85 % at 100 μM for SRO-91

⁽ⁱ⁾ The maximum inhibition of cell proliferation was 70% at 100 μM for RBV and 80 % at 100 μM for SRO-91

We were also interested in ovarian cancer, for which the prognosis is quite unfavorable and the need for therapeutic improvement is important. The hallmark of cancer cells is their significant proliferative capacity. It therefore seemed interesting to study firstly the effect of SRO-91 on ovarian cancer cells (Fig. 3). For this, both cell lines (SKOV3 and IGROV1 cells) were incubated for 7 days in the presence of increasing concentrations of SRO-91 or RBV (0, 1, 10, 20, 30 or 50 μg/mL).

IGROV1



SKOV3

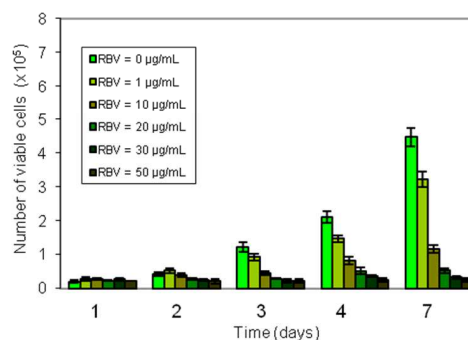
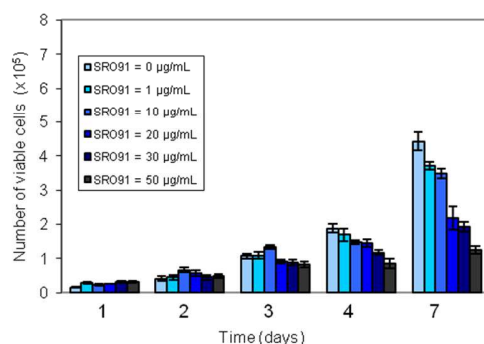


Fig. 3. SRO-91 or RBV effect on viability of ovarian cancer cell. IGROV1 and SKOV3 were exposed to SRO-91 or RBV at 0 to 50 µg/mL for 7 days of culture. The number of viable cells was measured daily by the trypan-blue exclusion test. Results are representative of 3 or more independent experiments done in triplicate, and are expressed as mean ± standard deviation.

These results demonstrate an inhibitory effect of SRO-91 on the proliferation of both cell lines with a different sensitivity to SRO-91. Indeed, it seems that for the same concentration of SRO-91, IGROV1 cells are sensitive earlier than SKOV3 cells but the effect fades over time. This observation is underlined by the IC₅₀ (Table 2).

Table 2

The IC₅₀ of SRO-91 vs RBV on the proliferation of the two ovarian cancer cell lines.

Time (days)	Compound	IC ₅₀ values (µg/mL)	
		SKOV3	IGROV1
3	SRO-91	24.5	23

3	RBV	9	25
4	SRO-91	25.5	22.8
4	RBV	10	21
7	SRO-91	19	23.8
7	RBV	10	16

2.2.2. Study of SRO-91 acute toxicity

SRO-91 acute toxicity studies were initially carried out by immunofluorescence staining of the nuclei and the actin cytoskeleton on the MeT-5A mesothelial cells treated or not with SRO-91 (Figure 4).

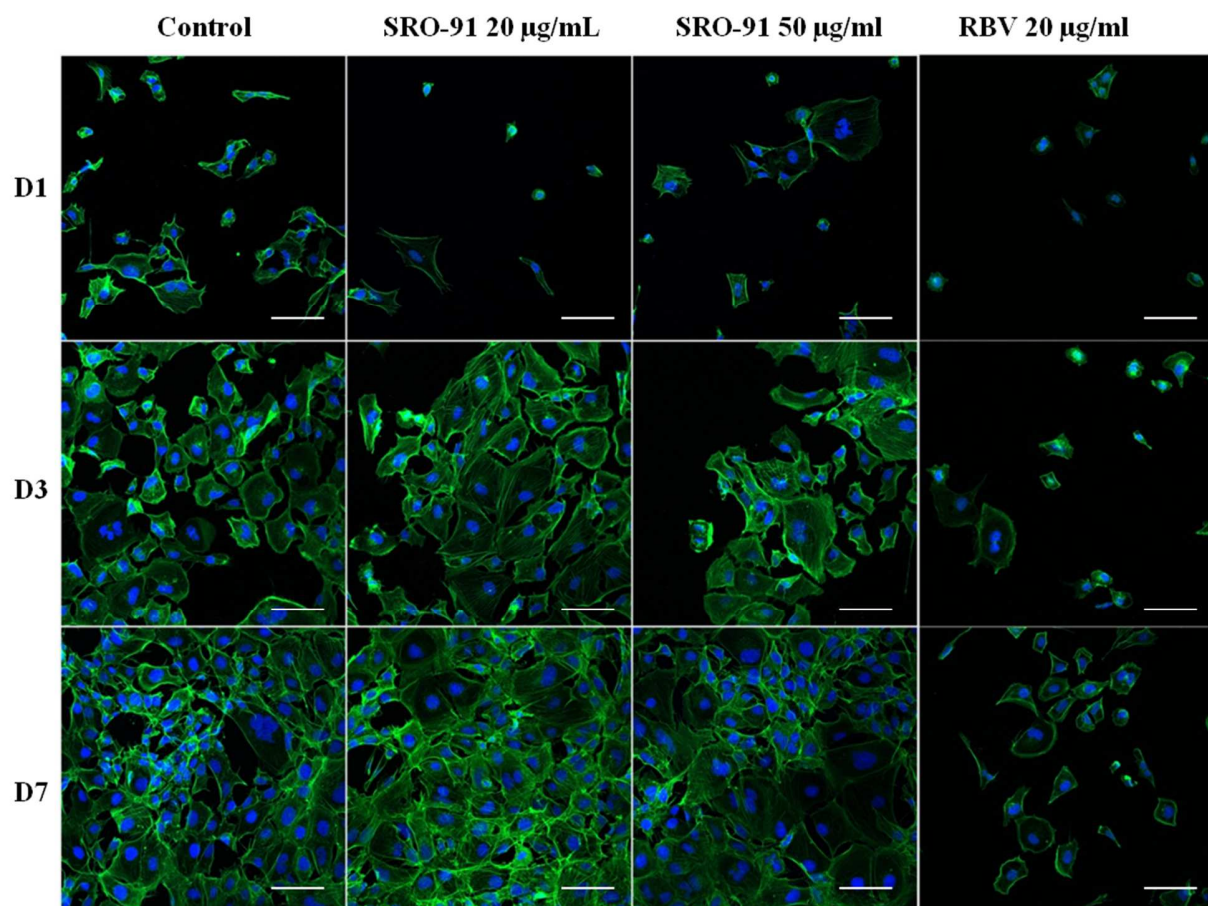


Fig. 4. Cell morphology of healthy mesothelial cells after SRO-91 or RBV treatment. Immunofluorescent staining of actin cytoskeleton (green) expressed by mesothelial cells after SRO-91 or RBV treatment or without treatment (control). Cell nuclei were stained with DAPI (blue). Staining was examined with laser

scanning confocal microscopy. Scale bar is 50 μ m. D1, D3, D7 refer to 1 day, 3 days and 7 days respectively.

As shown in Fig. 4, RBV causes significant mortality of MeT-5A cells, unlike SRO-91, even at higher concentrations of 50 μ g/mL. It therefore seems that SRO-91 is no more effective than RBV but has a much lower cytotoxicity.

The toxicity studies were then pursued *in vivo* to determine the effective and safe dosage using standard guidelines. Limit test was performed with six different dosages, that is, 100, 75, 50, 25, 10 and 5 mg/kg in six groups through single intraperitoneal (i.p.) injection in mice. The control group was housed and fed in similar conditions without injection of SRO-91. Mice were monitored during 24 first hours and daily thereafter for 7 days. Mice mortality and behavior were noted. The results of acute toxicity tests on mice are shown in Table 3.

Table 3

Acute toxicity data of SRO-91 on mice

Mice group	Number of mice	Dose (mg/kg)	Number of death mice after 24h	Number of death mice after 7 days
Control	3	0	0	0
G1	3	5	0	0
G2	3	10	0	0
G3	3	25	0	0
G4	3	50	0	0
G5	3	75	0	0
G6	3	100	0	0

Whatever SRO-91 test doses, no lethal effect was observed throughout the experiment. No abnormal appearance or behavior was noted in the first 24 h. All animals survived until the end of the study with no systemic signs of toxicity evident throughout the entire observation period. No drug-related changes were observed in animal body weight, food consumption and behavior during the 7 days study.

3. Conclusion

In this work the antitumor activity of SRO-91, a C-nucleoside analogue of RBV was studied. Firstly the chemical synthesis of this compound previously obtained in our group, was revisited according to a pivaloyloxymethyl-protecting approach. The study of the antitumor activity of SRO-91 was achieved and revealed interesting IC₅₀ (0.5-9.5 μM) on 9 cancer cell lines. In the case of ovarian cancer, SRO-91 presents a promising antitumor activity of the same order as RBV with a lower cytotoxicity in vitro on the MeT-5A mesothelial cells and with no lethal effect in vivo on mice.

4. Experimental

4.1. Chemistry

All reactions were conducted under an inert atmosphere of argon. Metallic indium was purchased from Alfa Aesar. Dichloromethane was distilled under argon over CaH₂. ¹H and ¹³C NMR spectra were recorded in CDCl₃ or CD₃OD on a Jeol 400 MHz ECX. High resolution mass spectra were obtained with a LTQ orbitrap spectrometer. Optical rotations were determined at 25 °C in CHCl₃ or MeOH, 589 nm, on an Anton Paar MCP200 instrument and IR spectra were recorded on a Bruker Tensor 27 spectrophotometer. Thin layer chromatography was carried out on silica gel plates (Macherey-Nagel), spots were detected with UV light and revealed with H₂SO₄ solution. Flash chromatography was performed with silica gel 60, 40-63 μm.

4.1.1. Ethyl 5-(5'-O-acetyl-2',3'-O-isopropylidene-β-D-ribofuranosyl)-1-((2,2-dimethylpropanoyl)methyl)-1,2,3-triazole-4-carboxylate (**5a**) and Ethyl 4-(5'-O-acetyl-2',3'-O-isopropylidene-β-D-ribofuranosyl)-1-((2,2-dimethylpropanoyl)methyl)-1,2,3-triazole-5-carboxylate (**5b**)

Ribosylpropiolate **4** [25,26] (2 g, 6.41 mmol) and azidomethyl 2,2-dimethylpropanoate (4.03 g, 25.64 mmol) in toluene (15 mL) were stirred under reflux for 24h. After cooling, the reaction mixture was concentrated under reduced pressure. The crude material was purified by flash column chromatography on silica gel (20-25% of EtOAc in cyclohexane) giving a mixture of **5a/5b** (2.86 g, 95%, 3/1 ratio) as yellowish oil. **5a**: [α]_D²⁵ -32.3 (c 1.0, CHCl₃). ¹H NMR (400

MHz, CDCl₃) δ 6.41 (AB, J_{AB} = 10.6 Hz, 2H, NCH₂O), 5.70 (d, $J_{1',2'}$ = 5.0 Hz, 1H, H_{1'}), 4.86 (dd, $J_{2',3'}$ = 7.0, $J_{2',1'}$ = 5.0 Hz, 1H, H_{2'}), 4.75 (dd, $J_{3',2'}$ = 7.0, $J_{3',4'}$ = 5.6 Hz, 1H, H_{3'}), 4.41 (q, J = 7.2 Hz, 2H, OCH₂CH₃), 4.32 (ABX, J_{AB} = 12.2, J_{AX} = 5.2, J_{BX} = 3.4 Hz, 2H, H_{5'}), 4.18 (m, 1H, H_{4'}), 2.07 (s, 3H, OAc), 1.59 (s, 3H, C(CH₃)₂), 1.40 (t, J = 7.2 Hz, 3H, OCH₂CH₃), 1.31 (s, 3H, C(CH₃)₂), 1.17 (s, 9H, C(CH₃)₃). ¹³C NMR (100 MHz, CDCl₃) δ 176.6 (C=O pivaloyloxymethyl), 170.6 (COCH₃), 160.8 (CO₂Et), 138.7 (C₅), 137.8 (C₄), 116.4 (C(CH₃)₂), 84.9 (C_{2'}), 82.8 (C_{4'}), 81.1 (C_{3'}), 76.5 (C_{1'}), 70.3 (NCH₂O), 63.3 (C_{5'}), 61.8 (OCH₂CH₃), 38.9 (C(CH₃)₃), 27.4 (C(CH₃)₂), 26.9 (C(CH₃)₃), 25.6 (C(CH₃)₂), 20.8 (OCH₂CH₃), 14.3 (OCOCH₃). HRMS (ESI) calcd for C₂₁H₃₂N₃O₉ [M+H]⁺: 470.2139; found 470.2133. **5b**: [α]_D²⁵ + 24.5 (c 1.0, CHCl₃). ¹H NMR (400 MHz, CDCl₃) δ 6.50 (AB, J_{AB} = 10.0 Hz, 2H, NCH₂O), 5.56 (d, $J_{1',2'}$ = 3.5 Hz, 1H, H_{1'}), 5.28 (dd, $J_{2',3'}$ = 6.4, $J_{2',1'}$ = 3.5 Hz, 1H, H_{2'}), 4.84 (dd, $J_{3',2'}$ = 6.4, $J_{3',4'}$ = 3.5 Hz, 1H, H_{3'}), 4.41 (q, J = 7.0 Hz, 2H, OCH₂CH₃), 4.28 (m, 2H, H_{5'}), 4.12 (m, 1H, H_{4'}), 2.05 (s, 3H, OAc), 1.59 (s, 3H, C(CH₃)₂), 1.40 (t, J = 7.0 Hz, 3H, OCH₂CH₃), 1.37 (s, 3H, C(CH₃)₂), 1.16 (s, 9H, C(CH₃)₃). ¹³C NMR (100 MHz, CDCl₃) δ 176.7 (C=O pivaloyloxymethyl), 170.8 (COCH₃), 157.7 (CO₂Et), 148.7 (C₄), 126.5 (C₅), 114.5 (C(CH₃)₂), 84.3 (C_{2'}), 83.6 (C_{4'}), 82.7 (C_{3'}), 77.9 (C_{1'}), 71.3 (NCH₂O), 64.2 (C_{5'}), 62.7 (OCH₂CH₃), 38.9 (C(CH₃)₃), 27.4 (C(CH₃)₂), 26.9 (C(CH₃)₃), 25.6 (C(CH₃)₂), 20.9 (OCH₂CH₃), 14.1 (OCOCH₃). HRMS (ESI) calculated for C₂₁H₃₂N₃O₉ [M+H]⁺: 470.2139; found 470.2133.

4.1.2. 5-(2',3'-O-isopropylidene- β -D-ribofuranosyl)-2H-1,2,3-triazole-4-carboxamide (**6**)

A solution of pivaloyloxymethyl-protected nucleosides **5a,b** (1.95 g, 4.15 mmol) in the mixture of 1,4-dioxane (8 mL) and aqueous ammonia (35% w/w, 24 mL) was placed into a sealed tube and stirred under reflux for 48h. Upon cooling, the mixture was concentrated under reduced pressure, co-evaporated several times with water under high vacuum to get rid of residual pivalamide. The crude material was then purified by flash column chromatography on silica gel (10% of MeOH in DCM) yielding the nucleoside **6** (510 mg, 43%) as a white crystalline solid. m.p. = 64-66°C. [α]_D²⁵ -7.8 (c 1.0, MeOH). ¹H NMR (400 MHz, MeOD) δ 5.50 (d, $J_{1',2'}$ = 4.4 Hz, 1H, H_{1'}), 4.92 (dd, $J_{2',3'}$ = 6.5, $J_{2',1'}$ = 4.4 Hz, 1H, H_{2'}), 4.77 (dd, $J_{3',2'}$ = 6.5, $J_{3',4'}$ = 3.9 Hz, 1H, H_{3'}), 4.10 (ddd, $J_{4',5'}$ = 4.5, $J_{4',5'}$ = 4.4, $J_{4',3'}$ = 3.9 Hz, 1H, H_{4'}), 3.67 (ABX, J_{AB} = 12.1, J_{AX} = 4.5, J_{BX} = 4.4 Hz, 2H, H_{5'}), 1.52 (s, 3H, C(CH₃)₂), 1.29 (s, 3H, C(CH₃)₂). ¹³C NMR (100 MHz, MeOD): δ 164.8 (CONH₂), 144.8 (C₅), 139.1 (C₄), 115.5 (C(CH₃)₂), 87.1 (C_{2'}),

86.3 (C_{4'}), 82.9 (C_{3'}), 79.2 (C_{1'}), 63.0 (C_{5'}), 27.7 (C(CH₃)₂), 25.7 (C(CH₃)₂). HRMS (ESI) calcd for C₁₁H₁₇N₄O₅ [M+H]⁺: 285.1199; found 285.1202.

4.1.3. 5-(β-D-ribofuranosyl)-2H-1,2,3-triazole-4-carboxamide (**1**)

Procedure A. To a solution of isopropylidene protected nucleoside **6** (114 mg, 0.4 mmol) in methanol/water (4:1, 5 mL) DOWEX 50WX8 (2 g) was added and the mixture was stirred at 50°C overnight. Upon cooling the mixture was filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (0-50% MeOH in EtOAc) affording the final nucleoside **1** (70 mg, 72%) as a white crystalline solid. **Procedure B.** Isopropylidene protected nucleoside **6** (85 mg, 0.3 mmol) was dissolved in TFA (3 mL) and stirred for 1 h at room temperature. Then the solvent was repeatedly co-evaporated with methanol under reduced pressure. The residue was purified by flash column chromatography on silica gel (0-50% MeOH in EtOAc) affording the final nucleoside **1** (84 mg, 86%) as a white crystalline solid. m.p. = 204-206 °C. $[\alpha]_D^{25} +13.8$ (c 1.0, CH₃OH). ¹H NMR (400 MHz, D₂O) δ 5.25 (d, $J_{1',2'} = 5.4$ Hz, 1H, H_{1'}), 4.26 (dd, $J_{2',1'} = 5.4$, $J_{2',3'} = 5.4$ Hz, 1H, H_{2'}), 4.16 (dd, $J_{3',2'} = 5.4$, $J_{3',4'} = 5.4$ Hz, 1H, H_{3'}), 4.06 (ddd, $J_{4',3'} = 5.4$, $J_{4',5'} = 4.7$, $J_{4',5'} = 3.0$ Hz, 1H, H_{4'}), 3.80 (ABX, $J_{AB} = 12.5$, $J_{AX} = 4.7$, $J_{BX} = 3.0$ Hz, 2H, H_{5'}). ¹³C NMR (100 MHz, CD₃OD) δ 165.6 (CONH₂), 144.1 (C₅), 138.4 (C₄), 86.1 (C_{2'}), 78.3 (C_{4'}), 77.5 (C_{3'}), 72.1 (C_{1'}), 62.8 (C_{5'}). HRMS (ESI) calcd for C₈H₁₂N₄O₅ [M+H]⁺, 244.0808; found, 244.0811.

4.2. Cells and cells culture

Lyophilized Ribavirin (Virazol®) was purchased from TCI EUROPE NV (Zwijndrecht, Netherlands).

MDA-MB-231, MDA-MB-435, B16F10, Caco2, HuH7 and DU145 cells were obtained from the American Type Culture Collection. The C6 and SNB75 cells were a gift from N. Bakalara (INM). These cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U mL⁻¹ streptomycin.

Cell viability was evaluated using the MTT microculture tetrazolium assay. 22 Cells were seeded at a density of 5x10⁴ cells/well in 96-well flat-bottom plates (Falcon, Strasbourg, France)

and incubated in complete culture medium for 24h. Then, medium was removed and replaced by 2% FCS (Fetal Calf Serum)-medium containing increasing concentrations of SRO-91 and RBV. After 72h incubation, cells were washed with phosphate buffered saline (PBS, Life Technologies) and incubated with 0.1 mL of MTT (2 mg/mL, Sigma-Aldrich) for additional 4h at 37°C. The insoluble product was then dissolved by addition of DMSO (Sigma-Aldrich). Optical density was measured at 570 nm using a Labsystems Multiskan MS microplate reader. The presented experiments were carried out at least in triplicate. Depending on the number of independent variables Student's or ANOVA statistical tests was applied, using PRISM software. EC₅₀ values and K_i were calculated using the Hill equation of the dose–log response curves.

The human ovarian adenocarcinoma cell line, IGROV1, was kindly provided by Dr. J. Bénard, and SKOV3 cell lines were kindly supplied by Dr L. Poulain from the BioTICLA team INSERM U1086 (Biologie et Thérapies Innovantes des Cancers Localement Agressifs, Caen, France). The cells were grown in RPMI-1640 glutaMAX (Life technologies, France) containing 0.07 % (v/v) sodium bicarbonate supplemented with 10 % fetal bovin serum (FBS, Biosera, France) in a humidified air atmosphere with 5 % CO₂ at 37°C. The two well characterized human ovarian cancer cell lines IGROV1 and SKOV3 were used for their ability to mimic the progression of ovarian carcinoma when injected into in vivo mouse models [23].

In proliferation assay, cells were seeded in 24-wells at 20 000 cells/well and exposed to various concentrations of SRO-91 or ribavirin (0, 1, 10, 20, 30 or 50 µg/mL) during 7 days of culture and the number of viable cells was evaluated each day by trypan exclusion test. Results are representative of at least three independent experiments performed in triplicate and are expressed as mean ± standard deviation.

4.3. Acute toxicity studies

Experiments were performed on male 6 week-old Swiss mice (Janvier Laboratories, France). All experiments were approved by the Charles Darwin Ethics Committee for Animal Experimentation and were carried out in accordance with Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 and French law (2013/118). All efforts were made to minimize the number of animals used and their suffering. The Swiss mice were randomly divided into seven groups (3 mice in each group). All the mice were acclimatized for

5-7 days before the experiment. A 7-day acute oral toxicity study was performed to evaluate the acute toxic effects of SRO-91. Mice were given single intraperitoneal injection of SRO-91 dose and the mice were observed for 7 days.

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