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Sudemycin K: a synthetic anti-tumor splicing inhibitor variant with improved activity and versatile chemistry

Kamil Makowski[§,≠,∫,†], Luisa Vigevani[§,‡,∫], Fernando Albericio[≠,#,ǂ,†], Juan Valcárcel[*, §, ‡, ¥], Mercedes Álvarez[*,≠,ǂ,∞]

[§]. Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Dr. Aiguader 88, 08003 Barcelona, Spain

[≠]. Institute for Research in Biomedicine (IRB-Barcelona), Baldiri i Reixac 10, 08028, Barcelona, Spain

[‡]. Universitat Pompeu Fabra (UPF), Dr. Aiguader 88, 08003 Barcelona, Spain

[#]. Department of Organic Chemistry, Faculty of Chemistry, University of Barcelona, Martí Franqués 1, 08028 Barcelona,Spain

[¥]. ICREA, Pg. Lluís Companys 23, 08010 Barcelona, Spain

ǂ. CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona, Spain

[∞]. Laboratory of Organic Chemistry, Faculty of Pharmacy, University of Barcelona, Joan XXIII s/n, 08028 Barcelona, Spain

∫. Equal contribution

* Corresponding authors

ABSTRACT: Important links exist between the process of pre-mRNA splicing and cancer, as illustrated by the frequent mutation of splicing factors in tumors and the emergence of various families of anti-tumor drugs that target components of the splicing machinery, notably SF3B1, a protein subunit of spliceosomal U2 small nuclear Ribonucleoprotein Particle (snRNP). Sudemycins are synthetic compounds that harbor a pharmacophore common to various classes of splicing inhibitors. Here we describe the synthesis and functional characterization of novel Sudemycin analogues that functionally probe key functional groups within this pharmacophore. Our results confirm the importance of a conjugated diene group and in addition reveal significant spatial flexibility in this region of the molecule. **Sudemycin K**, a derivative that replaces the pharmacophore's oxycarbonyl by an amide group, displays improved potency as an inhibitor of cancer cell proliferation, as a regulator of alternative splicing in cultured cells and as an inhibitor of *in vitro* spliceosome assembly. Sudemycin K displays higher stability, likely related to the replacement of the oxycarbonyl group, which can be substrate of esterases, by an amide group. The activity and special reactivity of **Sudemycin K** can pave the way to the synthesis and evaluation of a variety of novel Sudemycin derivatives.

The high incidence of cancer and severe limitations in current therapies (e.g. side effects and drug resistance) make the identification of new drugs and targets an area of intense investigation in oncology. Several small molecules targeting components of the RNA splicing machinery have been shown to display antitumor properties[1-3]. Of relevance, recent findings indicate that the splicing machinery can indeed be limiting for the proliferation of cancer cells and consequently splicing inhibition can confer therapeutic vulnerability to Myc oncogene-driven cancers[4, 5].

RNA splicing is the process by which introns are removed from messenger RNA precursors (pre-mRNAs) and is achieved by the spliceosome, composed of five small nuclear RiboNucleoProtein complexes (U1, U2, U4, U5 and U6 snRNPs) and more than 200 additional polypeptides[6, 7]. Introns are recognized via specific sequence signals located at their boundaries: a short (6-8 nucleotides) consensus at the 5' splice site (5'ss) and three sequence elements at the 3' splice site (3'ss). The latter include the branch point sequence (containing an adenosine involved in 2'-5' phosphodiester bond formation with the 5' end of the intron after the first catalytic step of the splicing reaction), a polypyrimidine tract and a conserved AG dinucleotide at the 3' end of the intron. The first steps of spliceosome assembly include the recognition of the 5'ss by U1 snRNP and of the branch point sequence by U2 snRNP, both involving base-pairing interactions between the corresponding small RNA components (snRNAs) and the pre-mRNA[6, 7].

SF3B1 is a protein component of SF3B, a subcomplex within U2 snRNP implicated in branch point recognition. Mutations in SF3B1, as well as in other 3' splice site-recognizing factors, are recurrent in cancer[1, 8]. SF3B1 mutations are particularly frequent in Myelodysplastic Syndromes with Refractory Anemia and Ring Sideroblasts (RARS)[9, 10] and in Chronic Lymphocytic Leukemia (CLL)[11-13]. In CLL, SF3B1 mutants correlate with resistance to chemotherapy and poor prognosis[11-13]. Notably, SF3B1 was identified as the physical target of drugs that display higher cytotoxicity in tumor cells than in normal cells and are therefore promising therapeutic candidates[1, 14-18].

Several natural compounds isolated from bacterial fermentation products display these properties, including FR901464, Pladienolides, FD-895, GEX1A, Herboxidiene and Thailanstatines[19-23]. Stabilized derivatives SSA (Spliceostatin A, FR901464-related) and E7107 (Pladienolide-related) were shown to inhibit splicing and bind tightly to the SF3B complex[17, 18]. Similar results were obtained for Herboxidiene[15]. Thus, the SF3B complex has emerged as a target of representative drugs in each of the three main classes of natural compounds (Spliceostatin, Pladienolides and Herboxidienes). The Spliceostatin analogue Meayamycin was shown to display antitumor effects at picomolar concentrations[14].

The drug Spliceostatin A binds SF3B1 and prevents its interaction with the pre-mRNA, concomitant with interactions of U2 snRNA with "decoy" sequences upstream of their productive binding site at the branch point sequence[24]. In addition, the drug E7107 alters the balance between alternative U2 snRNA conformations, also destabilizing U2 snRNP recruitment[25]. Interestingly, cancer-associated SF3B1 mutations induce cryptic 3' splice site selection through the use of upstream branch points[26, 27]. Thus, SF3B1 appears to be involved in multiple interactions important for U2 snRNP binding that are relevant for the control of cell proliferation and apoptosis.

How can drugs targeting a core component of the splicing machinery not result in general cellular toxicity? Tumor cells often display an altered balance of alternative isoforms that prevent apoptosis, promote proliferation and invasion[28]. Transcriptome-wide analyses have identified drug-induced changes in alternative splicing that particularly affect genes involved in cell division, apoptosis and cancer progression[24], suggesting that these compounds differentially affect alternative splice sites. Moreover, recent results indicate that these drugs can have beneficial therapeutic effects for chronic lymphocytic leukemia (CLL) and for melanoma cells displaying drug resistance[29, 30]. Notably, leukemic cells with spliceosomal mutations display also increased sensitivity to splicing inhibitors[31, 32].

A well-characterized alternative splicing event relevant for anti-tumor drug function involves inclusion/skipping of exon 2 in the three-exon gene coding for Myeloid Cell Leukemia 1 (MCL1) proteins. This protein belongs to the Bcl-2 family of apoptosis regulators, displays anti-apoptotic functions and is overexpressed in several tumors[33-35]. Due to its rapid turnover both at protein and RNA levels, MCL1 is highly affected by transcription and translation inhibitors, causing death of some tumor cells depending on the levels and activity of Bcl-X, another alternatively spliced apoptotic regulator[35]. Exon 2 skipping leads to the production of a pro-apoptotic protein[36] and could therefore facilitate therapeutic effects. Interestingly, recent studies show that *MCL1* is highly sensitive to splicing inhibition, as depleting several splicing factors induces *MCL1* exon 2 skipping[37-39]. Indeed, *MCL1* alternative splicing was found to be the most affected by SF3B1-targeting splicing inhibitors among a panel of alternative splicing events involved in proliferation and apoptosis control[39]. Indeed, Spliceostatin A induces apoptosis in chronic lymphocytic leukemia (CLL) cells through *MCL1* downregulation[29] and resistant cell lines reacquire sensitivity to Bcl-X-targeting drugs when treated with Meayamycin, due to *MCL1* regulation[40].

Despite their very different overall skeletons, SF3B-targeting molecules share a common pharmacophore[41], which includes a conjugated diene, an epoxide and an oxycarbonyl group (Figure 1). While the epoxide group was found not to be absolutely required for activity, it contributes to increase it[19, 42-44]. Based in this pharmacophore, a total synthetic compound series known as Sudemycins has been described[45]. In spite of their simplified structure, containing up to 6 sterocenters less that natural products, these drugs retain potent anticancer activity *in vitro* and *in vivo*[45], as well as the ability to target SF3B1[15]. Previous extensive structure-activity relationship (SAR) studies reported by the Webb's group led to the synthesis of stable active derivatives, described as **Sudemycin C1** (cyclohexane core) and **Sudemycin E** (dioxane core)[41, 45, 46] (Figure 1). Challenging synthetic hurdles included the development of a synthetic route for the heterocyclic spiro moiety with two stereocenters, present in all the Sudemycins, and the diene linker with *E,E* configuration. The sterocenter in position 2 of the pyrane ring was induced by organocatalic reduction of double bond using McMillan catalyst and the spiroepoxide was prepared by diastereoselective introduction of dimethylsulfoxonium methylide to the ketone. The key step for preparation of diene was the Julia-Kocienski olefination. In a recent study[47], the synthetic route was revised and the Julia-Kocienski step was optimized by shifting the sulfone and aldehyde group positions required for the olefination, which in comparison to the previously described procedures resulted in better diastereoselectivity and yield. Additionally, new Sudemycin derivatives, mostly with ester moiety modification were reported, among them **Sudemycin D6**, which is the most potent Sudemycin so far, displaying improved solubility[47], bearing a methylcarbamate group instead of the isobutyric group present in Sudemycins C1 and E (Figure 1).

With the aim of further exploring the chemical space of this family of drugs, we have designed and synthesized several novel Sudemycin analogues aimed to probe key chemical features and exploring possibilities for further derivatization of the structural frame.

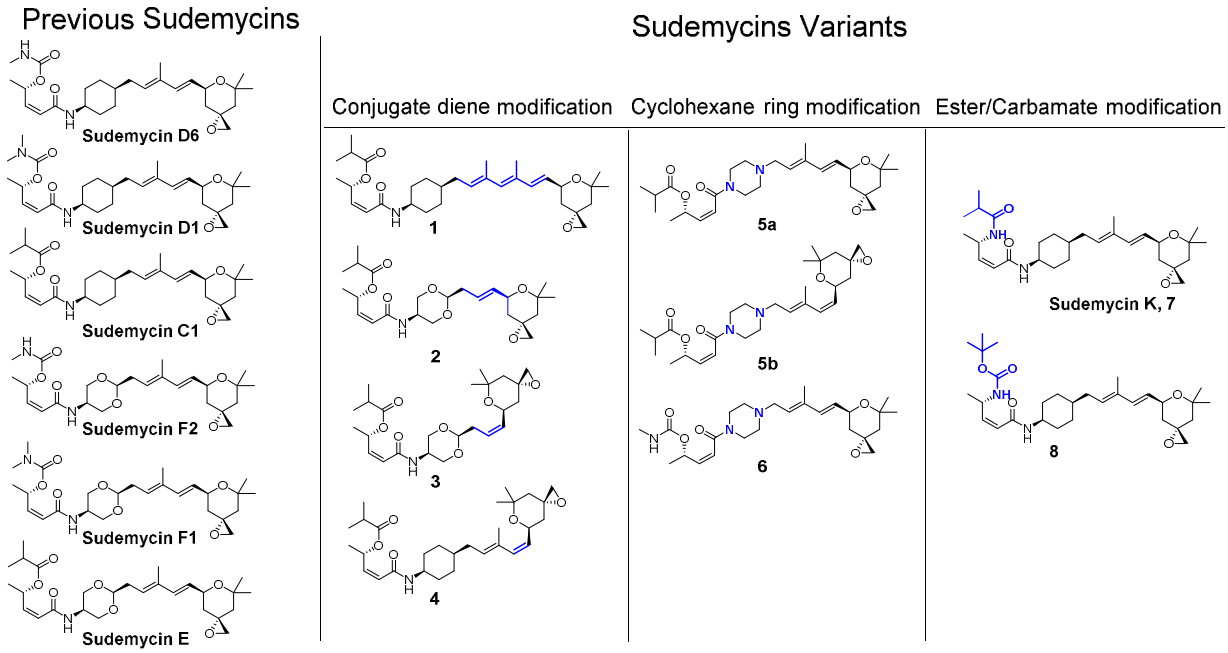


Figure 1. Chemical structure of Sudemycins and variants tested in this study. The general feature of each class of modification is indicated and modifications highlighted in blue. **Sudemycin F2** was previously described as compound **19n**[47].

RESULTS AND DISCUSSION

We aimed to test: a) the function of the conjugated diene, which is part of the common pharmacophore of three classes of splicing inhibitors, b) the function of the oxycarbonyl moiety, another key element of the pharmacophore, and c) the identity of the ciclohexane ring that links the previous two moieties. In addition, the most active previously described **Sudemycins D6**, **D1**, **C1**, **F1**, **F2**[47], and **E** (Figure 1), were prepared in parallel, using procedures reported by the Webb's group[47] and used for biological activity comparison with the new derivatives.The epoxide group was not modified in our study because previous work already showed that it contributes, but is not absolutely required for activity[19, 42-44].

The activity of the compounds was tested in *in vitro* biochemical assays of spliceosome (complex A) assembly and in cell culture assays by assessing *MCL1* alternative splicing regulation and cytotoxicity. The structure of the drug variants and their activities are summarized in Figure 1 and Table 1, respectively.

Conjugated diene modifications

Previous studies indicated that the conjugated diene is important for the activity of Sudemycins and other drugs[41]. To test the relevance of this moiety's length, **1,** the triene harboring three E conjugate double bonds, and **2**, a derivative harboring only one E double bond, were obtained. To evaluate the importance of the stereochemistry of double bonds for biological activity, additional compounds **3** and **4**, harboring a double bond in Z configuration, were also prepared.

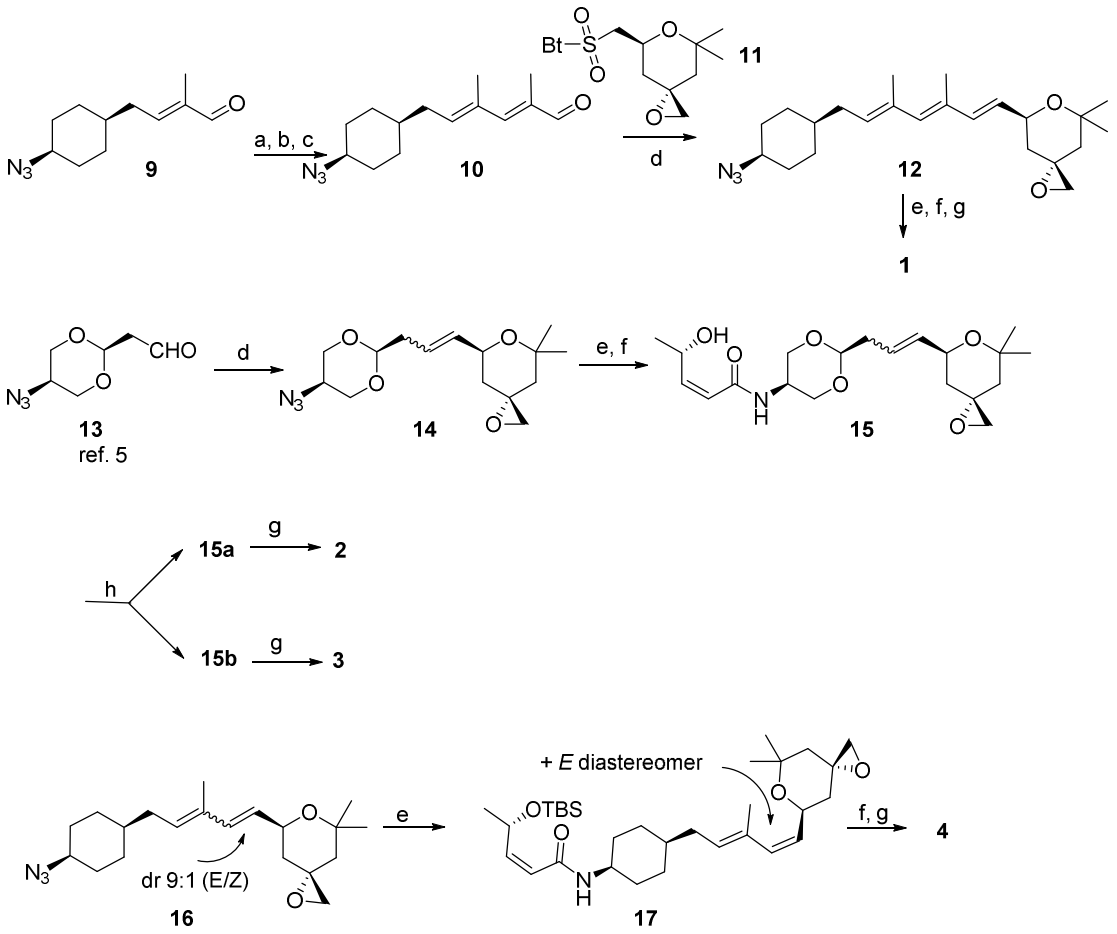
The synthesis of triene **1** was performed (Scheme 1) using the known aldehyde **9**[47] as starting material. Transformation of aldehyde **9** into **10** required three synthetic steps: Wittig elongation with Ph_3P=CH-CO_2Et prepared *in situ* from corresponding phosphonium salt; reduction of the ester to allylic alcohol; and oxidation of alcohol to generate aldehyde **10**. Modified Julia-Kocienski olefination between the aldehyde **10** and sulfone **11**[47] afforded triene **12** with excellent diastereoselectivity (E/Z ratio 96:4). The transformation of compound **12** into **1** required the following steps: chemoselective reduction of azide functional group of **12** to amine followed by coupling with (*S*,*Z*)-4-(*tert*-butyldimethylsilyloxy)pent-2-enoic acid[48]; alcohol deprotection; and formation of ester with isobutyric anhydride. During the amide formation, isomerisation of *Z* double bond in alpha position was produced *(Z*/*E* ratio 7:3). The *Z*/*E* mixture was purified by RP-HPLC and pure **1** was obtained.

The preparation of **2** with only one double bond between dioxane ring and spiro moiety started from aldehyde **13**[46] and follows a similar sequence of reactions as described for **1**. However, the generation of olefin **14** by Julia-Kocienski was not diastereoselective. Diasteromeric mixture of *E* and *Z* olefins was converted to alcohol **15** in three steps and then both diastereoisomers were separated using RP-HPLC semi-preparative technique. Stereochemistry of *E* and *Z* double was assigned by ^{13}C NMR. Sterically compressed carbon nuclei produce shielding effects[49], thus, in *Z* olefin shows 33.7 and 64.6 ppm chemical shift of carbons affected and *E* olefin 38.3 and 69.1 ppm respectively. Finally, esterification of both alcohols led to corresponding isobutyric esters **2** and **3**.

Improved protocol of preparation of diene **16** developed by Webb produces diasteromeric mixture *E,E* and *E,Z* with ratio 9:1. In our hands this mixture as well as TBS protected alcohol could be separated by column chromatography and a pure sample of **17** was collected and converted into **Sudemycin C1** diastereoisomer, compound **4** (Scheme 1)**.**

When these derivatives were tested for activity, reduction to a single double bond (in both *E* and *Z* diasteroisomer configurations, **2** and **3**, Figure 1) completely suppressed drug's activity, while the **1** triene displayed highly reduced but still detectable activity in splicing assays, but negligible in cytotoxicity assays (Table 1, Figure 2G and 2H, Figure 3). On the other hand, the *Z,E,Z* diastereomer of **Sudemycin C1**, harboring opposite stereochemistry at the double bond (**4**, Table 1, Figures 1, 2A, 2B and 3) displays lower but still significant activities (particularly in cytotoxic assays) despite the dramatic change in spatial orientation of key pharmacophore components (Table 1).

Scheme 1. Syntheses of 1, 2, 3 and 4



a) (1-Ethoxy-1-oxopropan-2-yl)triphenylphosphonium bromide, *t*-BuOK, CH_2Cl_2, 0 °C to rt, 50%; b) DIBALH, CH_2Cl_2, -78 °C, 93%; c) Dess-Martin periodinane, CH_2Cl_2, 0 °C, 70%; d) **11**, NaHMDS, THF, −78 °C to rt, 80% for **12** , 42% for **14**; e) 1) Ph_3P, benzene, 55 °C 2) (*S,Z*)-4-(*tert*-butyldimethylsilyloxy)pent-2-enoic acid, HBTU, NEt_3, ACN, 0 °C to rt 81% for **15**, 75% for **17**; f) TBAF, THF, 0 °C to rt, 77% for **15**, 80% for **4**; g) isobutyric anhydride, NEt_3, 4-DMAP, CH_2Cl_2, 0 °C 79% for **2**, 76% for **3**, 80% for **4**; h) RP-HPLC semi-preparative separation. Bt = 2-benzo[*d*]thiazole

Cyclohexane substitution by piperazine or 1,3-dioxane.

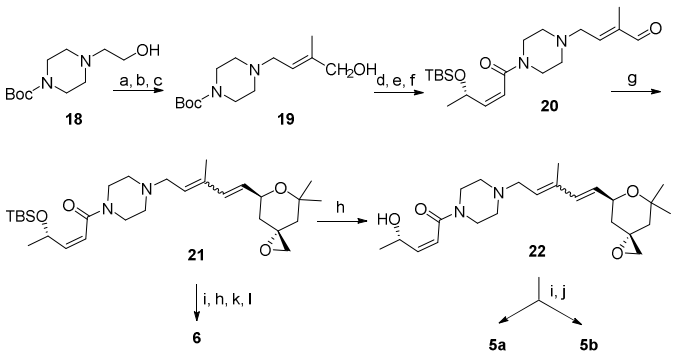
In previous studies, substitution of the cyclohexane ring by a dioxane improved drug solubility[46]. With the aim of further increasing aqueous solubility, three novel compounds harboring piperazine rings were synthetized (**5a**, **5b** and **6**, Figure 1).

The formation of the Sudemycins derivatives with piperazine core (Scheme 2) started from commercially available *tert*-butyl 4-(2-hydroxyethyl)piperazine-1-carboxylate (**18**). Chain elongation of **18** and transformation into alcohol **19** as unique *E* diastereoisomer required: Swern oxidation of **18**; Wittig olefination with $Ph_3P{=}CH\text{-}CO_2Et$; and reduction of ester using DIBALH. Confirmation of *E* stereochemistry was possible based on lack of signals when performing 1D-NOE irradiation at $\delta = 6.8$ ppm and $\delta = 1.8$ ppm (see the Supporting Information). At this point, BOC protecting group was removed with TFA and free secondary amine was coupled with (*S,Z*)-4-(*tert*-butyldimethylsilyloxy)pent-2-enoic acid and then alcohol was oxidized with Dess-Martin periodinane, generating **20**. This aldehyde and sulfone **5** were used for Julia-Kocienski olefination, rendering **21** (*dr* 8:2). Removal of TBS protecting group and esterification with isobutyric anhydride of **22** produce a mixture of final compounds **5a** and **5b** that were separated by semi-preparative RP-HPLC. In order to prepare car-

bamate **6**, the diastereomeric mixture of **21** was first purified, then TBS group was removed, the alcohol was activated as a carbonate with nitrophenylcholoroformate, and finally treated with methylamine to obtain the desired carbamate.

These compounds showed very low activity in splicing or cell proliferation tests and no detectable activity *in vitro* assays, revealing the requirement of the cyclohexane ring for Sudemycin's function (Table 1, Figures 2G, 2H and 3). In agreement with previous results[47], substitution of **Sudemycin D6** cyclohexyl group by a dioxane also reduced strongly the drug's activity (**Sudemycin F2**[47], Figure 1 and Table 1, Figure 3, Supplementary Figure 1A and 1C).

Scheme 2. Syntheses of 5a, 5b and 6



Reagents and conditions: a) $(COCl)_2$, DMSO, CH_2Cl_2, -78 °C, then Et_3N -78 °C to rt; b) (1-ethoxy-1-oxopropan-2-yl)triphenylphosphonium bromide, *t*-BuOK, CH_2Cl_2, 0 °C to rt 65% (2 steps); c) DIBALH, CH_2Cl_2, -78 °C, 78%; d) TFA, CH_2Cl_2, 0 °C to rt; e) (*S,Z*)-4-(*tert*-butyldimethylsilyloxy)pent-2-enoic acid, HBTU, NEt_3, ACN, 0 °C to rt, 90% (2 steps); f) Dess-Martin periodinane, CH_2Cl_2, 0 °C, 78%; g) **11**, NaHMDS, THF, −78 °C to rt, 70%; h) TBAF, THF, 0 °C to rt, 67%; i) semi-preparative RP-HPLC purification j) isobutyric anhydride, NEt_3, 4-DMAP, CH_2Cl_2, 0 °C, 98%; k) 4-nitrophenyl chloroformate, NEt_3, CH_2Cl_2, 0 °C to rt, 45%; l) methylamine, $ClCH_2CH_2Cl$, 0 °C to rt, 75%.

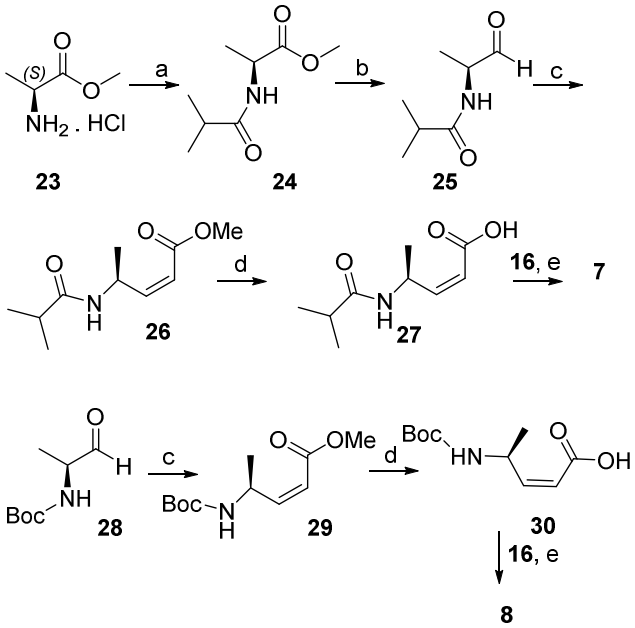
Oxycarbonyl group modifications: Sudemycin K

The oxycarbonyl group is another key element of the common pharmacophore. As reported by Webb and colleagues[46], a free alcohol at this position dramatically decreases the activity compared to ester derivatives. To further explore other chemical moieties at this position, we prepared compounds with amide or carbamate groups instead of ester (**Sudemycin K** or **7** and **8,** Figure 1). As amide groups are less susceptible to hydrolysis than ester groups, these compounds might display higher stability and efficacy.

Sudemycin K was obtained by reaction of the acid **27** with the amine previously obtained in the reduction of azide **16** (Scheme 3). The acid **27** was obtained from commercially available *L*-alanine methyl ester hydrochloride by transformation into amide **24** followed by ester reduction to aldehyde **25**. Olefination of **25** to give ***Z*-26** was achieved using Still-Gennari protocol with good diastereoselectivity (*dr* = 92:8). The hydrolysis of ester ***Z*-26** using conventional methods (LiOH, NaOH) led to decomposition; however, using milder reagents like trimethyltin hydroxide, the desired acid with moderate yield was obtained. Acid **30** needed for the preparation of carbamate **8** was obtained in a similar way (Scheme 3) starting from commercial aldehyde **28**. The main advantage of this synthetic process lies in greater convergence and fewer synthetic steps than previously described, with a similar yield.

Replacing the oxycarbonyl by an amide group (**Sudemycin K**, Figure 1) resulted in a compound with higher activity than **Sudemycin D6**, the most potent Sudemycin described so far, both in biochemical spliceosome assembly assays as well as in cellular assays for *MCL1* alternative splicing and for cytotoxicity (Table 1, Figures 2C-2F and 3). The solubility of Sudemycin K and D6 were found to be comparable (Supplementary Information). Because the higher activity of **Sudemycin K** was observed both in short-term alternative splicing regulation of *MCL1* and in long-term increased cytotoxicity assays, the improved activity is likely contributed both by direct effects on the splicing machinery and by improved cell permeability or *in vivo* stability. The replacement of the ester by an amide group could make **Sudemycin K** less sensitive to esterases, present for example in plasma and microsomes[50] and believed to be the main factor responsible for the observed short *in vivo* half-life of Sudemycin C1[45]. Indeed, we observed higher stability of this compound upon incubation in culture medium containing fetal bovine serum (Figure 4). Replacing the isopropyl by a carbamate (**8**, Figure 1) strongly reduced the compound activity to levels undetectable in *in vitro* biochemical assays and very low -but still detectable- in cytotoxicity and alternative splicing switching assays (Table 1, Figures 2G, 2H and 3).

Scheme 3. Synthesis of Sudemycin K and 8



Reagents and conditions a) Isobutyric anhydride, NEt_3, 4-DMAP, CH_2Cl_2, 0 °C, 99%; b) DIBALH, toluene, -78 °C, 71%; c) KHMDS, 18-crown-6, bis(2,2,2-trifluoroethyl) (methoxycarbonylmethyl)phosphonate, THF, -78 °C, 82% for **26**, 72% for **29** ; d) Me_3SnOH, $ClCH_2CH_2Cl$, 85 °C, 50% for **29**, 75% for **30**; e) 1) **16**, Ph_3P, benzene, 55 °C 2) acid **27** or **30**, HBTU, NEt_3, ACN, 0 °C to rt, 30% (2 steps) for **7**, 50% (2 steps) for **8**. The overall yield of Sudemycin K synthesis was 12%, comparable to previous yields obtained for other Sudemycin variants[45-47].

Structure-function insights

Webb and colleagues proposed that, despite their structural variety, natural compounds targeting the SF3B complex share a common pharmacophore structure[41]. The pharmacophore was repeated in the synthetic Sudemycins that, in contrast with natural compounds, are suitable for scalable production, and display improved stability and solubility[45, 47]. The common pharmacophore hypothesis is also supported by recent results arguing that Herboxidiene, Spliceostatin A and Pladienolide B bind to the same site in the SF3B complex and likely share a common inhibitory mechanism[51].

A conjugated diene is one of the three key features of the common pharmacophore[41]. Cyclopropyl modifications in this moiety were shown to reduce but not suppress the activity of Meayamycin's variants, suggesting that the diene needs to be in *trans* configuration[52]. We confirmed that conversion of the diene to a single double bond suppresses activity. Surprisingly, we also found that both the compound harboring a triene moiety and the stereoisomer displaying a *Z*,*E*,*Z* configuration retained some activity (particularly the latter in cytotoxicity assays). This result reveals spatial flexibility around the conjugated diene moiety, particularly regarding the relative orientation of the oxane ring and its associated epoxi group, as well as its spatial relationship with the oxycarbonyl moiety (another key feature of the pharmacophore) at the other end of the molecule. Interestingly, the *Z*,*E*,*Z* configuration opens the possibility of versatile modification routes through Diels-Alder reactions.

We also confirmed the need of cyclohexane or dioxane rings for activity, with cyclohexane-containing drugs being more active. The more planar structure of piperazine disrupts drug's activity, suggesting that the spatial configuration of this moiety is essential to display a proper orientation of the oxycarbonyl and conjugated diene groups in the functional pharmacophore. Substitution of the cyclohexyl group by a dioxane also reduced strongly the drug's activity, further supporting the importance of this structure.

The introduction of an amide group instead of the ester led to a compound with improved splicing inhibitory activity and cytotoxicity. While we only analyzed this variant in the context of Sudemycin molecules, we hypothesize that a similar modification can have similar enhancing effects on the activity of the other classes of compounds harboring a similar pharmacophore, including Pladienolides, Herboxidienes and FR901464.

Given that compounds from parallel synthesis of the various Sudemycin analogs were tested, the higher activity of **Sudemycin K** can be attributed to the amide group and be associated both with stronger direct effects on the splicing machinery, e.g. improved affinity for the target, and to higher solubility and/or stability. As the solubility was found to be comparable to that of Sudemycin D6, the results of Figure 4 indeed argue for improved stability, as expected if replacement of the oxycarbonyl by an amide group makes it no longer a substrate of esterases, believed to be the main factor responsible for the short half-life of Sudemycin C1 *in vivo*[45,47]. Jurica and colleagues recently showed that both active compounds and their inactive analogues compete for binding to the same site, suggesting that the compounds' activities may rely upon a conformational change within the SF3B complex induced (or prevented) only by the active variants[51]. Therefore the different activity of Sudemycin variants, including the higher activity of **Sudemycin K**, may be also due to more efficient modulation of such conformational changes.

The amide moiety makes **Sudemycin K** suitable for conjugation with ureas, amides and carbamates, potentially generating a large variety of chemical derivatives, which once again might be extrapolable to other families of splicing inhibitors, like Meayamycin, Spliceostatin and Pladienolides. Future work will focus on the generation and activity evaluation of such derivatives.

In summary, in addition to confirming the importance of the conjugated diene, our studies reveal that changes in the diene configuration only partially decrease drug activity, while replacement of a cyclohexane ring by piperazine abolishes it. Finally, we obtained a compound with improved activity, at least partly due to increased stability, **Sudemycin K**, by replacing the oxycarbonyl by an amide group. This variant offers reactivity possibilities that can potentially expand significantly the structural diversity of these drugs.

METHODS. Synthesis methods are summarized in the legends of Schemes 1-3 and fully detailed, along with the characterization of synthetic products by NMR and 2D correlation spectra, in Supplementary Information. Biochemical and cellular assays were described before[24, 39], and fully detailed in Supplementary Information.

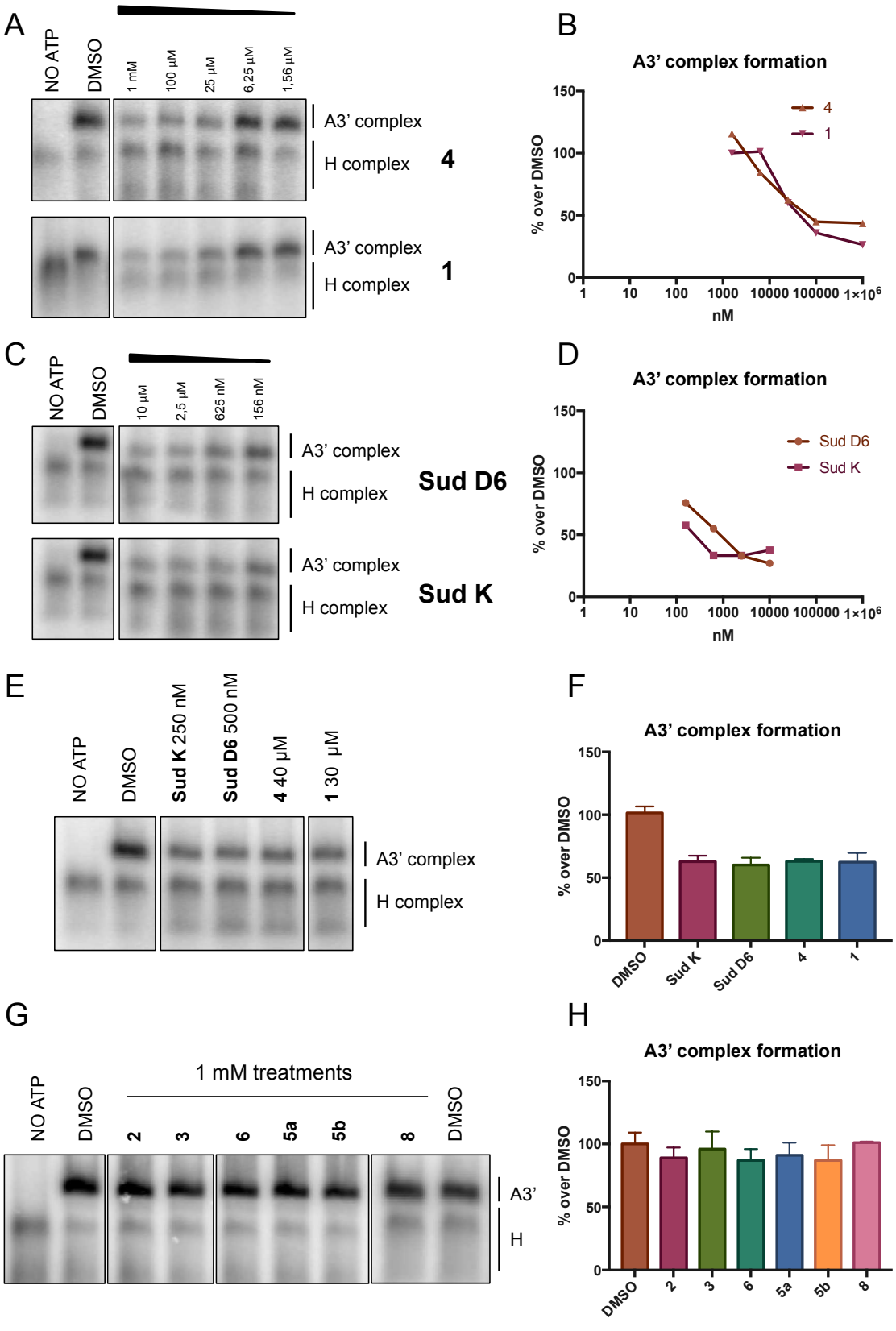
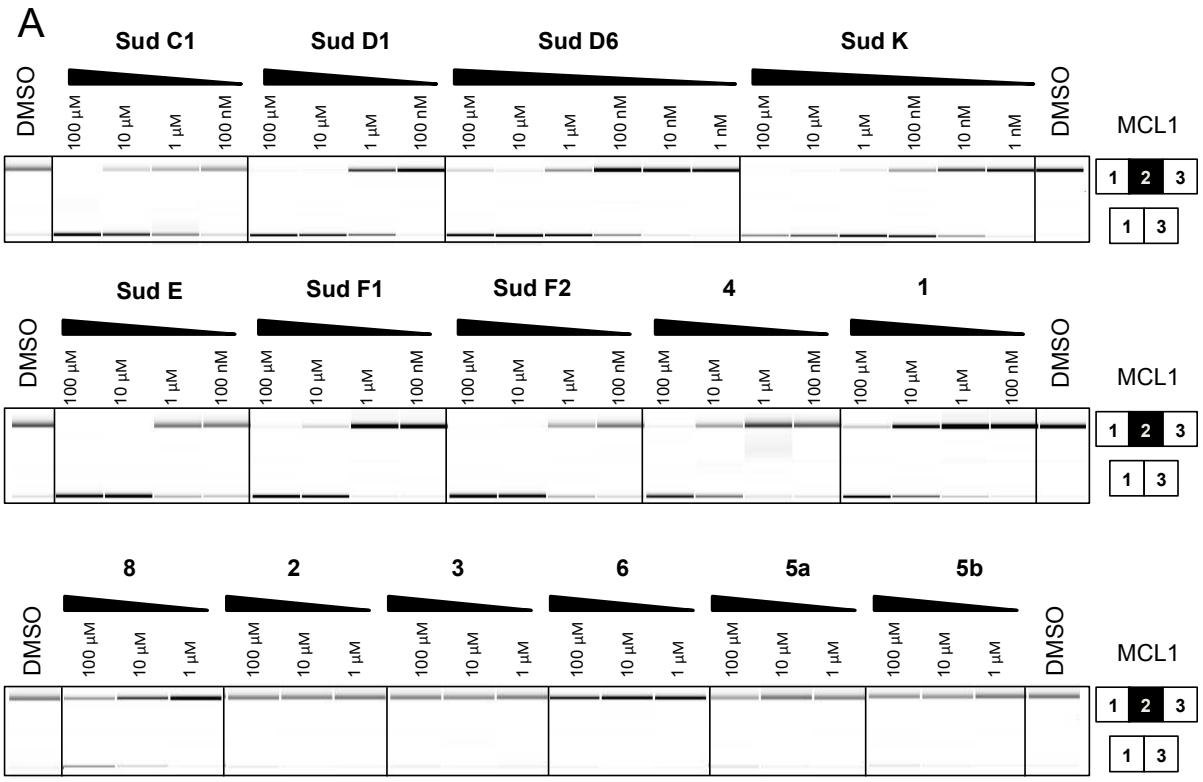


Figure 2. ***In vitro*** **spliceosome (A3' complex) formation assays.** A) Representative Phosphoroimager pictures of electrophoretic separation of H and A3' complexes assembled upon incubation of a radioactively labeled adenovirus major late promoter RNA (spanning sequences corresponding to 3' half of intron 1 and part of the following exon) in HeLa nuclear extracts and fractionation on non-denaturing agarose gels. The electrophoretic mobility of A3' and H complexes is indicated, as well as concentrations of the indicated drugs (**1** and **4**, conjugate diene variants) or DMSO as control. Only complex H is formed in the absence of ATP. B) Quantification of the percentage of

A3' complex formation for a range of concentrations of the indicated drugs, corresponding to the results reported in A. C) Results equivalent to those in A, for Sudemycins D6 and K. D) Quantification of the results reported in C, as in B. E) Analyses as in A for the indicated drugs and concentrations. The goal of the experiment was to compare in parallel the different concentrations of various drugs causing 50% decrease in A3' complex formation. F) Quantifications of results as in E, corresponding to triplicate experiments. Differences between drugs were not significant (t-test), while they were all significantly different from the control DMSO treatment (p-value < 0.01). Standard deviations are indicated. G) Analyses as in E at 1mM drug concentrations (maximal concentrations tested). H) Quantification of results as in G, corresponding to triplicate experiments. Drug effects were not significantly different from control DMSO treatment (t-test). Standard deviations are indicated.



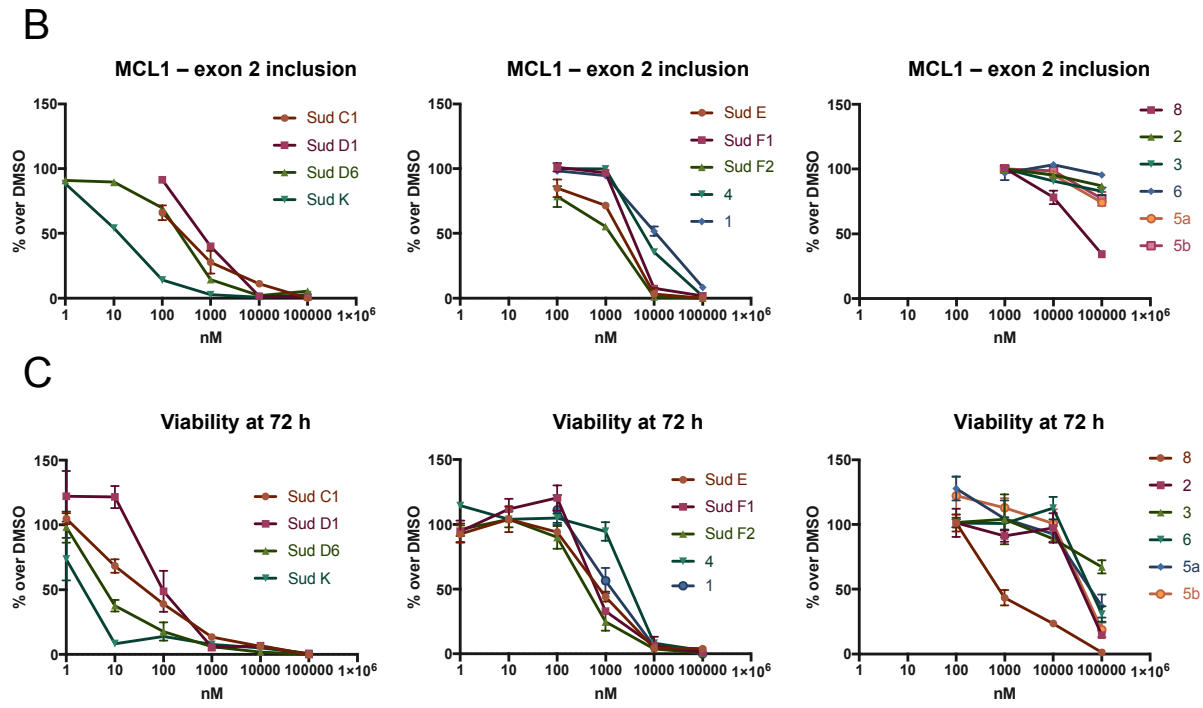


Figure 3. *MCL1* alternative splicing and cytotoxicity assays in HeLa cells. A) Capillary electrophoresis profiles of RT-PCR amplification of *MCL1* alternatively spliced products from RNA isolated 3 h after drug exposure. The positions of the products corresponding to exon 2 inclusion and skipping are indicated, along with the drug treatment and concentrations (DMSO, control without drug). One representative example per condition is shown. B) Quantification of data shown in A for duplicate experiments. Graphs represent % of *MCL1* exon 2 inclusion at different drug concentrations, as indicated. Drug treatments were clustered according to the different concentration ranges at which they induce exon 2 skipping. Standard deviations are shown. Higher activity of **Sudemycin K** than **Sudemycin D6** was significant (t-test, p-value < 0.01). C) Cytotoxicity assays. Cell viability was measured using Resazurin assays 72 h after drug exposure. Graphs indicate fraction of living cells compared to control DMSO treatment. All treatments were performed in triplicate and standard deviations are shown. Drug treatments were clustered according to the different concentration ranges at which they induce significant decreases in cell viability. Higher activity of **Sudemycin K** than **Sudemycin D6** was significant (t-test, p-value < 0.01).

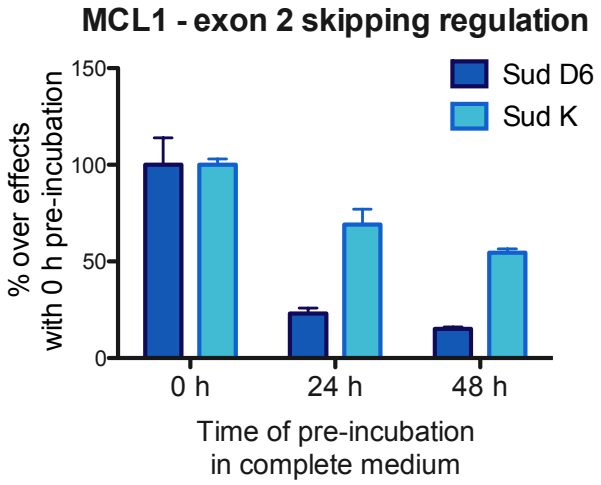


Figure 4. Stability of Sudemycin D6 and Sudemycin K upon incubation in culture medium with 10% fetal bovine serum. Complete culture medium containing 1 μM drug or the equivalent volume of DMSO was incubated at 37 °C for the indicated times and subsequently added to a lawn of HeLa cells. After 3 h of incubation RNA was isolated and *MCL1* alternative splicing was assessed as a measure of residual drug activity. Exon 2 inclusion levels upon DMSO treatment were used to normalize values across time points, and the levels of regulation induced at 0 h time point by each drug were set at 100%. The reduction of the effects at each time point is significantly lower for **Sudemycin K** compared to **Sudemycin D6** (p-value < 0.02 at 24h, p—value < 0.001 at 48 h by t-test comparison of duplicated treatments).

Table 1. Summary of Sudemycin variants' activities

DRUG	*In vitro* A3' complex formation – IC_{50} (nM)	*MCL1* alternative splicing regulation – IC_{50} (nM)	Cytotoxicity in HeLa cells – IC_{50} (nM)
Sud K (7)	≈250	≈15	2,3 ± 0,81
Sud D6	≈500	≈250	6,3 ± 0,82
Sud D1	≈750	≈630	109 ±48
4	≈40'000	≈6'300	12'703 ± 16'386
1	≈30'000	≈12'500	> 30'000
Sud C1	≈400	≈320	123 ± 154
Sud E	≈10'000	≈2'000	764 ± 113
Sud F1	≈12'000	≈3'500	646 ± 38
Sud F2	≈50'000	≈1'200	417 ± 0,00
8	n.d.	≈40'000	848 ± 200
2	n.d.	>100'000	> 30'000
3	n.d.	>100'000	n.d.
5a	n.d.	>100'000	> 30'000
5b	n.d.	>100'000	> 30'000
6	n.d.	>100'000	> 30'000

Table 1. Summary of activities of the compounds tested in this study. Biochemical assays to evaluate complex A3' formation, RT-PCR assays to evaluate effects on *MCL1* alternative splicing regulation and cytotoxicity assays were carried out and quantified as described in the Methods section (Supplementary Information file). Estimates of IC_{50} values are provided. Sud: abbreviation for Sudemycin. n.d.: not detected at the maximum concentration tested (100 μM for cytotoxicity assays, 1 mM for *in vitro* spliceosome assembly assay).

ASSOCIATED CONTENT

Supporting Information

Supporting Information 1 (PDF): Material and Methods, Supplementary Figure 1.

AUTHOR INFORMATION

Corresponding Authors

* E-mail: mercedesalvarez@ub.edu, juan.valcarcel@crg.eu

Present Addresses

†Prof. Fernando Albericio, School of Chemistry and Physics, University of KwaZulu-Natal, Durban , South Africa

†Dr. Kamil Makowski, School of Chemistry, YachayTech University, Hacienda San Jose SN, San Miguel de Urcuqui 100119 Ecuador

Author Contributions

∫ *These authors contributed equally.*

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Sud, Sudemycin; SAR, Structure-activity relationship; 4-DMAP, 4-(Dimethylamino)pyridine; ACN, Acetonitrile; DIBALH, Diisobutylaluminium hydride; Bt, 2-Benzo[d]thiazole; DMSO, Dimethylsulfoxide; NaHMDS, Sodium hexamethyldisilazane, KHMDS, Potassium hexamethyldisilazane; TBAF, Tetrabutylammonium fluoride; THF, Tetrahydrofuran

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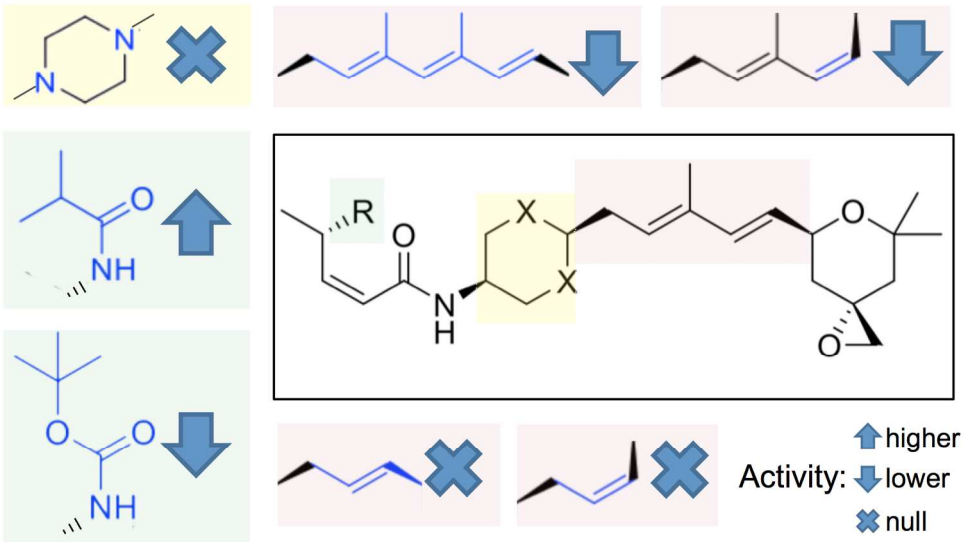

Several Sudemycin analogues have been synthetized. Sudemycin K displays improved potency as an inhibitor of cancer cell proliferation
Sudemycin K
359x200mm (143 x 143 DPI)