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Synthesis and cytotoxic potency of novel tris(1-alkylindol-3-yl)methylium salts: Role of *N*-alkyl substituents

Sergey N. Lavrenov [a], Yuriy N. Luzikov [a], Evgeniy E. Bykov [a], Marina I. Reznikova [a], Evgenia V. Stepanova [b], Valeria A. Glazunova [b], Yulia L. Volodina [b], Victor V. Tatarsky Jr. [b], Alexander A. Shtil [b], Maria N. Preobrazhenskaya [a,*]

[a] *Gause Institute of New Antibiotics, Russian Academy of Medical Sciences, 11B. Pirogovskaya Street, Moscow 119021, Russian Federation*
[b] *Blokhin Cancer Center, Russian Academy of Medical Sciences, 24 Kashirskoye shosse, Moscow 115478, Russian Federation*

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ABSTRACT

Novel derivatives of tris(indol-3-yl)methane and tris(indol-3-yl)methylium salts with the alkyl substituents at the N-atoms of the indole rings were synthesized. An easy substitution of indole rings in trisindolylmethanes for other indoles under the action of acids is demonstrated, and the mechanism of substitution is discussed. To obtain trisindolylmethylium salts, the environmentally safe method of oxidation of trisindolylmethanes with air oxygen in acidic conditions was developed. Tris(1-alkylindol-3-yl)methanes and tris(1-alkylindol-3-yl)methylium salts represent three-bladed molecular propellers whose physico-chemical and biological properties strongly depend on the *N*-alkyl substituent. The cytotoxicity of novel compounds increased with the number of C atoms in the alkyl chains, with optimal number $n = 3–5$ whereas the derivatives with longer side chains were less cytotoxic. The most potent novel compounds killed human tumor cells at nanomolar-to-submicromolar concentrations, being one order of magnitude more potent than the prototype antibiotic turbomycin A [tris(indol-3-yl)methylium salt]. Apoptosis in HCT116 colon carcinoma cell line induced by tris(1-pentyl-1*H*-indol-3-yl)methylium methanesulfonate was detectable at concentrations tolerable by normal blood lymphocytes. Thus, *N*-alkyl substituted tris(1-alkylindol-3-yl)methylium salts emerge as perspective anticancer drug candidates.

1. Introduction

The antibiotic turbomycin A originally isolated as a product of *Saccharomyces cerevisiae* fermentation is the salt of tris(indol-3-yl)methylium.[1] Eventually turbomycin A was obtained from soil microorganisms by a metagenomic approach using a 24,546-member DNA library expressed in *Escherichia coli*.[2] Several clones produced the dark brown colonies from which the trisarylmethylium cations designated turbomycin A [tris(indol-3-yl)methylium] and turbomycin B [bis(indol-3-yl)phenyl)methylium], respectively, were isolated (Fig. 1). Both these agents were capable of killing Gram-negative and Gram-positive microorganisms.[2]

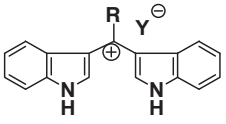
These results provided strong evidence that turbomycins comprise a perspective class of biologically active compounds. Given the complex role of triphenylmethyl motif in anticancer compounds,[3] we set out to synthesize new *N*-alkyl substituted trisindolylmethylium cations and test their potency against cultured human tumor cells.

The trisindolylmethylium salts (**1**) can be obtained by oxidation of the corresponding trisindolylmethanes (**2**). These have been synthesized by condensation of indol-3-carboxaldehyde (**3**) with indoles (**4**) in the presence of acetic acid,[4,5] $La(OSO_2CF_3)_3$ or triflates of other lantanoids,[6] acid-washed montmorillonite K10 clay,[7] ammonium chloride[8] and other low acidic catalysts (Scheme 1). Oxidation of trisindolylmethanes to produce the salts of trisindolylmethylium has been performed using oxidizing agents such as DDQ, chloranil[1,2], trityl perchlorate[9] or $FeCl_3$[10] (Scheme 1).

2. Results and discussion

2.1. Chemistry

To obtain the substituted trisindolylmethanes (**2**), we condensed *N*-alkylindol-3-carboxaldehydes (**3**) with corresponding N-substituted indoles (**4**) in ethanol in the presence of $La(OTf)_3$,



R = Indol-3-yl. Turbomycin A

R = Phenyl. Turbomycin B

Figure 1. Structure of turbomycin A and turbomycin B.

* Corresponding author. Tel.: +7499 245 3753; fax +7499 245 0295.
E-mail address: mnp@space.ru (M.N. Preobrazhenskaya).

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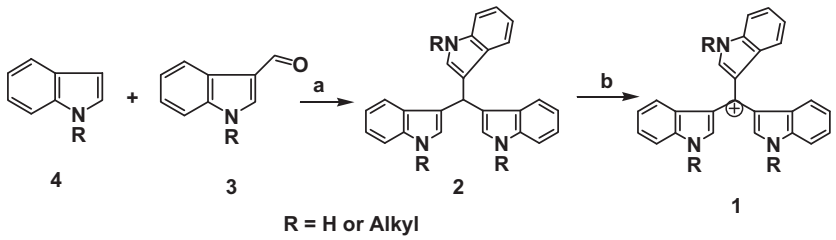
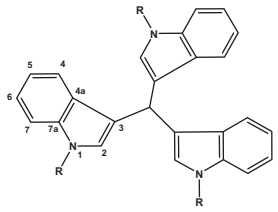


Scheme 1. Standard synthesis of tris(indol-1-yl)methylium salts. Reagents and conditions: (a) ethanol, weak acidic catalyst (AcOH, $La(OTf)_3$), reflux; (b) ethanol, DDQ, $FeCl_3$ or trityl perchlorate.

$Dy(OTf)_3$ or a mixture of acetic acid and acetic anhydride as the catalysts. The yields of trisindolylmethanes were >95%. The properties of compounds **2b–i** are presented in Table 1.

Chakrabarty and Sarkar have synthesized symmetrical and non-symmetrical trisindolylmethanes from 3-formylindole and mono- and dialkylindoles under the action of montmorillonite K10 clay.[7]

Table 1
Properties of tris(1-alkyl-indol-3-yl)methanes **2a–i**



N-substituent	Mass spectra m/z[b]	Combustion analysis	Mp (from ethanol) °C TLC R_f (solvents)[a]	^1H and ^{13}C NMR Chemical shifts (ppm) and J (Hz)
R = H **2a**	Calcd: 361.16. Found: 361 (M^+)[b]	Calcd ($C_{25}H_{19}N_3$): C, 83.08; H, 5.30; N, 1.63. Found: C, 83.78; H,5.59; N, 11.72	244–246 0.50 (B)	^1H (DMSO-d_6): 6.11 (1H; s; CH); 6.89 (3H; t; J = 7.69; H-5(6)); 6.99 (3H; d; J = 2.1; H-2); 7.05 (3H; t; J = 7.5; H-6(5)); 7.38 (3H; d; J = 8.1; H-7); 7.45 (3H; d; J = 7.9); 10.77 (3H; d; J = 2.1; N–*H*) ^{13}C (DMSO-d_6): 30.99 (3-*C*H); 111.43 (7-C); 118.01 (4-C); 118.32 (3-C); 119.36 (6-C); 120.70 (5-C); 123.26 (4a-C); 126.82 (2-C); 136.63 (7a-C)
R = methyl **2b**	Calcd: 403.2. Found: 403 (M^+)[b]	Calcd ($C_{28}H_{25}N_3$): C, 83.34; H, 6.24; N, 10.41. Found: C, 83.45; H, 6.27; N, 10.43	241–243 0.70 (B)	^1H (Pyr/d5): 4.46 (9H; s; N–CH_3); 6.51 (1H; s; CH); 6.98 (3H; s; H-2); 7.11 (3H; t; J = 7.6; H-6(5)); 7.28 (3H; t; J = 7.6; H-6(5)); 7.37 (3H; d; J = 8.0; H-7); 7.79 (3H; d; J = 7.9; H-4) ^{13}C (Pyr/d5): 31.60 (1-CH_3); 32.26 (3-*C*H); 109.76 (7-C); 119.04 (4-C); 119.07 (3-C); 120.34 (6-C); 121.71 (5-C); 128.33 (4a-C); 128.40 (2-C); 138.21 (7a-C)
R = ethyl **2c**	Calcd: 445.25. Found: 445 (M^+)[b]	Calcd ($C_{31}H_{31}N_3$): C, 83.56; H, 7.01; N, 9.43. Found: C, 83.67; H, 7.04; N, 9.43	225–227 0.47 (B)	^1H (Pyr/d5; 60 °C): 1.09 (9H; t; N–CH_2CH_3); 3.84 (6H; q; N–CH_2CH_3); 6.48 (1H; s; CH); 7.06 (3H; s; H-2); 7.07 (3H; t; J = 7.5; H-6(5)); 7.23 (3H; t; J = 7.5; H-6(5)); 7.36 (3H; d; J = 8.2; H-7); 7.74 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 15.37 (signal of N-ethyl group); 32.02 (3-*C*H); 40.78 (signal of N-ethyl group); 109.89 (7-C); 118.96 (3-C); 118.99 (4-C); 120.59 (6-C); 121.59 (5-C); 126.83 (2-C); 128.54 (4a-C); 137.22 (7a-C)
R = propyl **2d**	Calcd: 487.3. Found: 487 (M^+)[b]	Calcd ($C_{34}H_{37}N_3$): C, 83.74; H, 7.65; N, 8.62. Found: C, 83.56; H, 7.80; N, 8.72	214–216 0.62 (A)	^1H (Pyr/d5; 60 °C): 0.68 (9H; t; N–$CH_2CH_2CH_3$); 1.57 (6H; m; N–$CH_2CH_2CH_3$); 3.81 (6H; t; N–$CH_2CH_2CH_3$); 6.50 (1H; s; CH); 7.07 (3H; s; H-2); 7.09 (3H; t; J = 7.5; H-6(5)); 7.26 (3H; t; J = 7.5; H-6(5)); 7.40 (3H; d; J = 8.2; H-7); 7.76 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 10.84; 23.22 (signals of N-propyl group); 31.61 (3-CH); 47.26 (signal of N-propyl group); 109.59 (7-C); 118.15 (3-C); 118.44 (4-C); 120.22 (6-C); 121.14 (5-C); 127.15 (2-C); 127.97 (4a-C); 137.07 (7a-C)
R = *n*-butyl **2e**	Calcd: 529.8. Found: 529.5 (M^+)[b]	Calcd ($C_{37}H_{43}N_3$): C, 83.89; H, 8.18; N, 7.93. Found: C, 83.68; H, 8.20; N, 8.03	211–213	^1H (Pyr/d5; 60 °C): 0.71 (9H; t; N–$CH_2CH_2CH_2CH_3$); 1.11 (6H; m; N–$CH_2CH_2CH_2CH_3$);1.57 (6H; m; N–CH_2CH_2 CH_2CH_3); 3.81 (6H; t; N–$CH_2CH_2CH_2CH_3$); 6.50 (1H; s; CH); 7.07 (3H; s; H-2); 7.08 (3H; t; J = 7.5; H-6(5)); 7.25 (3H; t; J = 7.5; H-6(5)); 7.40 (3H; d; J = 8.2; H-7); 7.78 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 13.67, 20.15, 31.97 (signals of N-butyl group); 32.48 (3-*C*H); 45.78 (signals of *N*-butyl group); 110.12 (7-C); 118.46 (3-C); 118.88 (4-C); 120.69 (6-C); 121.91 (5-C); 127.60 (2-C); 128.28 (4a-C); 137.38 (7a-C)
R = *n*-pentyl **2f**	Calcd: 571.8. Found: 571.6 (M^+)[b]	Calcd ($C_{40}H_{49}N_3$): C, 84.01; H, 8.64; N, 7.35. Found: C, 83.81; H, 8.86; N, 7.56	193–195	^1H (Pyr/d5): 0.7–3.9 (37H, signals of N-pentyl group); 6.58 (1H; s; CH); 7.19 (6H; m; H-2 + H-6(5)); 7.33 (3H; t; J = 9.1; H-6(5)); 7.50 (3H; d; J = 9.5; H-7); 7.84 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 14.06, 22.45, 29.11, 30.18 (signals of N-pentyl group); 32.05 (3-CH); 46.08 (signal of N-pentyl group); 110.15 (7-C); 118.48 (3-C); 118.96 (4-C); 120.73 (6-C); 121.64 (5-C); 127.65 (2-C); 128.35 (4a-C); 137.41 (7a-C)
R = *n*-hexyl **2g**	Calcd: 613.44. Found: 612.997 (M^+)[c]	Calcd ($C_{43}H_{55}N_3$): C, 84.13; H, 9.03; N, 6.84. Found: C, 83.91; H, 9.24; N, 6.93	168–170 0.85 (A)	^1H (Pyr/d5): 0.7–3.9 (39H, signals of N-hexyl group); 6.58 (1H; s; CH); 7.19 (6H; m; H-2 + H-6(5)); 7.33 (3H; t; J = 9.1; H-6(5)); 7.50 (3H; d; J = 9.5; H-7); 7.84 (3H; d; J = 8.2; H-4)

Table 1 (*continued*)

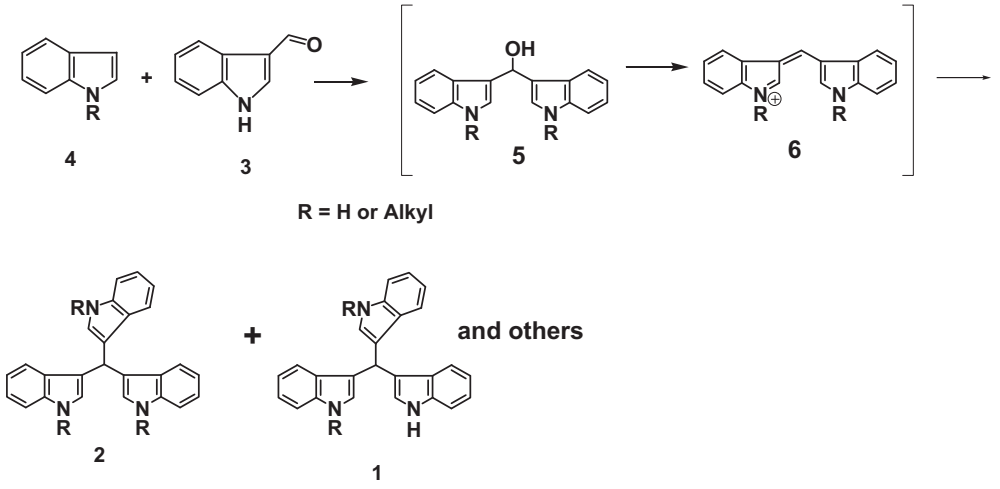
N-substituent	Mass spectra m/z[b]	Combustion analysis	Mp (from ethanol) °C TLC R_f (solvents)[a]	^{1}H and ^{13}C NMR Chemical shifts (ppm) and J (Hz)
				^{13}C (Pyr/d5): 14.09, 22.69, 26.63, 30.41, 31.47 (signals of N-hexyl group); 31.96 (3-*C*H); 46.09 (signal of N-hexyl group); 110.11 (7-C); 118.47 (3-C); 118.93 (4-C); 120.68 (6-C); 121.60 (5-C); 127.62 (2-C); 128.32 (4a-C); 137.37 (7a-C)
R = *n*-decyl **2i**	Calcd: 782.2. Found: (M^+) 781.5[b]	Calcd ($C_{55}H_{79}N_3$): C, 84.45; H, 10.18; N, 5.37. Found: C, 84.25; H, 10.10; N, 5.17	156–158	^{1}H (Pyr/d5): 0.94–4.01 (63H, signals of N-decyl group); 6.57 (1H; s; CH);7.19 (6H; m; H-2 + H-6(5)); 7.31 (3H; t; J = 9.1; H-6(5)); 7.49 (3H; d; J = 9.5; H-7); 7.83 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 14.20, 22.91, 27.17, 29.50, 29.52, 29.78, 29.79, 30.58, 32.11 (signals of N-decyl group); 32.24 (3-*C*H); 46.30 (signal of N-decyl group); 110.17 (7-C); 118.85 (3-C); 119.03 (4-C); 120.75 (6-C); 121.70 (5-C); 127.63 (2-C); 128.56 (4a-C); 137.67 (7a-C)
R = benzyl **2h**	Calcd: 631.8. Found: (M^+) 631.3[b]	Calcd ($C_{46}H_{37}N_3$): C, 87.45; H, 5.90; N, 6.65. Found: C, 87.17; H, 6.05; N, 6.71	236–238	^{1}H (Pyr/d5): 5.22 (6H; s; signals of N–C*H*$_2$Ph); 6.56 (1H; s; CH); 7.12–7.80 (30H; m; signals of N–CH$_2$*Ph* and indole rings) ^{13}C (Pyr/d5): 32.34 (3-*C*H); 49.97 (*C*H$_2$ of Bn group); 110.42; 119.27; 119.30; 120.77; 121.98; 127.07; 127.55; 128.18; 128.69; 128.87; 137.90; 138.75
7 R = Pr; Pr; Et	Calcd: 473.3. Found: (M^+) 473.1	Calcd ($C_{33}H_{35}N_3$): C, 83.68; H, 7.45; N, 8.87. Found: C, 83.35; H, 7.55; N, 8.67		^{1}H (Pyr/d5): 0.66 (6H; t; N–CH$_2$CH$_2$C*H*$_3$); 1.13 (3H; t; N–CH$_2$C*H*$_3$); 1.56 (4H; m; N–CH$_2$CH$_2$C*H*$_3$); 3.91 (4H; t; N–CH$_2$*CH*$_2$CH$_3$); 3.98 (2H; q; N–*CH*$_2$CH$_3$); 6.17 (1H; s; CH); 6.90 (3H; t; J = 7.5; H-6(5)); 6.97 (2H; s; H-2); 6.99(1H; s; H-2); 7.08 (3H; t; J = 7.5; H-6(5)); 7.36 (3H; d; J = 8.2; H-7); 7.49 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 12.67, 19.15, 30.95 (signal of N-ethyl and N-propyl group); 31.85 (3-*C*H); 44.83, 45.65 (signal of N-ethyl and N-propyl group); 109.55 (7-C); 118.73 (3-C); 118.81 (4-C); 120.48 (6-C); 121.92 (5-C); 126.86 (2-C); 128.44 (4a-C); 137.15 (7a-C)
8 R = Pr; Et; Et	Calcd: 459.27. Found: (M^+) 459.1	Calcd ($C_{32}H_{33}N_3$): C, 83.62; H, 7.24; N, 9.14. Found: C, 83.32; H, 7.54; N, 9.11		^{1}H (Pyr/d5): 0.91 (3H; t; N–CH$_2$CH$_2$C*H*$_3$); 1.11 (6H; t; N–CH$_2$C*H*$_3$); 1.58 (2H; m; N–CH$_2$*CH*$_2$CH$_3$); 3.92 (2H; t; N–CH$_2$CH$_2$CH$_3$); 4.05 (4H; q; N–CH$_2$CH$_3$); 6.21 (1H; s; CH); 6.92 (3H; t; J = 7.5; H-6(5)); 6.99 (2H; s; H-2); 6.99(1H; s; H-2); 7.08 (3H; t; J = 7.5; H-6(5)); 7.36 (3H; d; J = 8.2; H-7); 7.49 (3H; d; J = 8.2; H-4) ^{13}C (Pyr/d5): 11.82; 15.39; 23.24 (signal of N-ethyl and N-propyl group); 31.82 (3-CH); 44.78, 45.59 (signal of N-ethyl and N-propyl group); 109.79 (7-C); 118.85 (3-C); 118.89 (4-C); 120.46 (6-C); 121.59 (5-C); 126.78 (2-C); 128.48 (4a-C); 137.19 (7a-C)

[a] Analytical TLC was performed on Kieselgel F254 plates (Merck) in systems: petroleum ether–EtOAc 5:1 (A); 1:1 (B).
[b] Electron-impact mass spectra.
[c] Matrix-assisted laser desorption/ionization (MALDI) mass spectra.

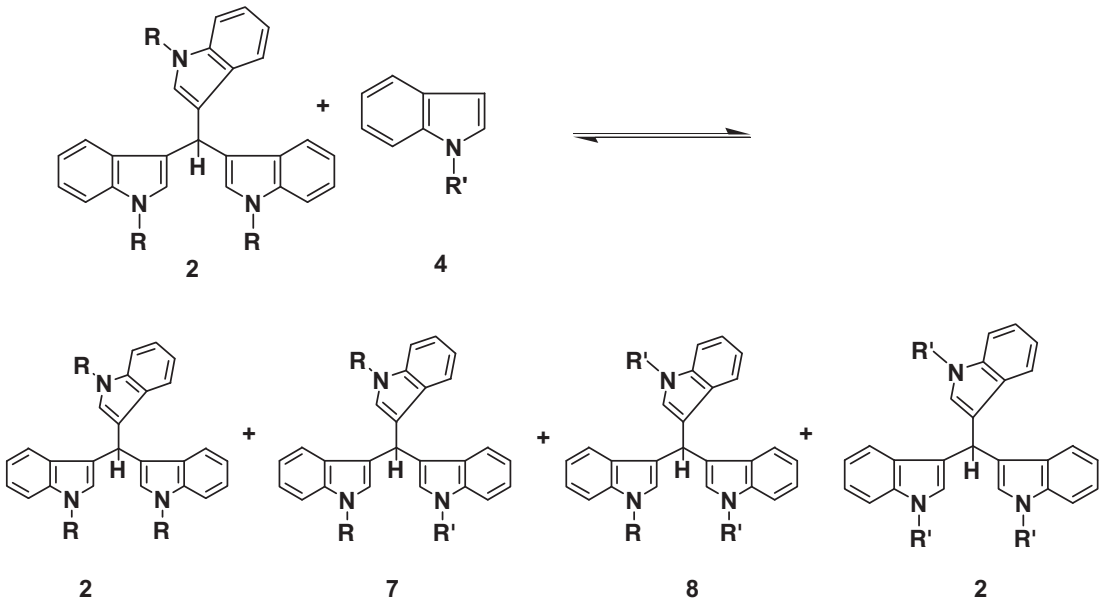
The authors suggested that the formation of symmetrical and non-symmetrical trisindolylmethanes proceeded via bis(indol-3-yl)methanol (**5**) which is transformed into *Z*-3-(indol-3-yl)methylene-3*H*-indolium (urorosein) (**6**). The latter compound interacts with indole molecules to produce a mixture of trisindolylmethanes (Scheme 2).[7]

We found that the reflux of tris(1-alkyl-indol-3-yl)methanes in ethanol in the presence of a catalyst (La(OTf)$_3$, Dy(OTf)$_3$ or NH_4Cl) with an excess of 1-alkylindole also leads to a mixture of symmetrical and non-symmetrical trisindolylmethanes (Scheme 3).

Table 1-SM (Supplementary data) shows the ratios of trisindolylmethanes formed by interaction of N-substituted 3-formylindole (**3**) with *N*′-substituted indole (**4**) or in the reaction of tris(1-alkylindol-3-yl)methane (**2**) with *N*′-alkylindole (**4**). The data demonstrate that isolation of a symmetrical trisindolylmethane (**2**) depends not only on the reaction conditions but also



Scheme 2. Interaction of indolcarboxaldehyde with N-alkylindoles under the action of montmorillonite K10 clay.[7]



Scheme 3. Interaction of tris(1-alkylindol-3-yl)methanes with indoles in the presence of acidic catalysts. R = C_nH_{2n+1}, R_1 = $C_{n+1}H_{2(n+1)+1}$; Compounds **2** and **4**: R, R_1 = H (a); CH_3 (b); C_2H_5 (c); *n*-C_3H_7 (d); *n*-C_4H_9 (e); *n*-C_5H_{11} (f); *n*-C_6H_{13} (g); $CH_2C_6H_5$ (h); $C_{10}H_{21}$ (i). Compounds **7** and **8**: R = C_2H_5, R′ = *n*-C_3H_7, R′ = R = C_2H_5, *n*-C_3H_7.

on the methods of isolation and purification of the reaction products. Figure 2 presents HPLC chromatograms of the products formed in ethanol solution (A) by refluxing tris(1-ethylindol-3-yl)methane (**2c**) with an excess of 1-propylindole (**4d**) and (B) by refluxing tris(1-propylindol-3-yl)methane (**2d**) with an excess of 1-ethylindole (**4c**).

In the interaction of trisindolylmethane with indole urorosein cannot be obtained since it is formed by dehydration of the intermediate bis(indol-3-yl)methanol. One can suggest that the substitution reaction starts with protonation of trisindolylmethane followed by substitution of the indole ring with *N*-alkyl indole (Schemes 4a and b). Protonation of one of indole rings in

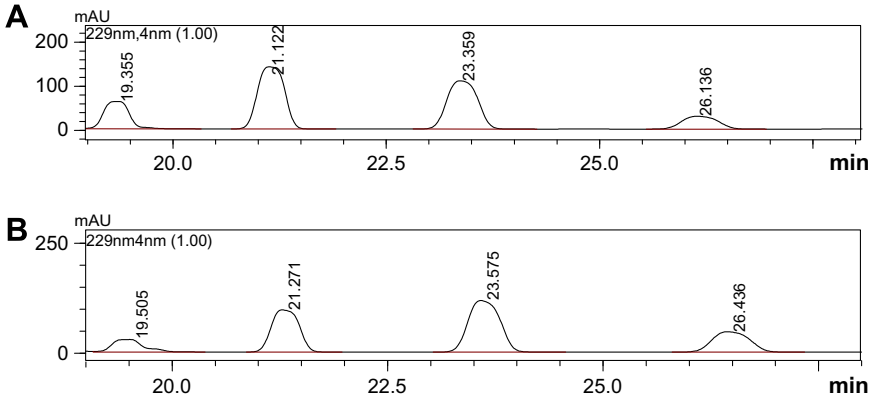
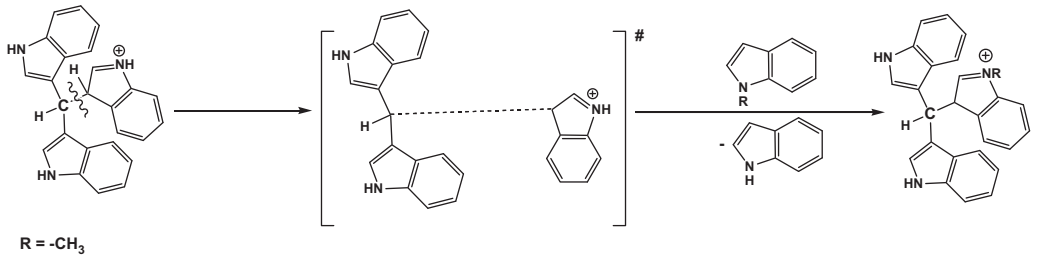
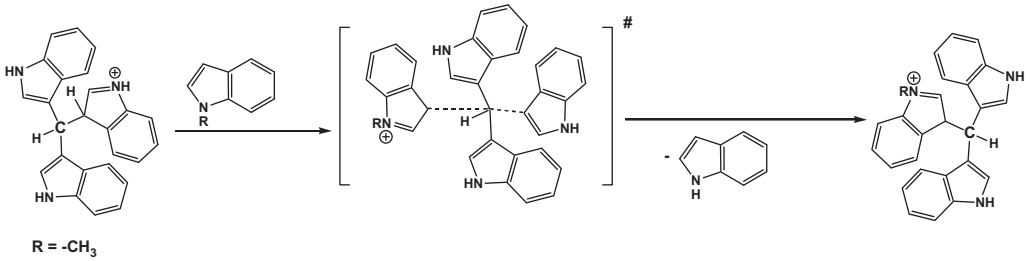


Figure 2. HPLC of the reaction products. (A) Trisindolylmethanes formed by the interaction of tris(1-ethylindol-3-yl)methane (**2c**) with 1-propylindole (**4d**). rt 26.13 min—tris(1-propylindol-3-yl)methane (**2d**); rt 19.55 min—tris(1-ethylindol-3-yl)methane (**2c**); rt 21.12 min—bis(1-ethylindol-3-yl)(1-propylindol-3-yl)methane (**7**) and 23.58 min—bis(1-propylindol-3-yl)(1-ethylindol-3-yl)methane (**7**). (B) Trisindolylmethanes formed by the interaction of tris(1-propylindol-3-yl)methane (**2d**) with 1-ethylindole (**4c**).



Scheme 4a. The suggested S_N1-type mechanism of nucleophilic substitution in trisindolylmethane.



Scheme 4b. The suggested S_N2-type of mechanism of nucleophilic substitution in trisindolylmethane.

trisindolylmethane leads to the removal of the protonated indole and formation of bisindolylmethyl cation (urorosein) attacked by a new indole molecule. These events resemble the reaction of substitution of a substituted phenyl ring for an unsubstituted one in triphenylmethanes under the action of super acids. The mechanism of substitution of a substituted phenyl ring in triphenylmethanes for a phenyl ring in the presence of strong acids has been interpreted as trans-alkylation of substituted triphenylmethanes.[11]

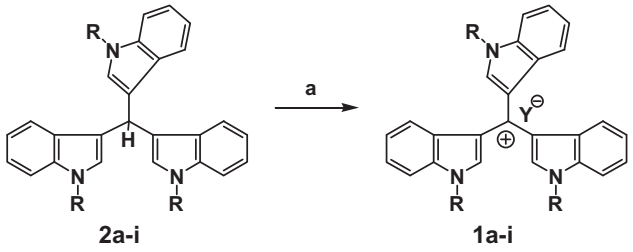
Quantum-chemical study using semi-empirical methods AM1 and DFT B3LYP/6-31G(d)[13,14] showed that the calculated activation barriers ($\Delta E^{\#}_{AM1}$ = 16.4 kcal/mol and $\Delta E^{\#}_{B3LYP/6\text{-}31G(d)}$ = 16.1 kcal/mol) are preferred for S_N1-type mechanism (Scheme 4a) than for S_N2-type mechanism (Scheme 4b) where $\Delta E^{\#}_{AM1}$ = 51.8 kcal/mol and $\Delta E^{\#}_{B3LYP/6\text{-}31G(d)}$ = 80.2 kcal/mol. $\Delta E^{\#}$ means activation barrier energy. The quantum chemical computation by density functional method B3LYP/6-31G(d) with considered influence of the solvent (method of overlaying spheres DPCM) showed that ΔE of the dissociation reaction of the protonated trisindolylmethane in ethanol as a solvent (Schemes 4a and b) was by 5 kcal/mol smaller than ΔE for this reaction in a gas phase. The calculated effect of solvatation is an additional argument in favor of S_N1-type mechanism of the investigated reaction.

The reaction of indole substitution in trisindolylmethanes is reversible and all four possible symmetrical and non-symmetrical trisindolylmethanes were formed. Presumably, the reversibility of the substitution reaction is due to similar values of Q_{Mal} and Fukui index of HOMO for C-3 atom of 1-alkylindoles (see Table 2 in Supplementary data).

Introduction of three indole rings into trisindolylmethanes generates three-bladed molecular propellers that consist of three rigid aromatic rings (blades) connected to the central atom (hub) in the propeller axis. The indole rings are rapidly twisted and adopt clockwise and counterclockwise arrangement. Due to unsymmetry within each indole ring, this gives rise to stereoisomeric conformations.[12] The NMR spectra of novel tris(indol-3-yl)methanes (Table 1) were well resolved at room temperature. The width of signals did not change with the increase of temperature, indicating that the indole rings undergo free rotation (flips) at room temperature.

To obtain trisindolylmethylium salts, we developed an environmentally safe method of oxidation of trisindolylmethanes with air oxygen in acidic conditions. Trisindolylmethanes were dissolved in butanol with the addition of an acid depending on the salt we aimed at (i.e., methanesulfonic or acetic acid). The solutions were stirred after the addition of activated charcoal to enhance the contact of the compound with air oxygen. These procedures resulted in trisindolylmethylium salts in good yields (Scheme 5). All tris(indol-3-yl)methylium salts **1a–i** were water soluble red or orange solids. The properties of novel tris(indol-3-yl)methylium methane sulfonates are summarized in Table 2.

In the NMR spectra of trisindolylmethylium salts the two sets of signals detectable at room temperature merged with the increase of temperature. At $\geqslant$100 °C only one set of signals was observed (Table 2). The ratio of intensities of two C_2-H signals at room temperature was 1:2. The ^{1}H NMR spectra (low field area) of tris(1-decylindol-3-yl)methylium methanesulfonate at 22 °C (A) and 100 °C (B) are shown (Fig. 3). NMR spectra of compounds **1a–1h** are broadened at room temperature and the area of rapid dynamics was reached at the temperature about 100 °C. This process is independent of the length of the *N*-alkyl substituent, demonstrating that the dynamic equilibrium is not dependent on steric hindrance. ^{13}C NMR spectrum of **1c** measured at room temperature and at 140 °C is presented in Supplementary data.



Scheme 5. Synthesis of tris(indol-3-yl)methylium salts (**1a**–**i**). R = H (a); CH_3 (b); C_2H_5 (c); *n*-C_3H_7 (d); *n*-C_4H_9 (e); *n*-C_5H_{11} (f); *n*-C_6H_{13} (g); $CH_2C_6H_5$ (h); *n*-$C_{10}H_{21}$ (i). Reagents and conditions: (a) butanol, O_2, activated charcoal, H^+Y^-, stirring 48 h.

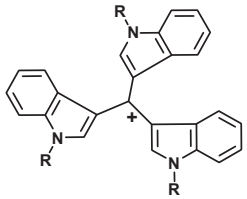
Four isomeric propeller conformations (A, A′, B, and B′) are possible for a molecule of this type including the stereoisomers that isomerize by flip mechanism (Scheme 6). The transition state of the one ring flip of trisindolylcarbonium ions is energetically favored due to resonance stabilization. The quantum chemical calculations using semi-empirical AM1 and DFT methods showed that the delocalized form of trisindolylmethylium cation (B) is more stable than the indolenine form (Scheme 7) by 1.4 kcal/mol.

2.2. Biological testing

We tested compounds **1a–i** for the ability to induce death of cultured human colon carcinoma HCT116 and leukemia K562 cell lines. In these experiments the cells were treated with each compound for 72 h followed by determination of cell viability using an MTT-test. Mean IC_{50} decreased progressively with the increase of the number of C atoms in the alkyl chain: from 3.2 μM (for Me substituted derivative **1b**) down to 0.05–0.15 μM (*n*-pentyl containing compound **1f**). The compounds **1g–i** with longer substituents at the N atom of the indole ring were less potent than **1f** (Table 3). The cytotoxicity of tris(1-alkylindol-3-yl)methylium methanesulfonates for K562 cells was generally higher than for HCT116 cell line; still, the dependence of the activity on the length of the alkyl chain was the same for both cell lines (Table 3). Turbomycin A induced tumor cell death at micromolar concentrations whereas some novel compounds were one order of magnitude more potent.

Thus, introduction of alkyl substituents at the N atoms of indoles produced the derivatives with more pronounced cytotoxicity compared with turbomycin A. Gradual increase of the cytotoxic

Table 2
Tris(1-alkylindol-3-yl)methylium methanesulfonates **1a–i**



N-Substituent	MW of methylium salt/ cation calculated, found by mass spectra**	HPLC data: (HPLC system), purity,%	^{1}H NMR Chemical shifts (ppm) and *J* (Hz)
1b R = methyl	Calcd: 497.18/402.2. Found: 402.51 [M$^+$]	33.32 (1A) 22.10 (2A) 95%	^{1}H (DMSO-d_6; 90 °C): 2.44 (3H; s; CH_3SO$_3$); 4.09 (9H; s; N–CH$_3$); 7.01 (3H; d; *J* = 8.0; 7(4)-H); 7.10 (3H; t; *J* = 8.0; 5(6)-H); 7.41 (3H; t; *J* = 7.9; 6(5)-H; 7.78 (3H; d; *J* = 8.2; 4(7)-H); 8.39 (3H; s; 2-H).
1c R = ethyl	Calcd: 539.22/444.24. Found: 444.6 [M$^+$]	39.18 (1A) 26.05 (2A) 97%	^{1}H (DMSO-d_6; 110 °C): 1.61 (9H; t, N–CH$_2$CH_3); 2.37 (3H; s; CH_3SO$_3$); 4.53 (6H; q, N–CH_2CH$_3$); 7.04 (3H; d; *J* = 8.0; 7(4)-H); 7.13 (3H; t; *J* = 8.0; 5(6)-H); 7.42 (3H; t; *J* = 8.2; 6(5)-H); 7.85 (3H; d; *J* = 8.2; 4(7)-H); 8.43 (3H; s; 2-H).
1d R = *n*-propyl	Calcd: 581.27/486.29. Found: 486.68 [M$^+$]	28.37(1A) 29.69 (2A) 95%	^{1}H (DMSO-d_6; 90 °C): 1.01 (9H; m; N–CH$_2$CH$_2$CH_3); 2.01 (6H; m; N–CH$_2$CH_2CH$_3$); 2.42 (3H;s; CH_3SO$_3$); 4.45 (6H; m; N–CH_2CH$_2$CH$_3$); 7.01 (3H; d; *J* = 7.9; 7(4)-H); 7.12 (3H; t; *J* = 7.8; 6(5)-H); 7.41 (3H; t; *J* = 7.9; 5(6)-H); 7.85 (3H; d; *J* = 8.1; 4(7)-H); 8.41 (3H; s; 2-H).
1e R = *n*-butyl	Calcd: 623.32/528.34. Found: 528.64 [M$^+$]	12.98 (1C) 7.78 (2B) 95%	^{1}H (DMSO-d_6; 50 °C): 0.94 (9H; m; N–CH$_2$CH$_2$CH$_2$CH_3); 1.37 (6H; m; N–CH$_2$CH$_2$CH_2CH$_3$); 1.92 (6H; m; N–CH$_2$CH_2CH$_2$CH$_3$); 2.31 (3H; s; CH_3SO$_3$); 4.45 (6H; m; N–CH_2CH$_2$CH$_2$CH$_3$); 6.78 (3H; d; *J* = 7.9; 7(4)-H); 7.11 (3H; t; *J* = 7.9; 5(6)-H); 7.40 (3H; t; *J* = 7.9; 6(5)-H); 7.86 (3H; d; *J* = 7.9; 4(7)-H); 8.43 (3H; s; 2-H).
1f R = *n*-pentyl	Calcd: 665.37/570.38. Found: 570.38 [M$^+$]	18.68 (1C) 10.57 (2B) 96%	^{1}H (DMSO-d_6; 90 °C): 0.96, 1.35, 1.44, 1.92 (27H; 4m; N–CH$_2$–C$_4$H$_9$); 2.33 (3H; s; CH_3SO$_3$); 4.46 (6H; m; N–CH_2–C$_4$H$_9$); 7.03 (3H; d; *J* = 8.0; 7(4)-H); 7.11 (3H; t; *J* = 8.0; 5(6)-H); 7.43 (3H; t; *J* = 7.9; 6(5)-H); 7.83 (3H; d; *J* = 8.0; 4(7)-H); 8.41 (3H; s; 2-H).
1g R = *n*-hexyl	Calcd: 707.41/612.43. Found: 612.92 [M$^+$]	17.05 (2B) 5.65 (3) 95%	^{1}H (DMSO-d_6; 90 °C): 0.98, 1.35, 1.44, 1.61, 1.95 (33H; 5m; N–CH$_2$–C$_5$H$_{11}$); 2.33 (3H; s; CH$_3$SO$_3$); 4.46 (6H; m; N–CH$_2$–C$_5$H$_{11}$) 7.02 (3H; d; *J* = 8.0; 7(4)-H); 7.10 (3H; t; *J* = 8.0; 5(6)-H); 7.43 (3H; t; *J* = 7.8; 6(5)-H); 7.84 (3H; d; *J* = 8.0; 4(7)-H); 8.42 (3H; s; 2-H).
1h R = benzyl	Calcd: 725.27/630.29. Found: 630.41 [M$^+$]	10.33 (1C) 6.33 (2B) 96%	^{1}H (DMSO-d_6; 90 °C): 2.34 (3H; s; CH_3SO$_3$); 5.72 (6H; s; N–CH_2Ph); 7.04 (3H; d; *J* = 8.0; 7(4)-H); 7.11 (3H; t; *J* = 8.0; 5(6)-H); 7.42 (18H; m; N–CH$_2$*Ph* + 6(5)-H); 7.79 (3H; d; *J* = 8.2; 4(7)-H); 8.57 (3H; s; 2-H)
1i R = *n*-decyl	Calcd: 875.6/780.62. Found: 780.83 [M$^+$]	7.78 (3) 95%	^{1}H (DMSO-d_6; 90 °C): 0.84, 1.29, 1.94, 2.09 (57H; 4m; N–CH$_2$–C$_9$H$_{19}$); 2.32 (3H; s; CH_3SO$_3$); 4.45 (6H; m; N–CH_2–C$_9$H$_{19}$); 7.02 (3H; d; *J* = 8.0; 7(4)-H); 7.10 (3H; t; *J* = 8.0; 5(6)-H); 7.41 (3H; t; *J* = 7.9; 6(5)-H); 7.84 (3H; d; *J* = 8.0; 4(7)-H); 8.38 (3H; s; 2-H).

For systems 1A–C: (A) 0.2% HCO_2NH_4, pH 4.5; (B) acetonitrile. The proportion of acetonitrile varied for system 1A from 30% to 90% for 30 min and then 90% for 15 min, for system 1B: 10–40% for 30 min, for system 1C: 70–90% for 10 min and then 90% for 20 min. The systems 2A, 2B: (A) 0.01 M H_3PO_4, pH 2.6; (B) acetonitrile. The proportion of acetonitrile varied for system 2A from 10% to 90% for 30 min and then 90% for 10 min, for system 2B: 65–90% for 25 min, for system 3: 95% CH_3OH and 5% acetonitrile for 15 min. For all systems the flow rate was 1 mL/min.

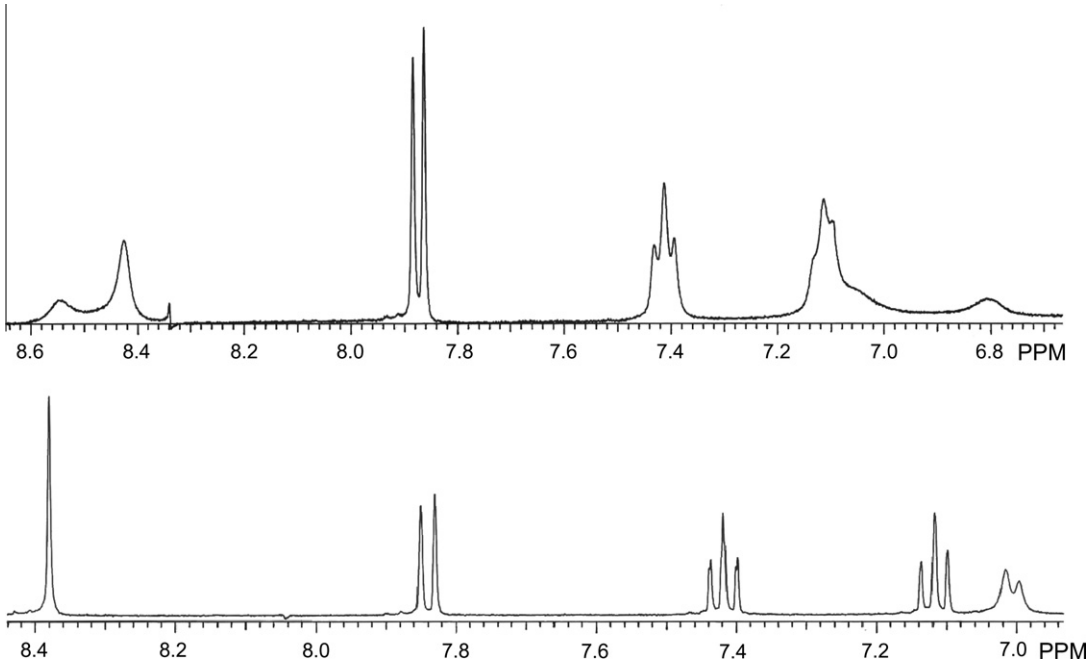
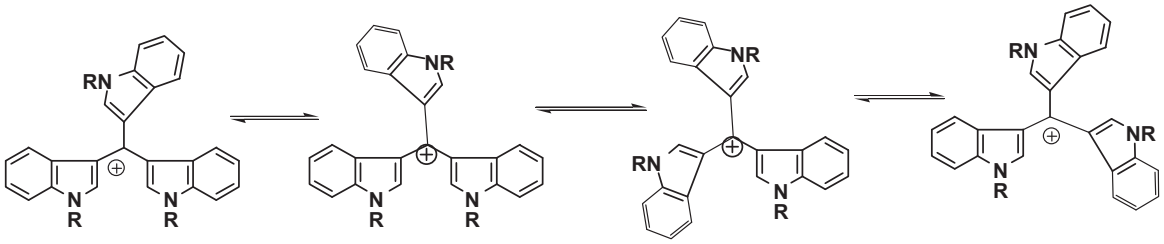
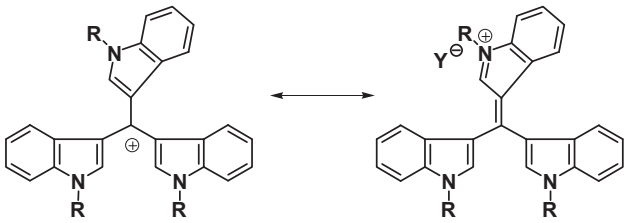


Figure 3. Low field areas of ^{1}H NMR spectra of **1i** in DMSO-d_6. A, 20 °C; B 100 °C.



Scheme 6. Isomeric propeller conformations of tris(1-alkylindol-3-l)methylium cations.



Scheme 7. Resonance stabilization of tris(1-alkylindol-3-yl)cation.

potency was documented if the length of the alkyl *N*-substituent changed from one C atom to a longer chain substituents, with $n = 3–5$ showing the highest potency. Further elongation of the alkyl chains was associated with lower cytotoxicity. Compounds **1c–g** were 2–3 orders of magnitude more potent than N-acetylated triindolylmetanes whose cytotoxicity has been reported to be at decimolar concentrations or higher.[5]

To investigate the cytotoxicity of tris(indol-3-yl)methylium salts in more detail, we selected **1f** as the most potent derivative. At submicromolar concentrations **1f** killed human leukemia, melanoma, breast carcinoma and ovarian carcinoma cell lines (Fig. 4). The Mel Kor cell line derived from disseminated skin melanoma[15] was the most sensitive to **1f**. Importantly, **1f** was less cytotoxic for peripheral blood lymphocytes (PBL) than for tested tumor cell lines (p <0.05 for all tumor cell lines compared with PBL; Fig. 4). These data suggest that tris(indol-3-yl)methylium salts might be preferentially toxic for proliferating cells. Furthermore, **1f** caused a programmed death as determined by characteristic traits of apoptosis, that is, the increase of Annexin V-positive cells and the loss of DNA integrity. The latter was determined as the increased percentage of hypodiploid (sub-G1) nuclei (bar diagram) and internucleosomal DNA fragmentation (Fig. 5). Apoptosis induced by **1f** was p53-independent since the drug was equally potent for HCT116 ($p53^{+/+}$) cell line and for its isogenic subline HCT116p53KO with deleted *p53* gene[16] (data not shown). Altogether, these results strongly suggest that tris(indol-3-yl)methylium salts are perspective for further development as anticancer drug candidates.

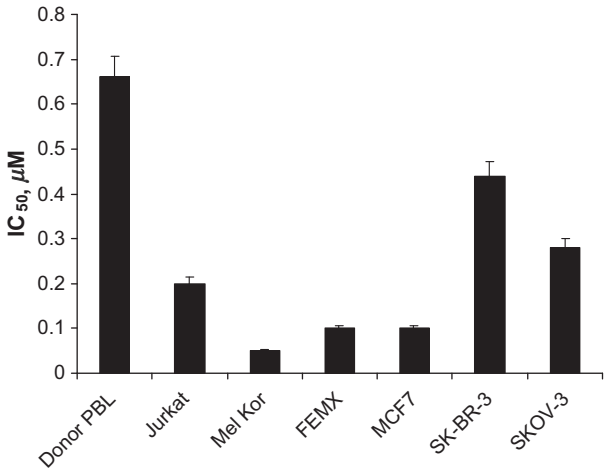


Figure 4. Cytotoxicity of compound **1f**. IC_{50} after a 72 h continuous exposure (MTT-test, mean ± S.D. of three replicates).

3. Conclusion

A novel environmentally safe and simple method of oxidation of tris(indol-3-yl)methanes yielded the corresponding tris(indol-3-yl)methylium salts. The N-substituted alkyl derivatives of tris(indol-3-yl)methylium salts with 2–6 carbon atoms in the side chain demonstrated a higher cytotoxicity for cultured human tumor cell lines than turbomycin A. The most potent compound tris(1-pentyl-*1H*-indol-3-yl)methylium methanesulfonate caused apoptosis of cultured tumor cells at concentrations that were less toxic for normal lymphocytes. These results expand the biological properties of turbomycins beyond antimicrobial activities, and demonstrate for the first time that tris(1-alkylindol-3-yl)methylium salts are promising chemical class for the search of anticancer drug candidates.

4. Experimental

4.1. Chemistry

4.1.1. Tris(1-ethyl-1*H*-indol-3-yl)methane (2c) (method A)

To a mixture of 1-ethylindole **4c** (2.9 g, 20 mmol) and 1-ethyl-3-formylindole **3c** (1.73 g, 10 mmol) in ethanol (50 mL) 50 mg $La(OTf)_3$ was added. After 3 h of refluxing the mixture was cooled to room temperature, the precipitate was filtered off, washed with 50 mL of ethanol and dried to give **2c** (4.25 g, 95% yield) as colorless crystals.

Table 3
Cytotoxicity (IC_{50} values, μM) of compounds **1a–i** for human tumor cell lines[a]

Cell line	Compound								
	1a H	**1b** Me	**1c** Et	**1d** Pr	**1e** Butyl	**1f** Pentyl	**1g** Hex	**1h** Bn	**1i** Decyl
Colon carcinoma HCT116	2.3 ± 0.2	3.2 ± 0.3	1.3 ± 0.3	1.0 ± 0.3	0.3 ± 0.1	0.15 ± 0.04	0.3 ± 0.1	15.0 ± 2.2	16.4 ± 2.3
Leukemia K562	1.6 ± 0.4	3.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.09 ± 0.04	0.05 ± 0.03	0.074 ± 0.03	9.3 ± 2.1	12.4 ± 2.1

[a] IC_{50} (μM), MTT-test after 72 h incubation. Data are mean ± SD of 3–5 measurements.

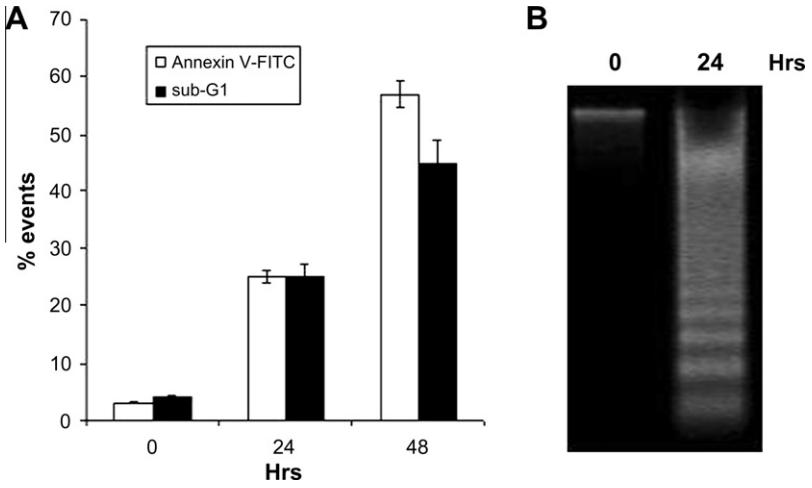


Figure 5. Apoptotic death of HCT116 cells treated with compound **1f**. (A) Cells were treated with 1 μM of **1f** for 24–48 h followed by flow cytometry-assisted analysis of Annexin V-FITC reactivity and DNA integrity. (B) Internucleosomal DNA fragmentation detected by electrophoresis in 1% agarose gel. The experiments were repeated three times with essentially the same results.

Tris(1*H*-indol-3-yl)methane (**2a**); tris(1-methyl-1*H*-indol-3-yl) methane (**2b**); tris(1-propyl-1*H*-indol-3-yl)methane (**2d**); tris (1-butyl-1*H*-indol-3-yl)methane (**2e**); tris(1-pentyl-1*H*-indole-3-yl) methane (**2f**); tris(1-hexyl-1*H*-indol-3-yl)methane (**2g**); tris (1-benzyl-1*H*-indol-3-yl)methane (**2h**); tris(1-decyl-1*H*-indol-3-yl) methane (**2i**) were also obtained by the same method using corresponding indoles **4a–i** and 3-formylindoles **3a–i**.

4.1.2. Tris(1-ethyl-1*H*-indol-3-yl)methane (2c) (method B)

A solution of 1-ethylindole **4c** (2.9 g, 20 mmol) and 1-ethyl-3-formylindole **3c** (1.73 g, 10 mmol) in 50 mL of AcOH/Ac_2O (10:1) mixture was refluxed for 2 h. Then the mixture was diluted with 10% aqueous $NaHCO_3$, the precipitate was filtered off, washed with 50 ml of ethanol and dried to give **2c** (4.25 g, 95% yield) as colorless crystals. Using substituted indoles **4b–i** and corresponding 3-formylindoles **3b–i**, compounds **2b–i** were obtained.

4.1.3. Tris(1-ethyl-1*H*-indol-3-yl)methylium methanesulfonate (1c)

To a suspension of **2c** (4.45 g, 10 mmol) in 100 mL of butanol, 0.5 g of activated charcoal Norit A and methanesulfonic acid (1.43 mL, 20 mmol) were added. After 24 h of stirring with an excess of air, the mixture was filtered, butanol solution was washed twice with water and evaporated in vacuo. Dry residue was pounded with ether, filtered and washed with ether to give **1c** (4.31 g, 80% yield) as amorphous dark red powder.

Tris(1*H*-indole-3-yl)methylium methanesulfonate (**1a**); tris(1-methyl-1*H*-indol-3-yl) methylium methanesulfonate (**1b**); tris (1-propyl-1*H*-indol-3-yl)methylium methanesulfonate (**1d**); tris (1-butyl-*1H*-indol-3-yl)methylium methanesulfonate (**1e**); tris(1-pentyl-*1H*-indol-3-yl)methylium methanesulfonate (**1f**); tris (1-hexyl-*1H*-indol-3-yl)methylium methanesulfonate (**1g**); tris(1-benzyl-*1H*-indol-3-yl)methylium methanesulfonate (**1h**); tris (1-decyl-*1H*-indol-3-yl) methylium methanesulfonate (**1i**) were obtained by similar method from corresponding **3a–i**.

4.1.4. 3,3′-((1-Ethyl-1*H*-indol-3-yl)methylene)bis(1-propyl-1*H*-indole) (7) and 3,3′-((1-propyl-1*H*-indol-3-yl)methylene)bis (1-ethyl-1*H*-indole) (8)

A mixture of **4d** (0.19 g, 1.2 mmol), **3c** (0.1 g, 0.6 mmol) and NH_4Cl (0.033 g, 0.6 mmol) was heated at 110 °C for 5 h. Then 5 mL water was added to dissolve NH_4Cl, the mixture was filtered and 30 mL of ethanol was added to the residue what represents the mixture of **7**, **8**, **2c** and **2d** (HPLC data, Table 2). The individual **7** and **8** were purified by preparative TLC and mixture 10:1 of dioxane/petroleum ether as eluent.

4.2. Biological testing

Unless specified otherwise, the cell lines were from American Type Culture Collection, and chemicals were purchased from Sigma-Aldrich. The HCT116 human colon carcinoma (wild type p53), its isogenic subline HCT116p53KO with deletion of both alleles of the *p53* gene (generated in B. Vogelstein lab, Johns Hopkins University, Baltimore, MD; gift of B. Kopnin), leukemia K562 and Jurkat, melanoma FEMX (gift of M. Krasil'nikov) and Mel Kor, breast carcinoma MCF-7 and SK-BR-3, as well as ovarian carcinoma SKOV-3 cell lines were cultured in Dulbecco modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C, 5% CO_2. Cells in logarithmic phase of growth were used in all experiments. Human lymphocytes were freshly isolated from peripheral blood of four healthy donors and pooled before the experiments. Novel compounds were dissolved in DMSO as 10 mM stock solutions followed by serial dilutions in water immediately before experiments.

The cytotoxic potency of novel compounds was determined in a formazan conversion assay (MTT-test). Cells (5×10^3 in 190 μL of culture medium) were plated into a 96-well plate (Costar) and treated with 0.1% DMSO (vehicle control) or with increasing concentrations of tested compounds (each dose in triplicate) for 72 h. After the completion of drug exposure, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were added into each well for an additional 2 h. Formazan was dissolved in DMSO, and the absorbance at $\lambda = 540$ nm was measured. Cell viability at a given drug concentration was calculated as the percentage of absorbance in wells with drug-treated cells to that of vehicle control cells (100%). The IC_{50} (50% inhibitory concentration) was defined as the concentration of the compound that inhibited cell viability by 50%.

To analyze apoptosis, the HCT116 cells were plated on 6-well plates (5×10^4 cells in 3 ml of culture medium) overnight. Then compound **1f** was added up to the final concentration 1 μM, and cells were incubated for another 24–48 h at 37 °C, 5%CO_2. The control wells contained no drug. After the completion of drug exposure cells were washed with cold saline, harvested and divided into

three portions. One aliquot of cells was stained with Annexin V-fluorescein isothiocyanate (FITC) as recommended by the manufacturer (Molecular Probes). The second portion was lysed in the solution containing 0.1% sodium citrate, 0.3% NP-40, 50 μg/ml RNAse A and 10 μg/ml propidium iodide and analyzed by flow cytometry on a FACSCanto II (Becton Dickinson) on FL2. The percentage of hypodiploid nuclei (a sub-G1 peak on histograms of cell cycle distribution) was calculated using FACSDiva software. The third portion of cells was lysed in the buffer (350 mM NaCl, 20 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 1 mM dithiotreitol, 0.5% NP-40) for 30 min on ice. DNA was isolated with phenol–chloroform (1:1 v/v) mixture, precipitated with 0.3 M sodium acetate and ethanol, and resolved by electrophoresis in a 1% agarose gel.

Statistical analysis was performed using Student's *t*-test.

Acknowledgment

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Supplementary data

Chemistry; General; Table 1-SM. Reaction products formed by the interaction of N-substituted 3-formylindole (3) with N′-substituted indole (4) or by the interaction of tris(1-alkylindol-3-yl) methane (2) with N′-substituted indole (4); Table 2-SM. Malliken charges and Fukui indexes of HOMO for 1-alkylindoles; Figure1-SM. ^{13}C NMR spectrum of **1c** measured at room temperature and at 110 °C. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.025.

References and notes

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