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Graphical Abstract

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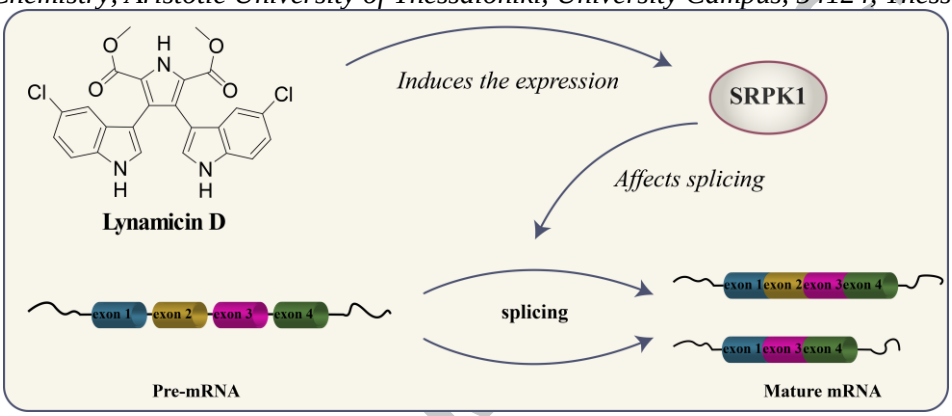

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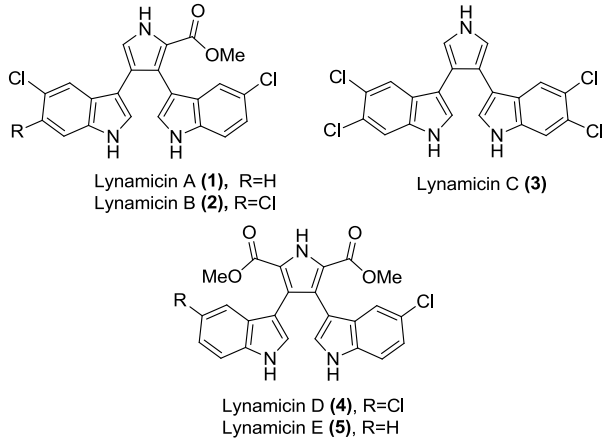
ABSTRACT

The first total synthesis of the antimicrobial natural product lynamicin D has been developed using a Suzuki coupling to construct the bisindole pyrrole skeleton. An evaluation of the biological activity of lynamicin D reveals that it has a minor effect on cell viability but it can modulate splicing of pre-mRNAs. We provide evidence that this effect is mainly due to the ability of lynamicin D to alter the levels of SRPK1, the key kinase involved in both constitutive and alternative splicing.

1. Introduction

Bisindolyl alkaloids represent a large family of natural products that display potent and diverse biological activities such as anti-inflammatory, antimicrobial, antiviral and antitumor.[1,2] Representative examples of this category, staurosporine[3] a potent inhibitor of protein kinase C and rebecamycin,[4] a topoisomerase I inhibitor are currently evaluated in clinical trials for the treatment of various cancer types.[5,6,7] Recently, new bisindole pyrroles, the lynamicins, were isolated from the marine actinomycete, NPS12745.[8] These compounds exhibit a broad-spectrum antimicrobial activity with MIC values in the low micromolar range. Molecular docking simulation studies by Saurav and coworkers revealed that lynamicins A and D and the related spiroindimicins A-D, isolated from deep marine sea derived Streptomyces sp. SCSIO 03032,[9] could potentially interact with topoisomerase II, cathepsin K, cytochrome P4503A4, aromatase P450, protein kinases and histone deacetylases, suggesting that these compounds may exert a broader biological activity.[10] In this respect, spiroindimicin C exhibited inhibitory effects against HepG2 and H460 cells with IC_{50} values of 6 and 15 μg/mL and spiroindimicin D was moderately active against HepG2, B16, and H460 cells. In another study, the structurally related indimicin B was cytotoxic towards the MCF-7 cell line with an IC50 of 10 μM.[11]

In this study, the first total synthesis of lynamicin D (**4**) is described via a Suzuki coupling as the key step in the assembly of bisindole pyrrole skeleton. Furthermore, it is shown that this natural product influences splicing of pre-mRNAs. SRPK1 is the key kinase involved in both constitutive and alternative splicing.[12,13] We provide evidence that lynamicin D exerts its effects on splicing mainly by upregulating SRPK1 without affecting the activity or localization of the kinase. Thus, lynamicin D may be considered as a new SRPK1 inducer and consequently as a new small molecule that affects splicing. Small molecule splicing modulators such as spliceostatin A, pladienolide B, pladienolide D, sudemycin and isoginkgetin have recently emerged as valuable tools to study and regulate aberrant splicing linked to various human diseases including neuromuscular, neurodegenerative diseases (myotonic dystrophy, spinal muscular atrophy), different types of cancer and pediatric malignancies (acute myeloid leukemia, neuroblastoma).[14,15]

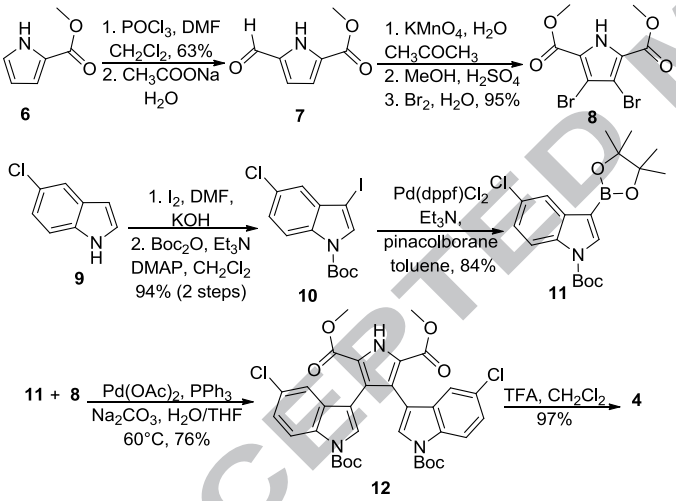


Scheme 1. The lynamicins

2. Results and discussion

2.1. Chemistry

Our synthetic studies towards the synthesis of lynamicin D were inspired on Fürstner's approach towards the synthesis of lycogalic acid A dimethyl ester.[16] Key reaction of this method is the Suzuki coupling of dimethyl 3,4-dibromo-1*H*-pyrrole-2,5-dicarboxylate **8** with boronate **11**. Dibromide **8** was synthesized from the bromination of dimethyl 1*H*-pyrrole-2,5-dicarboxylate, which in turn was obtained by the oxidation of aldehyde **7** following by the esterification of 5-(methoxycarbonyl)-1*H*-pyrrole-2-carboxylic acid.[17,18] To prepare its coupling partner, 5-chloro-1*H*-indole **9** was initially treated with I_2 and KOH in DMF and the corresponding iodide was protected with Boc_2O to give *tert*-butyl 5-chloro-3-iodo-1*H*-indole-1-carboxylate **10** in 94% over 2 steps.[19] Subsequently, boronate **11** was prepared from iodide **10** by using pinacolborane with $Pd(dppf)Cl_2$ as catalyst and Et_3N in toluene.[20] The Suzuki coupling of **8** and **11** was performed after treatment with $Pd(OAc)_2$, PPh_3, Na_2CO_3 in a mixture of THF/H_2O. Careful control of the reaction temperature (60°C) must be assured, since higher reaction temperature or other solvents lead to the decomposition of the coupling product **12**. Final deprotection of the Boc groups of **12** with TFA provided lynamicin D in 97% yield. The spectral and analytical data (^1H, ^{13}C NMR, MS) of synthetic **4** were identical to those of the natural lynamicin D.[9]



Scheme 2. Synthesis of lynamicin D

2.2. Biological studies

2.2.1. Effect on cell viability

Based on previous reports showing that lynamicin D exhibited antimicrobial activity,[8] while the structurally related bisindole alkaloids, spiroindimicin C and D and indimicin B, displayed moderate cytotoxicity,[11] we initially sought to assess the effect of lynamicin D on the viability of various tumor cell lines by MTT assays. In this respect, three human tumor cell lines were used: HeLa (human cervix adenocarcinoma), A549 (human lung carcinoma), and T98G (human brain glioblastoma multiforme). As shown in Fig. 1, treatment of HeLa, A549 and T98G cells with increasing concentrations of lynamicin D for 48 h had a very limited effect on cell viability (82, 88 and 91% viability of HeLa, A549 and T98G cells, respectively, at a concentration of 30 μM).

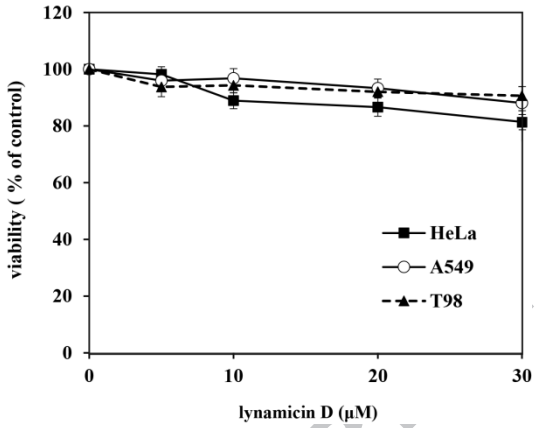


Figure 1. Lynamicin D does not affect significantly the cell viability of HeLa, A549 and T98 cells. Cells were treated with increasing concentrations of lynamicin D for 48 h and the number of viable cells was evaluated with MTT assays.

2.2.2. Lynamicin D modulates alternative splicing of various RNA transcripts similarly to SRPK1

Research from various laboratories during the last years revealed that several low-molecular mass natural compounds could modify both constitutive and alternative pre-mRNA splicing in mammalian cells.[21,22,23] Many of these molecules interfere with the phosphorylation of splicing factors thus modulating spliceosome assembly/disassembly. Based on the structure of lynamicin and prompted by the studies of Tazi and coworkers on the biological activities of NB-506, an indolocarbazole topoisomerase I inhibitor,[24] we initially tested whether lynamicin D could affect pre-mRNA splicing. In this respect, we transfected HeLa cells with various minigene plasmids in the presence of increasing concentrations (10, 20 and 30 μM) of lynamicin D or with DMSO only (control), and after 24 h of treatment we performed splicing assays. As reporter genes we used for constitutive splicing a rat insulin minigene that contains exons 1, 2 and 3 and the respective introns and for alternative splicing the SRp20 and the survival motor neuron-2 (SMN2) minigenes that contain alternative spliced exons flanked by introns. More specifically, the SRp20 minigene contains exons 3 and 5 and the alternatively spliced exon 4, while the survival motor neuron-2 (SMN2) minigene that contains exons 6 and 8 and the alternatively included exon 7.

As shown in Fig. 2 (left panels), lynamicin D was able to modulate splicing of the reporter genes. A significant retention of introns in the insulin minigene (pSVIRB) and inclusion of exon 4 in the SRp20 minigene was observed. The percentage of exon inclusion of exon 7 in the SMN2 minigene was also higher suggesting that lynamicin D might affect splicing of various mRNA targets. As many of the small molecules were reported to influence splicing by interfering with the phosphorylation of splicing factors and as a consequence by modulating spliceosome assembly/disassembly, we then tested on the same splicing assays the effect of SRPK1, which is the major kinase involved in the phosphorylation of the RS domains of splicing factors.[12,25] Co-transfection of increasing concentrations of SRPK1 with the minigene plasmids resulted in a more or less similar retention of introns and inclusion of exons as observed with lynamicin D (Fig. 2, right panel). This suggests that lynamicin D may exert its effect on splicing by modulating the activity and/or the levels of SRPK1. The effect of lynamicin D was not cell type specific as we were able to observe similar results with the pSVIRB and the pSRp20 minigenes in T98G and A549 cells (data not shown).

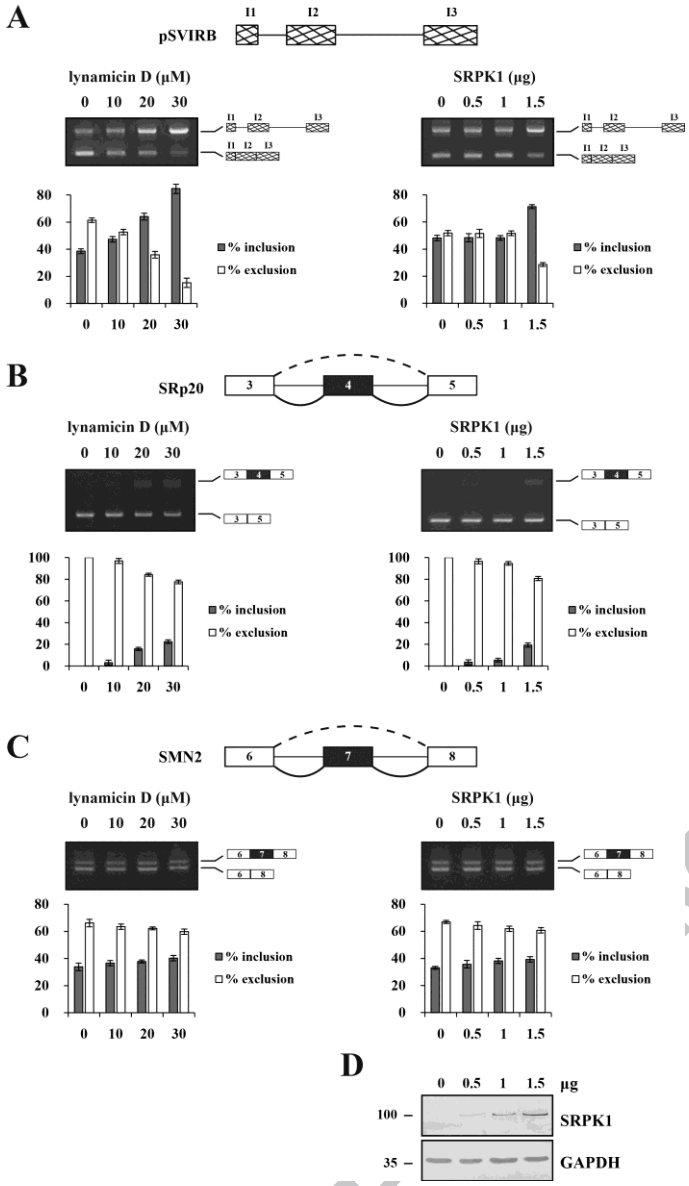


Figure 2. Lynamicin D affects splicing of the insulin, SRp20 and SMN2 minigenes similarly to SRPK1. Increasing concentrations of lynamicin D (left panels) and pFLAG-CMV2-SRPK1 (right panels) resulted in retention of introns of the insulin mRNA (A), inclusion of exon 4 of SRP20 mRNA (B) and inclusion of exon 7 of SMN2 mRNA (C). In control lanes (0) of the right panels, cells were co-transfected with the respective minigene plasmids and the empty vector pFLAG-CMV2. In each case representative agarose gels of the splicing assays are shown, while the ratio of the upper and lower spliced bands were quantified using ImageJ software. Data represent the means ± SE of three independent experiments. On top of each panel a schematic representation of the respective minigene is shown. (D) Western blotting analysis of the overexpressed SRPK1. GAPDH was used as an internal loading control for the western blot.

To address whether the effect of lynamicin D is mediated solely by SRPK1, we repeated the splicing assays with the insulin minigene (pSVIRB) but in the presence of a well-known inhibitor of SRPK1, SRPIN340.[26] As shown in Fig.3, SRPIN340 could effectively reverse the effect of overexpressed SRPK1 on the retention of intron, while a less efficacious reversion was observed in the case of lynamicin D. These data suggest that though the contribution of SRPK1 on the splicing effects of lynamicin D is significant, other mechanisms may also be involved.

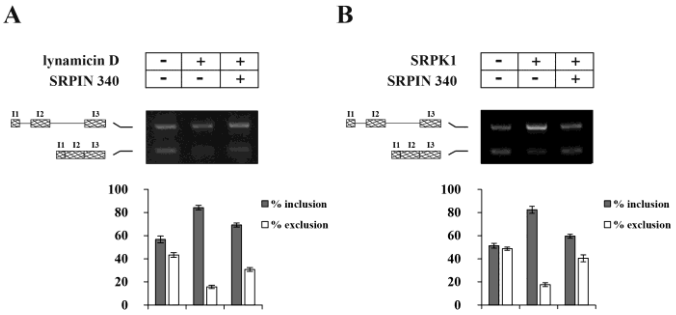


Figure 3. Effect of lynamicin D and overexpressed SRPK1 on splicing of the insulin minigene in the presence of SRPIN340. HeLa cells were transfected with pSVIRB and treated with 30 μM lynamicin D and 5 μM SRPIN340 (A) or co-transfected with the insulin minigene plasmid and 1.5 μg pFLAG-CMV2-SRPK1 and treated with 5 μM SRPIN340 (B). In the control lane (0) of the right panel, cells were co-transfected with the respective minigene plasmid and the empty vector pFLAG-CMV2. In each case representative agarose gels of the splicing assays are shown, while the ratio of the upper and lower spliced bands were quantified using ImageJ software. Data represent the means ± SE of three independent experiments. At left of each panel a schematic representation of the respective minigene is shown.

2.2.3. Lynamicin D does not affect the activity and the subcellular localization of SRPK1

SRPK1 is considered as a constitutively active kinase.[27] Yet, there are also reports showing that conformational changes of the non-catalytic regions of SRPK1, such as the N-terminus and a small helical segment of the spacer domain, may affect phosphorylation of its substrates.[28] In this respect, we initially tested whether lynamicin D had any effect on kinase activity. *In vitro* assays using recombinant GST-SRPK1 and a well-known substrate of the kinase, GST-LBRNt(62-92),[29] revealed that SRPK1 activity remained unaltered in the presence of increasing concentrations of lynamicin D (Fig.4A). On the contrary, SRPIN340 had a profound effect on the phosphorylation of GST-LBRNt(62-92) (Fig. 4B).

A critical factor in SRPK1 regulation is its subcellular partitioning. It has been demonstrated that SRPK1 is primarily located in the cytoplasm of interphase cells and, to a lesser extent, in the nucleus.[30] The nuclear accumulation of SRPK1 often leads to splicing inhibition, presumably due to aggregation of SR splicing factors.[30,31] We therefore examined in a following step whether lymamicin D could affect the intracellular localization of SRPK1. Treatment of HeLa cells with 30 μM lynamicin D for 24 h did not alter the localization of SRPK1 (Fig.4C).

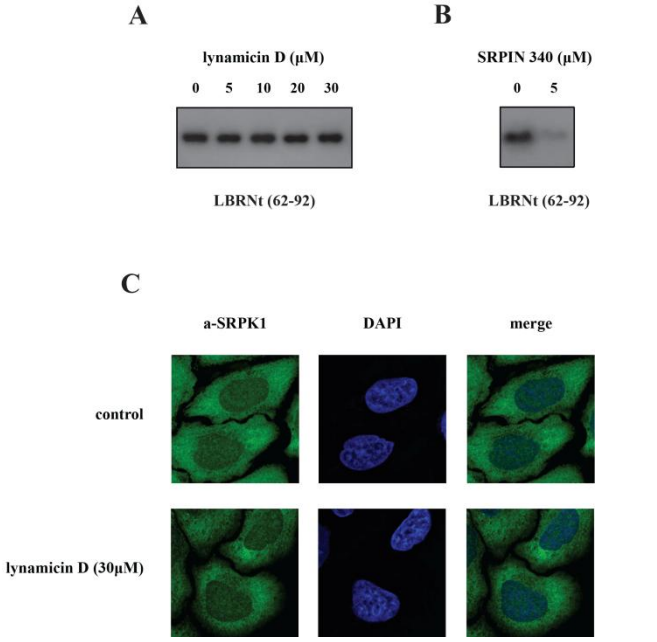


Figure 4. Lynamicin D does not affect the activity, and subcellular localization of SRPK1. Phosphorylation of 1.5 μg GST-LBRNt(62-92) by 0.5 μg GST-SRPK1 in the presence of increasing concentrations of lynamicin D (A) or 5 μM SRPIN340 (B). The samples were analyzed by SDS-PAGE on 12% gels, stained with Coomassie Blue and autoradiographed. Only the relevant part of the autorad corresponding to the phosphorylated recombinant protein is shown. (C) Fluorescence pattern of SRPK1 in control and lynamicin D-treated HeLa cells. SRPK1 was detected using the mouse monoclonal anti-SRPK1 antibody (FITC, green), while nuclei were stained with DAPI (blue). Scale bar, 10 μm.

2.2.4. Lynamicin D induces the expression of SRPK1

We then hypothesized that lynamicin D might induce splicing via an induction of the levels of SRPK1 in cells. At first, we decided to assess by RT-PCR the relative expression of SRPK1 mRNA in the presence of increasing concentrations of lynamicin D. As shown in Fig.5A, treatment of HeLa cells with lynamicin D resulted in a ~3-fold increase of the levels of SRPK1 mRNA. The increased expression of SRPK1 was further confirmed by Western blotting analysis using a mouse monoclonal antibody targeting SRPK1 (Fig.5B). Addition of lynamicin D increased ~1.9-fold SRPK1 levels. Similar results both at the mRNA and protein level were also obtained with T98G and A549 cells (data not shown). The fact that SRPK1 RNA and protein upregulation do not correlate in a dose responsive manner to the effects seen in splicing may be indicative of the involvement of other mechanisms of action of lynamicin that remain to be elucidated.

3. Conclusions

Collectively, the first total synthesis of lynamicin D was achieved. Lynamicin D did not show cytotoxic effects on three human tumor cell lines as assessed by MTT assays, yet our experiments clearly demonstrate that it influences splicing of pre-mRNAs. Its splicing effects are mediated to a significant extent by upregulating SRPK1, the key kinase involved in both constitutive and alternative splicing, while other mechanisms of action may also be involved. Although the molecular basis of the induction of SRPK1 expression, as well as the other effects of lynamicin D on the splicing machinery, remain indecipherable as the exact molecular targets of lynamicin D are currently unknown, this work sheds light on the mechanism-of-action of this indole-based natural product. More extensive research is required not only because small molecule modulators of pre-mRNA splicing are valuable tools for scientific purposes, but also because lynamicin D might prove to be an attractive small molecule to treat diseases in which aberrant splicing has been implicated.

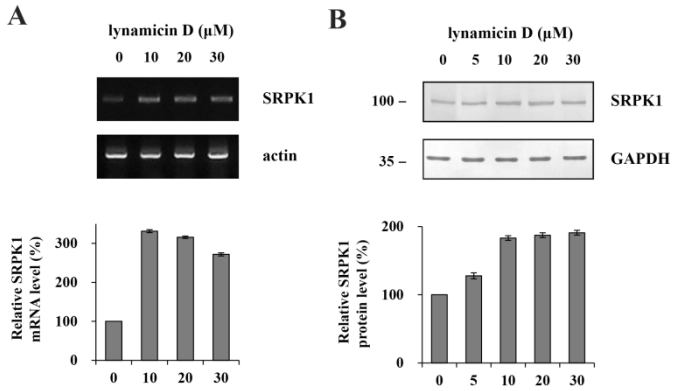


Figure 5. Lynamicin D induces the expression of SRPK1. Qualitative RT-PCR analysis of SRPK1 mRNA expression (A) and Western blotting analysis of SRPK1 protein (B) in lynamicin D-treated HeLa cells. Actin mRNA was used as a control for the RT-PCR analysis, while GAPDH was used as an internal loading control for the western blots. The relative to actin/GAPDH values of SRPK1 mRNA/protein were quantified using ImageJ software.

4. Materials and Methods

4.1. Chemistry

4.1.1. General Experimental Details.

All reactions were carried out under an atmosphere of Ar unless otherwise specified. Commercial reagents of high purity were purchased and used without further purification, unless otherwise noted. Reactions were monitored by TLC and using UV light as a visualizing agent and aqueous ceric sulfate/phosphomolybdic acid, ethanolic *p*-anisaldehyde solution, potassium permanganate solution, and heat as developing agents. The ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, and 300 and 75 MHz with tetramethylsilane as an internal standard. Chemical shifts are indicated in δ values (ppm) from internal reference peaks (TMS ^{1}H 0.00; $CDCl_3$ ^{1}H 7.26, ^{13}C 77.00; DMSO-*d6* ^{1}H 2.50, ^{13}C 39.51). Melting points (mp) are uncorrected. High-resolution mass spectra (HRMS) were recorded on a mass spectrometer at a 4000 V emitter voltage.

4.1.1.1. Methyl 4-(formyl)-1H-pyrrole-2-carboxylate, 7

Phosphoryl chloride (4.39 mmol, 0.4mL) was added dropwise to 0.34 mL DMF at 0°C. The reaction was allowed to reach room temperature and 2.2 mL of dry CH_2Cl_2 were subsequently added. The mixture was cooled again to 0°C and a solution of *6* (500 mg, 4 mmol) was added dropwise within 1 h. The reaction mixture was then refluxed for 4 h, cooled to 0 °C and hydrolysed with a solution of sodium acetate (1.8 g, 22 mmol) in water (5.5 mL). The phases were separated and the aqueous phase was extracted with ethyl acetate. The combined organic extracts were dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 5/1) to afford 387 mg of compound **7** (yield = 63%). Characterization

was in agreement with literature data.[17] ^{1}H NMR (500 MHz, *d*6-DMSO) δ 13.07 (s, 1H), 9.70 (s, 1H), 6.97 (d, *J* = 3.9 Hz, 1H), 6.90 (d, *J* = 3.9 Hz, 1H), 3.83 (s, 3H).

4.1.1.2. 5-(methoxycarbonyl)-1H-pyrrole-2-carboxy lic acid, ***S1***

To a solution of aldehyde **7** (50 mg, 0.326 mmol) in 10 mL acetone was added dropwise a solution of $KMnO_4$ (155 mg, 1 mmol) in 10 mL of acetone:water (1:1). After 3 h, the reaction was quenched with a solution of 10% $NaHSO_3$ and acidified with 1 N HC1. The solution was extracted several times with ethyl acetate (10 X 3 mL). The combined organic extracts were dried with Na_2SO_4 and concentrated. The residue was used to the next step without further purification. Characterization was in agreement with literature data.[18] **S1:** ^{1}H NMR (300 MHz, *d*6-DMSO) δ 12.28 (s, 1H), 6.80 (d, *J* = 3.9 Hz, 1H), 6.75 (d, *J* = 3.9 Hz, 1H), 3.78 (s, 3H).

4.1.1.3. Dimethyl 1H-pyrrole-2,5-dicarboxylate, ***S2***

To a solution of acid **S1** (282 mg, 1.667 mmol) in 7 mL of dry MeOH was added H_2SO_4 (1 mL) and the mixture was refluxed overnight. After the end of the reaction the solution was neutralized by the addition of sat. aq. $NaHCO_3$ and the product was extracted with CH_2Cl_2. The combined organic extracts were dried with Na_2SO_4 and concentrated. The residue was used to the next step without further purification. Characterization was in agreement with literature data.[32,33] **S2:** ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.68 (s, 1H), 6.87 (s, 2H), 3.89 (s, 6H).

4.1.1.4. Dimethyl 3,4-dibromo-1H-pyrrole-2,5-dicarboxylate, ***8***

Bromine (1.2 mL, 23.4 mmol) was added dropwise to a suspension of **S2** (400 mg, 2.18 mmol) in water (16 mL) at 0 °C. The reaction mixture is stirred for 5 min at room temperature. Then, sat. aq. Na_2SO_3 was added and stirring was continued until complete reduction of bromine was achieved. The product was extracted with CH_2Cl_2. The combined organic extracts were dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 3/1) to afford 707 mg of compound **8** (yield = 95%). Characterization was in agreement with literature data.[16] ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.93 (s, 1H), 3.95 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 159.0, 122.9, 107.8, 52.5.

4.1.1.5. tert-Butyl 5-chloro-3-iodo-1H-indole-1-carboxylate, ***10***

A solution of I_2 (506 g, 2 mmol) in DMF (2.3 mL) was added into a solution of 5-chloro-1*H*-indole 9 (302 mg, 2 mmol) and KOH (447 mg, 8 mmol) in DMF (2.3 mL) at room temperature and the mixture was stirred for 1h. Then, the reaction was quenched with sat. aq. $NaHSO_3$. The crude product was extracted with ethyl acetate. The organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. The obtained crude product was then protected. Crude 3-iodo-5-chloro-1*H*-indole (544 mg, 1.96 mmol) was dissolved in 9 mL CH_2Cl_2 and treated with Boc_2O (471 mg, 2.2 mmol), NEt_3 (0.8 mL), and DMAP (24 mg, 0.2 mmol). The mixture was stirred for 2 h. Then, the solution was washed with sodium metabisulphite (5% aqueous solution), dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 20/1) to afford 695 mg of compound 10 (yield = 94%). Characterization was in agreement with literature data.[19] ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.06 (d, *J* = 8.8 Hz, 1H), 7.73 (s, 1H), 7.39 (d, *J* = 1.9 Hz, 1H), 7.31 (dd, *J* = 8.8, 1.9 Hz, 1H), 1.66 (s, 9H).

4.1.1.6. tert-Butyl 5-chloro-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indole-1-carboxylate, ***11***

Pd(dppf)Cl_2 (29 mg, 0.04 mmol, 3 mol-%) and *tert*-butyl 5-chloro-3-iodo-1*H*-indole-1-carboxylate **10** (500 mg, 1.32 mmol) were placed in a dry screw-cap vessel with a septum. Then, dry toluene (6.5 mL) was added, and the mixture was degassed with argon (5 min). Dry triethylamine (0.7 mL) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.3 mL, 2 mmol) were successively added to the mixture, which was then stirred at 80 °C (preheated oil bath) for 3h (monitored by TLC). Subsequently the mixture was cooled to room temperature and filtered through Celite. The filtrate was concentrated and the residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 10/1) to obtain 418 mg of **11** (total yield= 84%). Characterization was in agreement with literature data.[34, 35] ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.08 (d, *J* = 8.8 Hz, 1H), 8.00 (s, 1H), 7.95 (d, *J* = 2.1 Hz, 1H), 7.26 (dd, *J* = 8.8, 2.1 Hz, 1H), 1.65 (s, 9H), 1.38 (s, 12H).

4.1.1.7. Dimethyl 3,4-bis(1-(tert-butoxycarbonyl)-5-chloro-1H-indol-3-yl)-1H-pyrrole-2,5-dicarboxylate, ***10***

A mixture of dimethyl 3,4-dibromo-1*H*-pyrrole-2,5-dicarboxylate **8** (69 mg, 0.20 mmol), *tert*-butyl 5-chloro-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indole-1-carbo xylate **11** (380 mg, 1.00 mmol) were dissolved in 7 mL THF. Then, Na_2CO_3 (1.68 g, 15.85 mmol) in H_2O (7.3 mL), PPh_3 (60 mg, 0.229 mmol) and $Pd(OAc)_2$ (17 mg, 0.076 mmol) were added and the mixture was degassed with argon. The mixture was heated at 60 °C overnight. Subsequently, after cooling to rt the product was extracted with dichloromethane. The organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 4/1) to afford 104 mg of compound 10 (yield = 76%). **10**: mp=210-212 °C, ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.11 (br , 1H), 7.99 (br, 2H), 7.35 (br, 2H), 7.17 (m, 4H), 3.74 (s, 6H), 1.57 (s, 18H); ^{13}C NMR (75 MHz, $CDCl_3$) 160.3, 149.1, 133.3, 131.9, 128.3, 126.6, 124.3, 123.3, 122.5, 119.6, 116.0, 112.4, 84.0, 51.9, 28.1; FT-IR: 3431, 3290, 3135,2979, 2928, 2850, 1736, 1713, 1689,1636, 1528, 1474, 1436, 1453, 1370, 1289, 1258, 1207, 1234, 1156, 1118, 1063, 1052, 1033, 805; HRESIMS *m/z* for $C_{34}H_{32}Cl_2N_3O_8$ $[M-H]^+$ calcd 680.1566, found 680.1567.

4.1.1.8. Lynamicin D, ***4***

Compound **12** (107 mg, 0.157 mmol) was dissolved in CH_2Cl_2 (5 mL) and cooled to 0 °C. Then, TFA (0.5 mL) was added dropwise at the same temperature and the mixture was stirred for 5h at rt. The reaction was quenched with saturated $NaHCO_3$ and the product was extracted with dichloromethane. The organic layers were dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by flash chromatography (eluent; hexane/ethyl acetate = 1/1) to afford 73 mg of compound **4** in 97% yield.[9] **4:** mp=132-135 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.96 (s, 1H), 8.01 (s, 2H), 7.15 (d, *J* = 1.6 Hz, 2H), 7.13 (d, *J* = 8.6 Hz, 2H), 7.02 (dd, *J* = 8.6, 1.9 Hz, 2H), 6.94 (d, *J* = 2.5 Hz, 2H), 3.75 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) 160.7, 133.8, 128.6, 125.7, 125.3, 124.3, 122.5, 121.9, 119.5, 111.9, 108.5, 51.8; FT-IR: 3416, 2951, 2923, 2851, 1701, 1620, 1569, 1521, 1460, 1434, 1406, 1316, 1236, 1107,1050, 999**;** HRESIMS *m/z* for $C_{24}H_{16}Cl_2N_3O_4$ $[M-H]^+$ calcd 480.0518, found 480.0516. Characterization was in agreement with literature data.[9] ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.98 (s, 1H), 8.01 (s, 2H), 7.14 (d, *J* = 1.0 Hz, 2H), 7.05 (d, *J* = 8.5 Hz, 2H), 6.98 (dd, *J* = 8.5, 2.0 Hz, 2H), 6.85 (d, *J* = 2.5 Hz, 2H), 3.75 (s, 6H); ^{13}C NMR (126 MHz,

$CDCl_3$) 160.7, 133.8, 128.5, 125.8, 125.2, 124.4, 122.4, 121.9, 119.4, 111.9, 108.3, 51.8.

4.2. Biology

4.2.1. Cell culture and treatment

HeLa (human cervical cancer), T98G (human glioblastoma), and A549 (human lung carcinoma) cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2. Cell growth was monitored by determining the cell number/ml with the use of a Coulter counter model ZBI.

4.2.2. MTT assay

Cells were seeded in 96-well plates (10^4 cells per well) and after 24 h they were exposed to increasing concentrations of lynamicin D (0-20 μM) for 48 and 72 h. Following treatment, the cells were incubated in culture medium with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) for 4 h. The crystals formed were solubilized by adding 100 μl of sodium dodecyl sulphate solution (10% SDS diluted in 0.01 N HCl) and absorbance was measured at 570 nm using an Elisa Biotek Microplate Reader. Absorbance values were blanked against DMSO and the absorbance of cells exposed to medium only was taken as 100% cell viability. Values shown represent the means ±SE of three independent experiments.

4.2.3. RT-PCR

RNA was extracted from control or lynamicin D-treated (for 24 h) HeLa, T98G, and A549 cells with TRIZOL according to standard procedures, and 1 μg of RNA was reverse transcribed with random hexamers and Reverse Transcriptase M-MLV (Invitrogen). To assess the expression of SRPK1 the following cycling protocol was designed: 95 °C for 2 min, followed by 27 cycles at 95 °C for 40 s, 66 °C for 40 s and 72 °C for 60 s, with sense primer 5'-CGGGAATTCTATGGAGCGGAAAGTGCTTGCG-3' and antisense 5'-GAATAATTTTTTTGACACCAGGCAGTGGAAGCCCCTG -3'. The same protocol was also used to assess the expression of actin, except that the annealing temperature was set to 60 °C for 40 s. For actin the sense primer was 5'-GTGGGGCGCCCCAGGCACCAG-3' and the antisense 5'-CTCCTTAATGTCACGCACGATTTCCC-3'. The cDNA products were separated by electrophoresis on 1.0 % (w/v) agarose gels and visualised under UV following ethidium bromide staining.

4.2.4. In vivo splicing of a reporter gene

The following two minigene plasmids, kindly provided by Stefan Stamm (University of Kentucky) were used for alternative splicing assays: the pSMN2 minigene (exons 6–8) and the pSRp20 minigene (exons 3–5),[36] while the pSVIRB vector, containing rat inslulin exons 1-3, was kindly provided by Athena Andreadis (University of Massachusetts Medical School) and was used for constitutive splicing assays.[37] Splicing assays were performed essentially as described.[36,37] Briefly, 0.5 μg of pSMN2 and pSRp20 and 1 μg of pSVIRB were transfected into 2 x 10exp4 HeLa cells, using the XfectTM transfection kit. Following 4 h incubation, nanoparticle complexes were removed by aspiration and 2 mL fresh complete growth medium were added, containing increasing concentrations of lynamicin D (0-20 μM). To test the effect of SRPK1 on the in vivo splicing assays, HeLa cells were co-transfected with the minigene plasmids and 0.5, 1 and 1.5 μg pFLAG-CMV2-SRPK1, respectively. As control, cells were transfected with the empty vector pFLAG-CMV2. SRPIN340 (Sigma-Aldrich) was added following medium change, concurrently with lynamicin D. RNA was isolated, 24 h following transfection, using the RNeasy mini kit (Qiagen) and one μg of it was reverse transcribed using the M-MLV RT kit (Invitrogen), according to the manufacturer's instructions. Part of this reaction mixture (1 μL) was diluted to a final volume of 50 μL, the concentrations of the buffer and dNTPs were adjusted for PCR and the mixture was initially denaturated at 94 °C for 2 min and then amplified for 30 cycles using the DyNAzyme polymerase (Finnzymes OY). PCR conditions were: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min (for SRp20 the annealing temperature was 55 °C), extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The primers used were: pSVIRB: sense, 5'-CAGCTACAGTCGGAAACCATCAGCAAGCAG-3' and antisense, 5'-CACCTCCAGTGCCAAGGTCTGAAGGTCACC-3'; pSMN2: sense, 5'-GGTGTCCACTCCCAGTTCAA-3' and antisense, 5'-GCCTCACCACCGTGCTGG- 3'; pSRP20: sense, 5'-TAATACGACTCACTATAGGG-3' and antisense, 5'-CCTGGTCGACACTCTAGATTTCCTTTCATTTGACC-3'; Amplified products were resolved by electrophoresis through 1% agarose gel and ethidium bromide staining. The obtained bands (upper band intron/exon inclusion, lower band intron/exon exclusion) were quantified using ImageJ software.

4.2.5. Western Blotting

Cells were lysed in 200 μL of 1% Triton buffer (1% Triton X-100, 20 mM Hepes-KOH, pH 7.5, 150 mM NaCl, and 1 mM PMSF) for 30 min on ice and passed 10 times through a 27-gauge needle. Whole cell extracts were clarified by centrifugation at 13,000 *g* for 15 min in a microcentrifuge and analyzed on 10% SDS-PAGE. Gel loading was adjusted to give equivalent amount of protein in each lane. Endogenous SRPK1 and GAPDH were detected by Western blotting, using an anti-SRPK1 (Clone12/SRPK1, BD Biosciences) and an anti-GAPDH mouse monoclonal antibody (Acris), respectively, an alkaline phosphatase-coupled goat anti-mouse secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate.

4.2.6. Kinase assay

Kinase assays were carried out in a total volume of 25 μL containing 12 mM Hepes pH 7.5, 10 mM $MgCl_2$, 25 μM ATP, 1 μg of GST-LBRNt(62-92) (encoding a fragment (aa 62-92) of the N-terminal domain of Lamin B Receptor (LBR) which contains the RS dipeptides) as substrate, 0.5 μg GST-SRPK1 and increasing concentrations of lynamicin D (0-30 μM) or 5 μM SRPIN340. Incorporation of radioactivity was measured by excising the radioactive bands from an SDS-PAGE gel and scintillation counting.

4.2.7. Immunofluorescence

Cells were cultured on glass coverslips for 48 h in the presence of 30 μM lynamicin D or DMSO only (control). The samples were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 for 20 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 12 min. We used 0.5% fish skin gelatin in PBS to block nonspecific binding during antibody staining. DNA staining (propidium iodide) and probing with the primary (anti-SRPK1 monoclonal antibody, BD Biosciences, diluted 1:150) and secondary (FITC-conjugated goat anti-mouse, diluted 1:400) antibodies, was performed using standard methods. After 3x washing, the coverslips were mounted in 90% glycerol and visualized in a Zeiss LSM 780 confocal microscope using the Zen 2011 software.

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