SUPPLEMENTARY INFORMATION

Synthesis and Biological Evaluation of Unprecedented Ring-Expanded Nucleosides (RENs) Containing an Imidazo[4,5-d][1,2,6]oxadiazepine Ring System

Stefano D’Errico, a Giorgia Oliviero, a,* Jussara Amato, a Nicola Borbone, a Vincenzo Cerullo, b Akseli Hemminki, c Vincenzo Piccialli, d Sabrina Zaccaria, d Luciano Mayo, a and Gennaro Piccialli a

a. Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, Via D. Montesano 49, 80131, Napoli, Italy.
b. Laboratory of Immunovirotherapy, Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland
c. Cancer Gene Therapy Group, University of Helsinki & Helsinki University Central Hospital, Haartmaninkatu 8, Helsinki, Finland
d. Dipartimento di Scienze Chimiche, Università degli Studi di Napoli Federico II, Via Cintia 21, 80126, Napoli, Italy.

golivier@unina.it

General Methods S3
Experimental procedures S3
Copies of 1H NMR Spectra

Compound 4a S9
Compound 4b S10
Compound 4c S11
Compound 6a S12
Compound 6b S13
Compound 6c S14
Compound 7a S15
Compound 7b S16
Compound 7c S17

Copies of 13C NMR Spectra S1
Compound 4a  S18
Compound 4b  S19
Compound 4c  S20
Compound 6a  S21
Compound 6b  S22
Compound 6c  S23
Compound 7a  S24
Compound 7b  S25
Compound 7c  S26

Copies of UV Spectra

Compounds 4a and 6a  S27
Compounds 4b and 6b  S28
Compounds 4c and 6c  S29

Antitumor activity of compounds 7a-c  S30

Figure S1. Cytotoxic effect of 7a-c and cisplatin in MCF-7 cell line  S31
Figure S2. Cytotoxic effect of 7a-c and cisplatin in A549 cell line  S32
General Methods

All the reagents were obtained from commercial sources (Sigma-Aldrich) and were used without further purification. $^1$H and $^{13}$C-NMR spectra were acquired on a Varian Mercury Plus 400 MHz and on a Varian Unit Inova 700 MHz in CD$_3$OD or CDCl$_3$. Chemical shifts are reported in parts per million (δ) relative to the residual solvent signals: CD$_2$HOD 3.31 and CHCl$_3$ 7.27 for $^1$H-NMR; CD$_2$HOD 49.0 and CHCl$_3$ 77.0 for $^{13}$C-NMR. $^1$H-NMR chemical shifts were assigned by 2D NMR experiments. The abbreviations s, bs, d, dd and m stand for singlet, broad singlet, doublet, doublet of doublets and multiplet, respectively. HPLC analyses and purifications were carried out on a Jasco UP-2075 Plus pump equipped with a Jasco UV-2075 Plus UV detector using a 4.60 x 150 mm LUNA (Phenomenex) silica column (particle size 5 µm) eluted with a linear gradient of MeOH in AcOEt (from 0 to 5% in 15 min, flow 1.0 mL min$^{-1}$, system A), with a linear gradient of AcOEt in n-hexane (from 0 to 100% in 30 min, flow 1.0 mL min$^{-1}$, system B) or using a 4.8 x 150 mm C-18 reverse-phase column (particle size 5 µm) eluted with a linear gradient of MeOH in H$_2$O (from 0 to 100% in 30 min, flow 1.3 mL min$^{-1}$, system C). UV spectra were recorded on a Jasco V-530 UV spectrophotometer. High Resolution MS spectra were recorded on a Bruker APEX II FT-ICR mass spectrometer using electrospray ionization (ESI) technique in positive mode. Elemental analyses were performed on a Thermo Finnigan Flash EA 1112 CHN analyser. IR spectra were recorded on a Jasco FT-IR 430 spectrophotometer. Optical rotations were determined on a Jasco polarimeter using a 1 dm cell at 25 ºC; concentrations are in g/100 mL. Preparative PLC chromatography was performed using F254 silica gel plates (0.5 mm thick, Merck). Analytical TLC analyses were performed using F254 silica gel plates (0.2 mm thick, Merck). TLC spots were detected under UV light (254 nm). For MTS assays the UV absorbance at 490 nm was read using a Beckman Anthos 96 well Microplate Reader.

Experimental Procedures

General procedure for the preparation of compounds 3a-c
See ref. 15 in main text.

General procedure for the preparation of compounds 4a-c
The mixtures of diastereomers 2a-c (0.030 mmol), prepared as previously described (ref 15a in main text) were dissolved in pyridine (0.5 mL) and stirred at 70 ºC for 18 h. Pyridine was evaporated under reduced pressure and the crudes were purified on silica gel plates (developing
system: AcOEt/MeOH, 95:5). The bands were scratched from the plates and the products were eluted with AcOEt/MeOH, 7:3 (50 mL), affording compounds 4a-c, the purity of which was checked by HPLC (system A, see General Methods).

2',3',5'-Tri-O-(tert-butyldimethylsilyl)-6-ethyl nebularine N-1 oxide 4a
Oil (15.0 mg, 0.023 mmol, 78%). [α]D -14.0 (c =0.9 ,CH3OH). 1H-NMR (400 MHz, CD3OD) ppm 8.98 (s, 1H, H-2), 8.80 (s, 1H, H-8), 6.11 (d, J = 5.0 Hz, 1H, H-1’), 4.80-4.75 (m, 1H, H-2’), 4.46-4.42 (m, H-3’), 4.20-4.15 (m, 1H, H-4’), 4.05 (dd, J = 11.5, 4.3 Hz, H-5’a), 3.87 (dd, J = 11.5, 2.9 Hz, H-5’b), 3.37 (q, J = 7.5 Hz, 2H, CH2CH3), 1.14 (t, J = 7.5 Hz, 3H, CH2CH3), 0.97 (s, 9H, C(CH3)3), 0.96 (s, 9H, C(CH3)3), 0.81 (s, 9H, C(CH3)3), 0.17 (s, 3H, CH3), 0.16 (s, 3H, CH3), 0.15 (s, 6H, 2 x CH3), 0.10 (s, 3H, CH3), -0.21 (s, 3H, CH3). 13C-NMR (100 MHz, CDCl3) ppm 154.5, 145.6 (two C), 141.6, 132.9, 88.3, 86.0, 76.1, 72.0, 62.5, 25.6, 25.8, 19.8, 18.5, 18.0, 17.8, 10.3, -4.4, -4.6, -4.7, -5.2, -5.4. m/z (HRESIMS) 661.3620 ([M+Na] +, C30H58N4NaO5Si3, requires 661.3613). IR (neat) νmax 2923, 1470, 1251, 1122, 834, 781 cm−1; UV (MeOH) λmax 226, 325 nm, shoulders 240, 267 nm.

2',3',5'-Tri-O-(tert-butyldimethylsilyl)-6-methyl nebularine N-1 oxide 4b
Amorphous white solid (14.4 mg, 0.023 mmol, 78%). [α]D -19.0 (c =0.2 ,CHCl3). 1H-NMR (400 MHz, CD3OD) ppm 8.99 (s, 1H, H-2), 8.80 (s, 1H, H-8), 6.11 (d, J = 5.0 Hz, 1H, H-1’), 4.78-4.74 (m, 1H, H-2’), 4.45-4.40 (m, H-3’), 4.19-4.15 (m, 1H, H-4’), 4.05 (dd, J = 11.5, 4.3 Hz, H-5’a), 3.86 (dd, J = 11.5, 2.8 Hz, H-5’b), 2.85 (s, 3H, CH3), 0.96 (s, 9H, C(CH3)3), 0.95 (s, 9H, C(CH3)3), 0.81 (s, 9H, C(CH3)3), 0.16 (s, 3H, CH3), 0.15 (s, 6H, 2 x CH3), 0.14 (s, 3H, CH3), 0.020 (s, 3H, CH3), -0.21 (s, 3H, CH3). 13C-NMR (100 MHz, CD3OD) ppm 152.0, 148.5, 146.2, 144.0, 134.5, 90.0, 87.2, 77.2, 73.2, 63.5, 26.5, 26.4, 26.2, 19.3, 18.9, 18.7, 12.3, -4.1, -4.3, -4.8, -5.2, -5.3. m/z (HRESIMS) 647.3463 ([M+Na] +, C29H56N4NaO5Si3, requires 647.3456). IR (neat) νmax 2927, 1475, 1248, 1126, 831, 787 cm−1; UV (MeOH) λmax 226, 325 nm, shoulders 240, 266 nm.

2',3',5'-Tri-O-(tert-butyldimethylsilyl)-6-phenyl nebularine N-1 oxide 4c
Pale yellow oil (15.0 mg, 0.022 mmol, 75%). [α]D -38.2 (c =1.9, CH3OH). 1H-NMR (400 MHz, CD3OD) ppm 9.01 (s, 1H, H-2), 8.81 (s, 1H, H-8), 8.20-8.16 (m, 2H, HPh), 7.65-7.60 (m, 3H, HPh), 6.15 (d, J = 4.9 Hz,1H, H-1’), 4.81-4.76 (m, 1H, H-2’), 4.48-4.43 (m, 1H, H-3’), 4.21-4.16 (m, 1H, H-4’), 4.07 (dd, J = 11.5, 4.2 Hz, 1H, H-5’a), 3.87 (dd, J = 11.5, 2.7 Hz, 1H, H-5’b), 0.98 (s, 9H, C(CH3)3), 0.95 (s, 9H, C(CH3)3), 0.84 (s, 9H, C(CH3)3), 0.17 (s, 3H, CH3), 0.15 (s, 9H, 3 x CH3), 0.050 (s, 3H, CH3), -0.15 (s, 3H, CH3). 13C-NMR (100 MHz, CD3OD) ppm 152.4, 149.3, 148.9,
147.5, 145.3, 133.6, 132.6, 132.2 (two C), 129.2 (two C), 127.8, 90.0, 87.1, 77.3, 73.1, 63.4, 26.5, 26.3, 26.2, 19.3, 18.9, 18.7, -4.1, -4.3, -4.8, -5.2, -5.3. $m/z$ (HRESIMS) 709.3624 ([M+Na]$^+$, C$_{34}$H$_{58}$N$_4$NaO$_5$Si$_3$, requires 709.3613). IR (neat) $\nu_{\text{max}}$ 2950, 2928, 2851, 1253, 1157, 836, 776 cm$^{-1}$; UV (MeOH) $\lambda_{\text{max}}$ 258, 346 nm, shoulder 300 nm.

General procedure for the preparation of compounds 6a-c

In a flamed round bottom flask charged with dry nitrogen, 4a-c (0.020 mmol), dissolved in dry THF (0.5 mL), were added via cannula. To the flasks, fresh Grignard reagents (0.080 mmol) were quickly added in one portion and the mixtures were stirred for 2 h (TLC monitoring: AcOEt/MeOH, 95:5) at room temperature. The reactions were quenched by addition of a 1 M solution of NH$_4$Cl (1 mL), diluted with AcOEt (10 mL) and extracted with brine (2 x 10 mL). The organic layers were separated, dried (Na$_2$SO$_4$), filtered and concentrated under evaporation. Compound 5a was purified as indicated for 4a-c, while compounds 5b-c were used for the next reaction step without further purification. 5a-c were dissolved CCl$_4$ (0.5 mL) and then tBuOOH (10 equiv.) was added in one portion. The systems were gently refluxed for 1 h (TLC monitoring: AcOEt/MeOH, 95:5) and then evaporated under reduced pressure. The crudes were purified by HPLC (system A for 6a-b, system B for 6c, see General Methods) affording compounds 6a-c.

5-(E,Z)-Ethylideneamino-1-[2,3,5-tri-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3H-imidazo-4-(E,Z)-propanoxime 5a (major isomer)

Oil (7.8 mg, 0.012 mmol, 60%). Element. Anal. Calcd. for C$_{31}$H$_{62}$N$_4$O$_5$Si$_3$ C, 56.84; H, 9.54; N, 8.55; found C, 56.81; H, 9.57; N, 8.59; $^1$H-NMR (500 MHz; pyridine-d$_5$): $\delta$ 12.80 (s, 1H, NOH), 8.91 (q, $J = 4.8$ Hz, 1H, N=CHCH$_3$), 8.31 (s, 1H, H-2), 6.25 (d, $J = 5.0$ Hz, 1H, H-1'), 4.81-4.77 (m, 1H, H-2'), 4.56-4.52 (m, 1H, H-3'), 4.33-4.29 (m, 1H, H-4'), 4.07 (dd, $J = 11.3$, 4.1 Hz, 1H, H-5'a), 3.91 (dd, $J = 11.3$, 2.7 Hz, 1H, H-5'b), 3.35 (q, $J = 7.4$ Hz, 2H, CH$_2$CH$_3$), 1.93 (d, $J = 4.8$ Hz, 3H, N=CHCH$_3$), 1.38 (t, $J = 7.4$ Hz, 3H, CH$_2$CH$_3$), 0.98 (s, 18H, 2 x C(CH$_3$)$_3$), 0.95 (s, 9H, C(CH$_3$)$_3$), 0.19 (s, 3H, CH$_3$), 0.18 (s, 3H, CH$_3$), 0.17 (s, 3H, CH$_3$), 0.16 (s, 3H, CH$_3$), 0.13 (s, 3H, CH$_3$), 0.06 (s, 3H, CH$_3$). $^{13}$C-NMR (175 MHz; pyridine-d$_5$) ppm 168.0, 157.4, 139.5, 133.5, 125.9, 88.8, 85.6, 77.2, 73.0, 63.6, 26.60 (two C), 26.58, 26.43 (four C), 26.38 (two C), 23.6, 21.3, 19.0, 18.8, 18.6, 12.0, -3.8, -4.1, -4.2, -4.3, -4.89, -4.93. $m/z$ 654.4032 (HRESIMS) ([M+Na]$^+$, C$_{31}$H$_{62}$N$_4$NaO$_5$Si$_3$, requires 654.4028). UV (MeOH) $\lambda_{\text{max}}$ 232 nm, shoulder 264 nm.

8-Ethyl-5-methyl-3-[2,3,5-tri-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3H-imidazo[4,5-d][1,2,6]oxadiazepine 6a

Amorphous white solid (7.1 mg, 0.011 mmol, 92%). Element. Anal. Calcd. for C$_{31}$H$_{62}$N$_4$O$_5$Si$_3$ C,
57.01; H, 9.26; N, 8.58; found C, 57.02; H, 9.28; N, 8.60. [α]D -37.7 (c = 0.3, CH3OH). 1H-NMR (400 MHz, CD3OD) ppm 8.72 (s, 1H, H-2), 6.10 (d, J = 4.9 Hz, H-1’), 4.80-4.76 (m, 1H, H-2’), 4.45-4.42 (m, 1H, H-3’), 4.19-4.14 (m, 1H, H-4’), 4.07 (dd, J = 11.9, 5.4 Hz, 1H, H-5’a), 3.86 (dd, J = 11.9, 2.6 Hz, 1H, H-5’b), 3.37 (q, J = 7.6 Hz, 2H, CH2CH3), 2.82 (s, 3H, CH3), 1.41 (t, J = 7.6 Hz, 3H, CH2CH3), 0.15 (s, 9H, 3x CH3), 0.019 (s, 3H, CH3), -0.20 (s, 3H, CH3). 13C-NMR (100 MHz, CD3OD) ppm 157.3, 156.2, 147.8, 143.7, 133.0, 90.3, 87.2, 77.4, 73.3, 63.7, 26.8, 26.6, 26.4, 21.3 (two C), 19.6, 19.1, 18.9, 11.1, -3.8, -4.2, -4.6, -4.9, -5.1. m/z 675.3764 (HRESIMS) ([M+Na]+, C31H60N4NaO5Si3, requires 675.3769). IR (neat) νmax 2923, 1470, 1251, 1119, 834, 773 cm⁻¹. UV (MeOH) λmax 226, 324 nm.

5-Ethyl-8-methyl-3-[2,3,5-tri-0-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3H-imidazo[4,5-d][1,2,6]oxadiazepine 6b
Amorphous white solid (7.2 mg, 0.011 mmol, 55% over two steps). Element. Anal. Calcd. for C31H60N4O5Si3 C, 57.01; H, 9.26; N, 8.58; found C, 57.04; H, 9.29; N, 8.61. [α]D -40.2 (c = 0.1, CH3OH). 1H-NMR (400 MHz, CD3OD) ppm 8.74 (s, 1H, H-2), 6.12 (d, J = 5.4 Hz, H-1’), 4.86-4.81 (m, 1H, H-2’), 4.44-4.39 (m, 1H, H-3’), 4.32 (q, J = 7.4 Hz, 2H, CH2CH3), 2.85 (s, 3H, CH3), 1.43 (t, J = 7.4 Hz, 3H, CH2CH3), 0.97 (s, 9H, C(CH3)3), 0.96 (s, 9H, C(CH3)3), 0.80 (s, 9H, C(CH3)3), 0.17 (s, 3H, CH3), 0.16 (s, 3H, CH3), 0.15 (s, 3H, CH3), 0.14 (s, 3H, CH3), 0.0040 (s, 3H, CH3), -0.23 (s, 3H, CH3). 13C-NMR (100 MHz, CD3OD) ppm 160.2, 151.6, 147.6, 143.1, 132.9, 89.6, 86.9, 76.6, 73.2, 63.6, 27.1, 26.3, 26.2, 25.9, 19.1, 18.7, 18.5, 12.7, 10.4, -4.3, -4.5, -4.9, -5.3, -5.5. m/z (HRESIMS) 675.3760 ([M+Na]+, C31H60N4NaO5Si3, requires 675.3769). IR (neat) νmax 2923, 1470, 1251, 1119, 834, 773 cm⁻¹. UV (MeOH) λmax 226, 320 nm.

5-Ethyl-8-phenyl-3-[2,3,5-tri-O-(tert-butyldimethylsilyl)-β-D-ribofuranosyl]-3H-imidazo[4,5-d][1,2,6]oxadiazepine 6c
Oil (7.7 mg, 0.011 mmol, 54% over two steps). Element. Anal. Calcd. for C36H62N4O5Si3 C, 60.46; H, 8.74; N, 7.83; found C, 60.48; H, 8.72; N, 7.81. [α]D -26.3 (c = 0.2, CH3OH). 1H-NMR (400 MHz, CD3OD) ppm 8.73 (s, 1H, H-2), 8.08-8.04 (m, 2H, HPh), 7.63-7.59 (m, 3H, HPh), 6.17 (d, J = 5.3 Hz, H-1’), 4.87-4.84 (m, 1H, H-2’), 4.45-4.41 (m, 1H, H-3’), 4.20-4.16 (m, 1H, H-4’), 4.07 (dd, J = 11.3, 4.9 Hz, 1H, H-5’a), 3.88 (dd, J = 11.3, 3.0 Hz, 1H, H-5’b), 3.27 (q, J = 7.4 Hz, 2H, CH2CH3), 1.47 (t, J = 7.4 Hz, 3H, CH2CH3), 0.98 (s, 9H, C(CH3)3), 0.95 (s, 9H, C(CH3)3), 0.82 (s, 9H, C(CH3)3), 0.18 (s, 3H, CH3), 0.16 (s, 3H, CH3), 0.15 (s, 6H, 2x CH3), 0.030 (s, 3H, CH3), -0.18.
(s, 3H, CH₃). ¹³C-NMR (100 MHz, CD₃OD) ppm 161.4, 149.5, 148.3, 144.4, 132.3 (two C), 132.1 (two C), 129.2 (two C), 128.6, 89.8, 87.1, 76.9, 73.4, 63.7, 26.5, 26.2, 25.4, 24.9, 19.4, 18.9, 18.8, 10.7, -4.1, -4.3, -4.7, -5.1, -5.2. m/z 737.3942 (HRESIMS) ([M+Na]⁺, C₃₆H₆₂N₄NaO₅Si₃, requires 737.3926). IR (neat) νmax 2955, 2931, 2858, 1255, 1160, 1124, 1075, 836, 777 cm⁻¹. UV (MeOH) λmax 252, 296, shoulder 346 nm.

General procedure for the desilylation of compounds 6a-c. Synthesis of 7a-c
Compounds 6a-c (0.010 mmol) were dissolved in 1.0 mL of MeOH, and then NH₄F (0.10 mmol) was added in one portion. The systems were refluxed for 5 h (TLC monitoring: AcOEt/MeOH, 7:3). The solvent was removed under reduced pressure and the crudes were dissolved in water, filtered and then purified by HPLC (system C, see General Methods) affording compounds 7a-c.

8-Ethyl-5-methyl-3-(β-D-ribofuranosyl)-3H-imidazo[4,5-d][1,2,6]oxadiazepine 7a
Amorphous white solid (3.0 mg, 0.0097 mmol, 97%). Element. Anal. Calcd. for C₁₃H₁₈N₄O₅ C, 50.32; H, 5.85; N, 18.06; found C, 50.30; H, 5.83; N, 18.05. [α]D -3.2 (c = 0.1, CH₃OH). ¹H-NMR (400 MHz, CD₃OD) ppm 8.78 (s, 1H, H-2), 6.09 (d, J = 5.1 Hz, 1H, H-1’), 4.70-4.65 (m, 1H, H-2’), 4.38-4.34 (m, 1H, H-3’), 4.17-4.11 (m, 1H, H-4’), 3.88 (dd, J = 12.2, 3.0 Hz, 1H, H-5’a), 3.36 (q, J = 7.5 Hz, 2H, CH₂CH₃), 2.81 (s, 3H, CH₃), 1.41 (t, J = 7.5 Hz, 3H, CH₂CH₃). ¹³C-NMR (100 MHz, CD₃OD) ppm 157.1, 155.8, 148.0, 143.7, 132.8, 90.4, 87.3, 75.9, 71.8, 62.6, 21.0 (two C), 10.9. m/z 333.1170 (HRESIMS) ([M+Na]⁺, C₁₃H₁₈N₄NaO₅ requires 333.1175). IR (neat) νmax 3329, 2917, 2848, 1246, 1118 cm⁻¹. UV (MeOH) λmax 226, 325 nm.

5-Ethyl-8-methyl-3-(β-D-ribofuranosyl)-3H-imidazo[4,5-d][1,2,6]oxadiazepine 7b
Amorphous white solid (3.0 mg, 0.0096 mmol, 96%). Element. Anal. Calcd. for C₁₃H₁₈N₄O₅ C, 50.32; H, 5.85; N, 18.06; found C, 50.33; H, 5.82; N, 18.04. [α]D -7.9 (c = 0.1, CH₃OH). ¹H-NMR (400 MHz, CD₃OD) ppm 8.76 (s, 1H, H-2), 6.11 (d, J = 5.1 Hz, 1H, H-1’), 4.77-4.73 (m, 1H, H-2’), 4.42-4.38 (m, 1H, H-3’), 4.15-4.11 (m, 1H, H-4’), 3.88 (dd, J = 12.2, 3.3 Hz, 1H, H-5’a), 3.77 (dd, J = 12.2, 3.4 Hz, 1H, H-5’b), 3.36 (q, J = 7.5 Hz, 2H, CH₂CH₃), 2.81 (s, 3H, CH₃), 1.43 (t, J = 7.4 Hz, 3H, CH₂CH₃). ¹³C-NMR (100 MHz, CD₃OD) ppm 160.3, 151.7, 148.1, 143.5, 133.0, 90.3, 87.1, 75.7, 71.8, 62.7, 27.2, 12.9, 10.3. m/z 333.1187 (HRESIMS) ([M+Na]⁺, C₁₃H₁₈N₄NaO₅ requires 333.1175). IR (neat) νmax 3329, 2917, 2848, 1245, 1117 cm⁻¹. UV (MeOH) λmax 225, 320 nm.

5-Ethyl-8-phenyl-3-(β-D-ribofuranosyl)-3H-imidazo[4,5-d][1,2,6]oxadiazepine 7c
Amorphous white solid (3.5 mg, 0.0094 mmol, 94%). Element. Anal. Calcd. for C$_{18}$H$_{20}$N$_4$O$_5$ C, 58.06; H, 5.41; N, 15.05; found C, 58.03; H, 5.40; N, 15.07. $[\alpha]_D = -10.4$ (c = 0.1, CH$_3$OH). $^1$H-NMR (700 MHz, CD$_3$OD) ppm 8.78 (s, 1H, H-2), 8.08-8.05 (m, 2H, HPh), 7.61-7.59 (m, 3H, HPh), 6.16 (d, $J = 5.0$ Hz, 1H, H-1’), 4.80-4.77 (m, 1H, H-2’), 4.43-4.41 (m, 1H, H-3’), 4.16-4.13 (m, 1H, H-4’), 3.88 (dd, $J = 12.1$, 3.3 Hz, 1H, H-5’a), 3.79 (dd, $J = 12.1$, 3.8 Hz, 1H, H-5’b), 3.26 (q, $J = 7.4$ Hz, 2H, CH$_2$CH$_3$), 1.47 (t, $J = 7.4$ Hz, 3H, CH$_2$CH$_3$). $^{13}$C-NMR (175 MHz, CD$_3$OD) ppm 161.3, 149.4, 148.7, 144.7, 132.3, 133.2, 132.1 (two C), 129.2 (two C), 128.8, 94.4, 87.1, 75.8, 71.9, 62.7, 27.5, 10.4. m/z 395.1323 (HRESIMS) ([M+Na]$^+$, C$_{18}$H$_{20}$N$_4$NaO$_5$, requires 395.1331). IR (neat) $\nu_{\text{max}}$ 3335, 2914, 2846, 1245, 1124 cm$^{-1}$. UV (MeOH) $\lambda_{\text{max}}$ 252, 296, 342 nm.

Procedures used for the MTS assays

Cell viability was assessed by MTS assay as described elsewhere.$^3$ Breast (MCF-7 cell line) and lung (A549 cell line) cancer cells were seeded at the concentration of $0.5 \times 10^4$ cells per well on 96-well plate and maintained overnight under appropriate condition (1% sodium pyruvate MEM or DMEM, respectively, completed with 10% FCS, 2 mmol L-glutamine and 100 units/mL of penicilllin. Cells in quadruplicated were than cultured in 5% FCS media containing different concentrations of tested compounds (0.1 µM, 1 µM, 10 µM and 100 µM) except for control wells that only received 5% FCS media. Every second day cells were washed with PBS and media were replaced. At the indicated time point cell viability was assessed reading the absorbance of treated and control cells at 490 nm using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), i.e., the 3-(4,5,dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay.
$^{1}$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (400 MHz, CD$_3$OD)
$^1$H-NMR (700 MHz, CD$_3$OD)
$^{13}$C-NMR (100 MHz, CDCl$_3$)
$^{13}$C-NMR (100 MHz, CD$_3$OD)
$^{13}$C-NMR (100 MHz, CD$_3$OD)
\[ ^{13}\text{C}-\text{NMR (100 MHz, CD}_3\text{OD)} \]
$^{13}$C-NMR (100 MHz, CD$_3$OD)
$^{13}$C-NMR (100 MHz, CD$_3$OD)
$^{13}$C-OBSERVE

Pulse Sequence: t2pu1
Solvent: d3od
Ambient Temperature
Ref: 1012_1temperaturct_one_basics100_080608
Mercury-4900B "mp0999"

Relax. delay 1.500 sec
Pulse 85.0 degrees
Width 25000.0 Hz
19632 repetitions
OBSERVE C13, 106.8698607 MHz
PSPME H2, 46.4271538 MHz
Power 40 dB
Continuous on
WALTZ-16 modulated
DATA PROCESSING
Line broadening 5.0 Hz
FT Size 8192
Total time 515600 hr, 10 min, 40 sec

$^{13}$C-NMR (100 MHz, CD$_3$OD)
$^{13}$C-NMR (175 MHz, CD$_3$OD)
Solvent: CH$_3$OH
Wavelength interval: 380-220 nm
Scan speed: 200 nm/min
Couvette with 1-cm path length
T: 25 °C

Solvent: CH$_3$OH
Wavelength interval: 380-220 nm
Scan speed: 200 nm/min
Couvette with 1-cm path length
T: 25 °C
Solvent: CH$_3$OH
Wavelength interval: 380-220 nm
Scan speed: 200 nm/min
couvette with 1-cm path length
T: 25 °C

Electronic Supplementary Material (ESI) for Chemical Communications
This journal is © The Royal Society of Chemistry 2012
Solvent: CH$_3$OH
Wavelength interval: 400-220 nm
Scan speed: 200 nm/min
cuvette with 1-cm path length
T: 25 °C
Antitumor activity of compounds 7a-c

MCF-7 breast cancer cell line and A549 lung cancer cell line were treated with different concentrations (0.1 μM, 1 μM, 10 μM and 100 μM) of novel RENs 7a-c. Cisplatin was used as control since its activity in these cell lines has been extensively studied.1,2 Cell viability was assessed by measuring the mitochondrial activity at day 1, 3 or 7 (MTS assay) in vitro.3 We found that all tested RENs increased significantly the mitochondrial activity of treated MCF-7 cells at day 1 and 3, with the exception of 7b that resulted inactive at day 1 (Fig. S1, panels A and B). This phenomenon is often reported as “starving state” leading up to cell death at later time points and/or at higher concentrations. Accordingly, at day 7 RENs 7b and 7c increased the mitochondrial activity at concentrations up to 10 μM, while at 100 μM they showed 38% and 40% cell death, respectively (Fig. 1, panel C). At the same time point 7a increased the mitochondrial activity at 0.1 μM and 1 μM, but showed 16% cell killing effect already at 10 μM and 55% cell death at 100 μM. It is noteworthy that at day 7 at 10 μM compound 7a proved to be as cytotoxic as cisplatin (16% and 15% cell death, respectively). 7a-c resulted less active in A549 cell line at almost all tested conditions (Fig. 2, panels A-C) and, in agreement with the “starving state” hypothesis, we did not observe any increased mitochondrial activity at day 3 and 7. The most active compound in A549 cell line was 7a that showed 21% cell death at day 7 at 10 μM. All together these data showed that the new synthesized compounds possess some degree of cytotoxic activity, whose manifestation in some cases was slower than the control. Further and more specific experiments would have to be performed in order to fully clarify the reason for such phenomenon. However, at this point we can speculate that only active metabolites formed around day 7 were capable to kill cells.

Fig. 1 Cytotoxic effect of RENs 7a-c and cisplatin in MCF-7 breast cancer-derived cell line. MCF-7 cell line was treated with four different concentrations (0.1 µM, 1 µM, 10 µM and 100 µM) of compound 7a-c and cisplatin. Cell viability (% referred to untreated cells) was assessed at day 1 (Panel A), day 3 (Panel B) and day 7 (Panel C). Columns, mean of quadruplicates; bars, SE (*p < 0.05; **p < 0.01; ***p < 0.001).
Fig. 2 Cytotoxic effect of RENs 7a-c and cisplatin in A549 lung cancer-derived cell line. A549 cell line was treated with four different concentrations (0.1 µM, 1 µM, 10 µM and 100 µM) of compound 7a-c and cisplatin. Cell viability (% referred to untreated cells) was assessed at day 1 (Panel A), day 3 (Panel B) and day 7 (Panel C). Columns, mean of quadruplicates; bars, SE (*p < 0.05; **p < 0.01; ***p < 0.001)