Electronic Supplementary Information

to the manuscript

AS1411-decorated niosomes as effective nanocarriers for Ru(III)-based drugs in anticancer strategies

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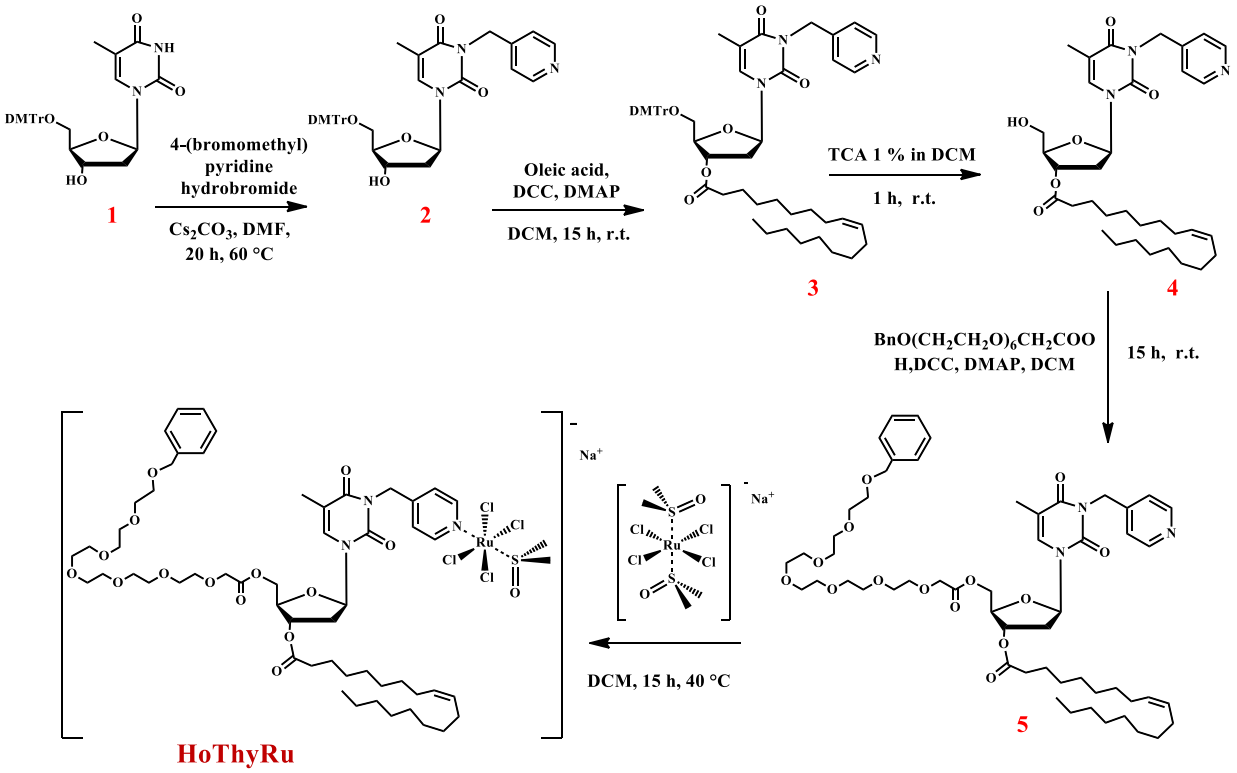
Synthesis and characterization of HoThyRu



Scheme S1. Synthetic procedure for the preparation of the nucleolipidic Ru(III)-complex HoThyRu. For all the synthesized compounds, the spectroscopic (^{1}H and ^{13}C) and spectrometric (ESI-MS) data were in perfect agreement with literature data (G. Mangiapia, G. D'Errico, L. Simeone, C. Irace, A. Radulescu, A. Di Pascale, A. Colonna, D. Montesarchio and L. Paduano, *Biomaterials*, 2012, **33**, 3770–3782).

- ***Synthesis of 3-(4-pyridylmethyl)-5'-O-(4,4'-dimethoxytriphenylmethyl)-thymidine (2)***

5'-O-(4,4′-dimethoxytriphenylmethyl)thymidine **1** (148 mg, 0.27 mmol) was dissolved in 6 mL of dry DMF. Cs_2CO_3 (531 mg, 1.63 mmol) and 4-(bromomethyl)pyridine hydrobromide (275 mg, 1.08 mmol) were then added to the reaction mixture, left at 60 °C under stirring. After 20 h, TLC analysis indicated the presence of the desired product and the reaction was quenched by removing the solvent under reduced pressure. First, a CH_2Cl_2/H_2O extraction was performed to remove the excess reagents. Then, the crude product was purified by chromatography on a silica gel column using AcOEt/MeOH (97:3, v/v, containing 2 % of TEA), as eluent, giving desired compound **2** in 67 % yield (114 mg, 0.18 mmol).

2: oil. R_f = 0.5 (AcOEt/MeOH, 95:5, v/v).

- ***Synthesis of 3-(4-pyridylmethyl)-3'-O-oleyl-5'-O-(4,4'-dimethoxytriphenylmethyl)-thymidine (3)***

Alkylated compound **2** (114 mg, 0.18 mmol) was dissolved in 2 mL of dry CH_2Cl_2 and then DMAP (122 mg, 0.54 mmol), oleic acid (50.1 mg, 0.18 mmol) and DCC (111 mg, 0.54 mmol) were sequentially added. After stirring for 15 h at r.t., TLC analysis indicated the complete disappearance of the starting materials. Thus, the reaction mixture was concentrated under reduced pressure; the crude product was purified by chromatography on a silica gel column using *n*-hexane/AcOEt (7:3, v/v, containing 2 % of TEA) as eluent, providing desired compound **3** in 41 % yield (64.1 mg, 0.07 mmol).

3: oil. R_f = 0.3 (*n*-hexane/AcOEt, 7:3, v/v).

- ***Synthesis of 3-(4-pyridylmethyl)-3'-O-oleyl-thymidine (4)***

Compound **3** (56.2 mg, 0.06 mmol) was dissolved in 4 mL of a 1 % TCA solution in CH_2Cl_2. Upon addition of the acid, the reaction mixture acquired an intense yellow-orange colour, typical of the trityl cation, and was left under stirring at r.t. After 1 h, TLC monitoring showed the complete disappearance of the starting compound. Thus the reaction was quenched by adding few drops of MeOH until complete decoloration, and finally TEA was added to neutralize the solution. Then the reaction mixture was concentrated *in vacuo* and purified by chromatography on a silica gel column eluted with $CHCl_3$/MeOH (99:1, v/v), giving the desired compound **4** in almost 71 % isolated yields (25.6 mg, 0.04 mmol).

4: yellow oil. R_f = 0.4 ($CHCl_3$/MeOH, 95:5, v/v).

- ***Synthesis of 3-(4-pyridylmethyl)-3'-O-oleyl-5'-O-(benzyloxy)hexaethylene glycol acetyl-thymidine (5)***

To compound **4** (21.1 mg, 0.04 mmol), dissolved in 3 mL of dry CH_2Cl_2, DMAP (12.8 mg, 0.11 mmol), BnO-HEG acetic acid **8** (16.6 mg, 0.04 mmol) and DCC (21.7 mg, 0.11 mmol) were sequentially added, and the resulting reaction mixture was left under stirring at r.t.. After 15 h TLC analysis indicated the formation of a new product, so the solvent was removed under reduced pressure and the crude product was purified by chromatography on a silica gel column eluted with AcOEt, giving the desired compound **5** in 51 % yield (17.2 mg, 0.02 mmol).

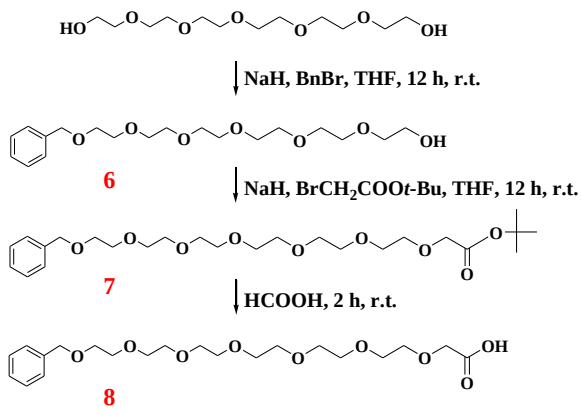
5: oil. R_f = 0.4 (AcOEt/MeOH, 95:5, v/v).

- ***Synthesis of HoThyRu***

Nucleolipid **5** (17.1 mg, 16.9 nmol) was dissolved in 2.5 mL of dry CH_2Cl_2 and then Na^+ [*trans*-$RuCl_4(DMSO)_2]^-$ (7.9 mg, 0.02 mmol) was added under stirring at 40 °C. After 15 h the reaction, monitored *via* TLC, indicated the complete disappearance of both starting materials and the concomitant formation of a new, more polar product. Thus, the solvent was removed under reduced pressure providing the target complex HoThyRu in almost quantitative yields (21.1 mg, 15.6 nmol).

HoThyRu: yellow oil. R_f = 0.2 (AcOEt/MeOH, 95:5 v/v).

Synthesis and characterization of BnO-HEG acetic acid



Scheme S2. Synthetic procedure for the preparation of BnO-HEG acetic acid (Bn = benzyl group). For all the synthesized compounds, the spectroscopic (^{1}H and ^{13}C) and spectrometric (ESI-MS) data were in perfect agreement with literature data (G. Mangiapia, et al. *Biomaterials*, 2012, **33**, 3770–3782).

- ***Synthesis of (monobenzyloxy)hexaethylene glycol (6)***

Hexaethylene glycol (2.0 g, 7.10 mmol) was dissolved in 8 mL of dry THF and NaH 60 % p.p. (170 mg, 4.25 mmol) and then benzylbromide (3.37 mL, 22.8 mmol) were sequentially added. The reaction mixture was stirred at r.t. for 12 h, then MeOH (1 mL) was added and the solvent removed *in vacuo*. The crude product was dissolved in $CHCl_3$, filtered on celite and then purified by chromatography on a silica gel column using AcOEt/MeOH (9:1, v/v) as eluent, yielding the desired compound **6** in 65 % yields (1.7 g, 4.60 mmol).

6: oil. R_f = 0.5 (AcOEt/MeOH, 9:1, v/v).

- ***Synthesis of tert-butyl (monobenzyloxy)hexaethylene glycol acetate (7)***

Alcohol **6** (155 mg, 0.42 mmol) was dissolved in 1 mL of dry THF and NaH 60 % p.p. (33.0 mg, 0.83 mmol) and *tert*-butyl bromoacetate (154 μL, 1.01 mmol) were sequentially added. The reaction mixture was stirred at r.t. for 12 h, then few drops of MeOH were added and the solvent removed *in vacuo*. The crude product was dissolved in $CHCl_3$, filtered on celite and then purified by chromatography on a silica gel column eluted with AcOEt, yielding the desired compound **7** in 86 % yields (175 mg, 0.36 mmol).

7: oil. R_f = 0.6 (AcOEt/MeOH, 95:5, v/v).

- ***Synthesis of (monobenzyloxy)hexaethylene glycol acetic acid (8)***

Ester **7** (95.0 mg, 0.19 mmol) was dissolved in 1.5 mL of HCOOH and stirred at r.t. for 2 h. The solvent was then removed *in vacuo* and the residue was coevaporated three times with $CHCl_3$ (3 x 3 mL), yielding the desired compound **8** in almost quantitative yields (82.0 mg, 0.19 mmol).

8: oil. R_f = 0.2 (AcOEt/MeOH, 95:5, v/v).

Concentration of niosome vesicles in AS1411/niosome formulations at different N/P charge ratios[a]	
N/P charge ratio	**niosome conc. (lipid + surfactant)**
1:0.5	6.25 μM
1:1	12.5 μM
1:3	37.5 μM
1:5	62.5 μM
1:8	100 μM
1:10	125 μM
1:12	150 μM
1:14	175 μM
1:16	200 μM

[a] these values are referred to fixed AS1411 concentration (0.5 μM).

Table S1. Concentration of niosome vesicles in AS1411/niosome formulations at different N/P charge ratios with fixed AS1411 concentration of 0.5 μM, used in gel electrophoresis and Z-potential assays.

N/P charge ratio	Z-potential (mV) ± SD
AS1411	-10.0± 1.5
1:0.5	-9.3 ± 0.8
1:1	1.0 ± 0.1
1:5	4.3 ± 0.7
1:8	10.7 ± 1.2
1:10	7.9 ± 1.6
1:12	12.7 ± 0.9
1:14	10.4 ± 0.9
1:16	11.6 ± 1.6

Table S2. Zeta potential values (mV) of AS1411/niosome formulations at different N/P charge ratios. Data are reported as an average of three measurements (mean ± SD).

N/P charge ratio	Z-average (r. nm) ± SD	PdI± SD
1:1	130 ± 20	0.39 ± 0.09
1:3	116 ± 2	0.36 ± 0.01
1:5	98 ± 4	0.40 ± 0.11
1:8	91.0 ± 0.2	0.27 ± 0.01
1:10	86.5 ± 0.1	0.27 ± 0.01
1:12	83.5 ± 0.5	0.34 ± 0.05
1:14	75.7 ± 0.1	0.35 ± 0.01
1:16	60.4 ± 0.6	0.38 ± 0.01

Table S3. Z-average size (r., nm) and associated polydispersity index (PdI) values determined for AS1411/niosome formulations at different N/P charge ratios. Data are reported as an average of three measurements (mean ± SD).

Concentration of niosome components in AS1411/niosome 1:12 N/P charge ratio formulations		
niosome	**HoThyRu**	**AS1411**
17.5 mM[a,c]	672 μM	58 μM
15 mM[b]	576 μM	50 μM
3.5 mM[c]	135 μM	12 μM
175 μM[c]	6.7 μM	585 nM
150 μM[d]	5.8 μM	500 nM
125 μM[d]	4.8 μM	416 nM
100 μM[d]	3.8 μM	333 nM
75 μM [d]	2.9 μM	250 nM
50 μM[d]	1.9 μM	166 nM
25 μM[d]	0.95 μM	83 nM
15 μM[d]	0.58 μM	50 nM
7.5 μM[d]	285 nM	33 nM
3.5 μM [d]	133 nM	16 nM
1.25 μM [d]	48 nM	8.3 nM

Table S4. Concentrations of niosome vesicles, HoThyRu and AS1411 in niosome formulations at the selected 1:12 oligonucleotide/lipid N/P charge ratio.
[a]concentration used in EPR measurements;
[b]concentration used in NMR measurements;
[c]concentration used in DLS particle size and storage stability measurements;
[d]concentration used in biological assays.

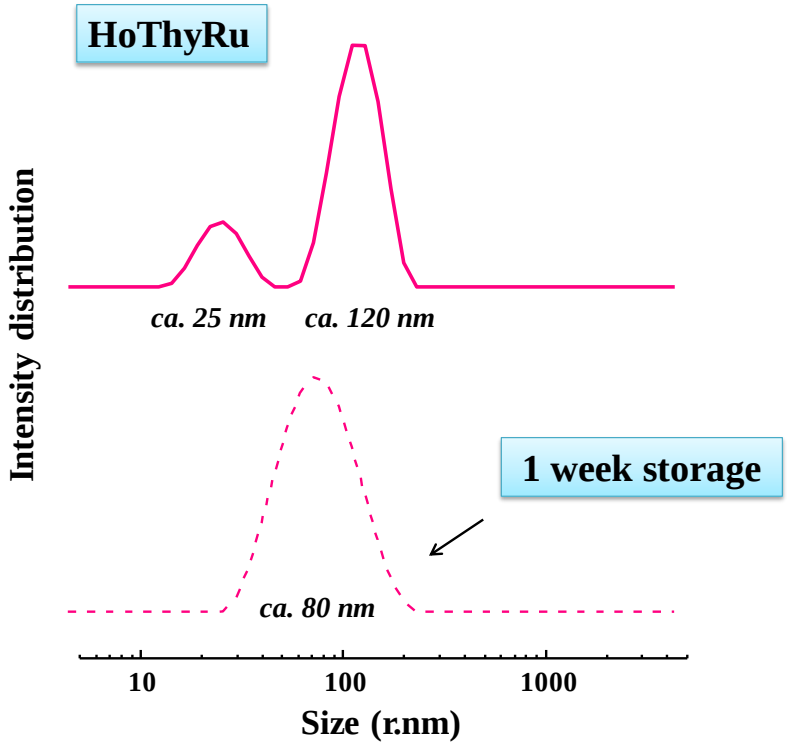


Figure S1. Size distribution by intensity of a 672 μM HoThyRu solution in PBS as a freshly prepared sample (up) and after one week storage at 4° C (down), reported as a representative example.

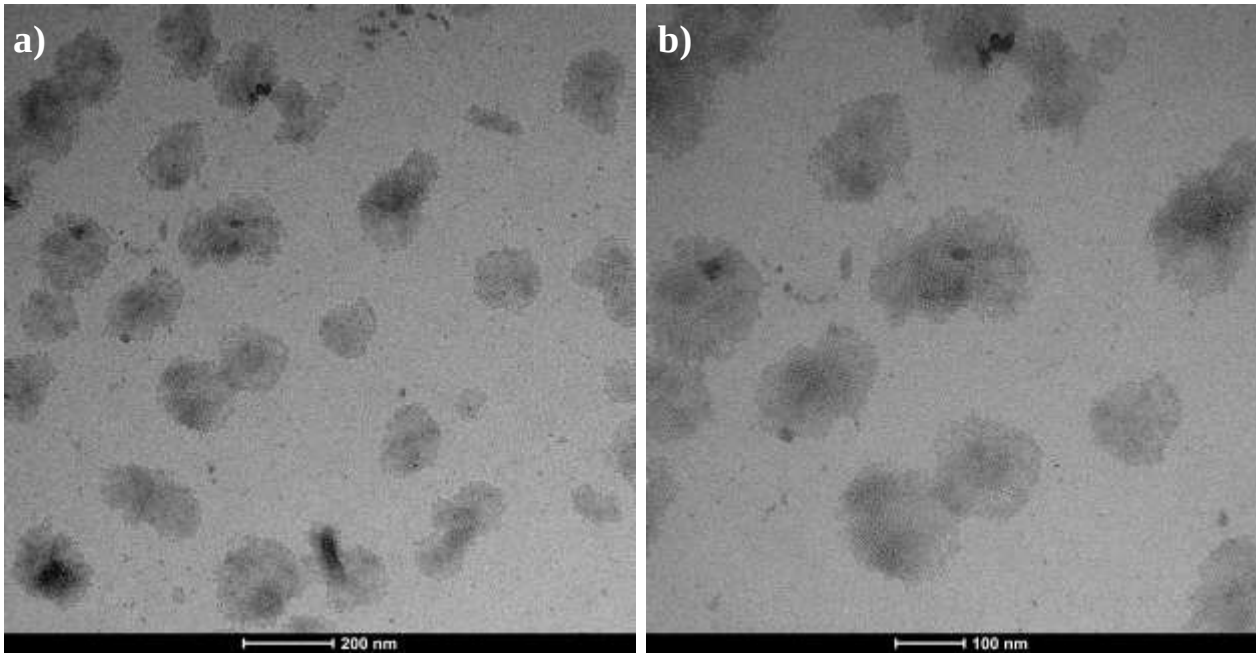


Figure S2. TEM images of unfunctionalized niosomes (cationic lipid and polysorbate 80 in 3:1 molar ratio); original magnification 29.000× and 43.000× respectively for panels **a** and **b**.

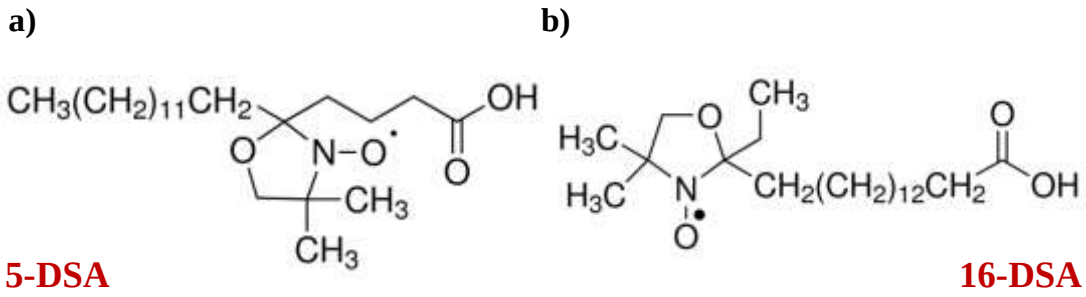


Figure S3. Molecular structures of (**a**) 5-DSA and (**b**) 16-DSA spin probes used in EPR measurements. DSA = doxyl stearic acid.

time (weeks)	17.5 mM		3.5 mM		175 µM	
	Pure niosome					
	Z-average (r. nm) ± SD	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**
0	69.8 ± 0.3	0.44 ± 0.01	62.9± 0.4	0.38 ± 0.01	78.7± 0.8	0.39 ± 0.10
1	85.7± 0.1	0.61± 0.10	65.9 ± 2.3	0.37 ± 0.01	79.4 ± 2.9	0.34 ± 0.02
3	86.5 ± 0.7	0.47 ± 0.03	69.0 ± 4.4	0.34 ± 0.03	84.5 ± 2.2	0.59 ± 0.11
4	74.7 ± 0.1	0.43 ± 0.02	79.5 ± 0.7	0.51 ± 0.06	76.9± 2.6	0.59 ± 0.12
	niosome_HoThyRu					
	Z-average (r. nm) ± SD	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**
0	56.8 ± 0.1	0.32 ± 0.01	76.1± 1.6	0.46 ± 0.02	56± 2	0.46 ± 0.12
1	52.9 ± 1.4	0.35 ± 0.07	87 ± 2	0.43 ± 0.03	107± 2	0.47 ± 0.03
3	54.8± 0.7	0.35 ± 0.01	68 ± 3	0.43 ± 0.03	93± 2	0.42 ± 0.02
4	59.1± 1.6	0.39 ± 0.01	103± 2	0.27 ± 0.03	106± 5	0.44 ± 0.02
	AS1411/niosome					
	Z-average (r. nm) ± SD	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**
0	81.4± 0.6	0.32 ± 0.02	66.5± 0.7	0.24 ± 0.01	67.8± 0.5	0.22 ± 0.01
1	85.6 ± 0.6	0.33 ± 0.01	77 ± 2	0.37 ± 0.01	68.3 ± 0.6	0.34 ± 0.02
3	80.6± 0.9	0.30 ± 0.03	68.3± 0.3	0.20 ± 0.01	72.9± 1.2	0.23 ± 0.01
4	87± 3	0.33 ± 0.01	80.2± 0.2	0.28 ± 0.01	75.9 ± 0.5	0.26 ± 0.01
	AS1411/niosome_HoThyRu					
	Z-average (r. nm) ± SD	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**	**Z-average (r. nm) ± SD**	**PdI± SD**
0	86.7± 0.6	0.32 ± 0.04	87± 5	0.37 ± 0.01	75.9 ± 0.5	0.39 ± 0.01
1	74.7± 0.4	0.26 ± 0.01	82.0 ± 1.3	0.36 ± 0.01	79.2 ± 0.3	0.28 ± 0.01
3	93.3 ± 0.6	0.38 ± 0.01	67.6 ± 0.4	0.21 ± 0.01	77.1 ± 0.1	0.30 ± 0.03
4	80.3 ± 0.1	0.29 ± 0.01	71.9 ± 0.1	0.26 ± 0.01	83.1 ± 0.7	0.36 ± 0.01

Table S5. Z-average size (r., nm) and corresponding PdI values, reported as a function of time for all the niosomal formulations (unfunctionalized niosomes, niosome_HoThyRu, AS1411/niosome and AS1411/niosome_HoThyRu) at different concentrations. Data are reported as an average of three measurements (mean ± SD).

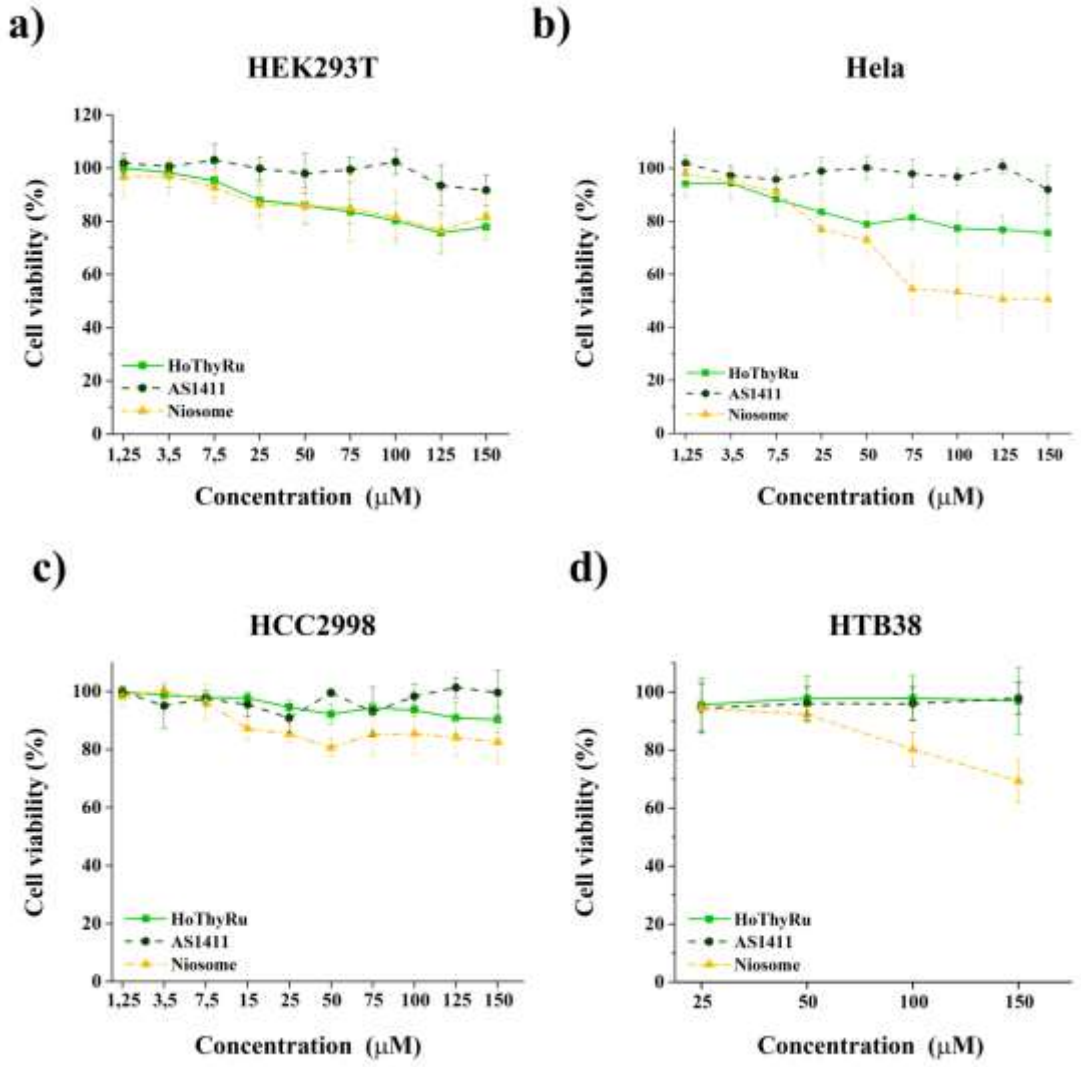


Figure S4. Normalized cell viability for AS1411, HoThyRu and unfunctionalized niosome in the four indicated cell lines. Cells were treated with increasing concentrations of the three tested samples ranging from 1.25 to 150 µM. **a**) HEK293T, **b**) HeLa, **c**) HCC2998 and **d**) HTB-38. Data are reported as mean values ± SD (error bar), for four independent experiments in triplicate (n = 15). All data are normalized to PBS used as control.