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New indolocarbazoles as antitumour active compounds: evaluation of the target by experimental and theoretical studies

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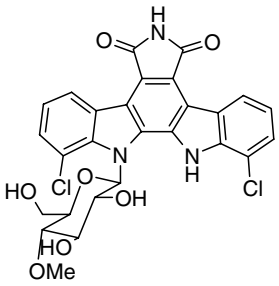
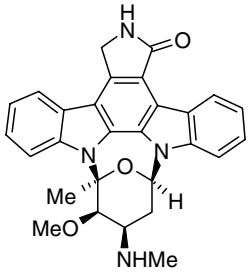
A series of indolocarbazoles was synthesized as congeners of the natural lead compounds rebeccamycin (**1**) and staurosporine (**2**) which reduce cell growth by inhibiting topoisomerase I and protein kinase C respectively. Two of the carbazoles (**3** and **4**) screened at the National Cancer Institute (NCI, USA) showed an interesting cytotoxic activity and were therefore further analysed. The mechanism of action of these two compounds was studied experimentally using different assays to determine the B-DNA binding ability and the inhibition of topoisomerase I and of protein kinase C. Theoretical molecular modelling studies were also performed to describe the possible interactions with protein kinase C and DNA.

1. Introduction

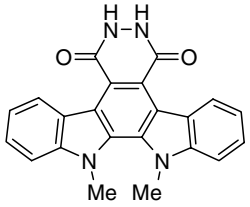
The indolocarbazole alkaloids rebeccamycin (**1**) and staurosporine (**2**) are of interest as lead structures for the development of new cytostatically active compounds. In spite of their closely related structures, they inhibit cell growth by different targeting. Rebeccamycin (**1**) exhibits antitumour properties probably via topoisomerase I inhibition [1], while staurosporine (**2**) is a non – selective protein kinase C inhibitor [2]. Continuing our research about DNA ligands as potential anticancer drugs, we have synthesized a great variety of indolocarbazoles by selective functionalization of 2,2′-bisindolyl derivatives [3–5]. A series of such compounds was tested for cytotoxicity at the NCI (NCI Primary Screening) and for PKC-inhibition activity. Two of these compounds (**3** and **4**) showed sufficient tumour cell growth inhibition and are now undergoing further biological screening at the NCI. To evaluate their biological targets we tried to rationalize the possible interaction of these compounds with the targets B-DNA and protein kinase C on the basis of experimental studies and computer molecular modelling. For the experimental studies different biophysical methods that focused on B-DNA as a possible target were used. Thus, the thermal denaturation experiments [6, 7], the ethidium bromide displacement assay [8–10], the DNA unwinding assay and the topoisomerase I-inhibition assay [11, 12], were performed to determine the DNA-affinity, the mode of binding to DNA and the ability to introduce the strand cleavage reaction by inhibition of the topoisomerase I.



Rebeccamycin (1)



Staurosporine (2)



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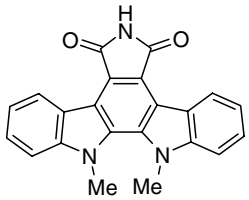

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2. Investigations, results and discussion

2.1. Cell biological antitumour screening

The *in vitro* sulforhodamine B (SRB) protein assay, an NCI routine primary screening method for the estimation of cell viability or growth, was used to evaluate the relative cytotoxicity of compounds **3** and **4** towards a panel of 60 different human tumour cell lines [3, 13]. Compared with the highly active substances staurosporine (average GI_{50} 8.2×10^{-9} M) or rebeccamycin (average GI_{50} 2.3×10^{-7} M) the compounds **3** and **4** showed only moderate average activity (average GI_{50} 1×10^{-4} M). Nevertheless both carbazoles showed significant cell growth inhibition towards many tested cell lines at almost the same concentration range as rebeccamycin or staurosporine (Table 1).

Table 1: GI_{50} growth inhibitory concentration [mol] of rebeccamycin, staurosporine and the indolocarbazoles 3 and 4

Cell line	Rebecc-amycin	stauro-sporine	**3**	**4**
Average GI_{50}	2.3×10^{-7}	8.2×10^{-9}	1×10^{-4}	1×10^{-4}
SR (leukaemia)	$10^{-7.5}$	n.d.	10^{-8}	10^{-8}
MCF 7 (breast cancer)	$10^{-7.1}$	n.d.	$10^{-6.79}$	$10^{-4.56}$
T-47 D (breast cancer)	$10^{-6.8}$	n.d.	$10^{-6.33}$	$10^{-4.44}$
OVCAR-3 (ovarian cancer)	$10^{-6.5}$	$10^{-8.1}$	n.d.	$10^{-7.26}$
RXF 393 (renal cancer)	$10^{-6.7}$	$10^{-8.6}$	n.d.	$10^{-7.38}$

SRB cell viability assay; primary screening method performed at the National Cancer Institute, Bethesda (USA) [13]; n.d. = not determined

2.2. Protein kinase C inhibition

Further studies were performed at the NCI (Bethesda, USA) to determine the inhibition of the protein kinase C_α by the two compounds using the protein kinase enzyme assay [3]. Considering the inhibition of the protein kinase C_α by staurosporine as 100%, compounds **3** and **4** showed 31% and 50% inhibition, respectively.

2.3. DNA binding studies

2.3.1. Thermal denaturation studies

An established easy method to monitor the interaction of a compound with the DNA is the determination of the melting curve of dsDNA in the presence of the compound. Substances which bind to dsDNA stabilize the double strand and raise the melting temperature (T_m) of the DNA [6, 7]. Both compounds **3** and **4** in 1 : 1 drug : DNA ratio showed no changes in the melting temperature of [Poly(dAdT) · Poly(dAdT)], which remained at 46 °C. Thus, on the basis of this method, no DNA-binding was observed.

2.3.2. Ethidium bromide displacement assay

The DNA binding affinity of the compounds was studied using the ethidium bromide displacement assay [8–10, 14]. The intercalation of ethidium bromide between the base pairs of the DNA results in the formation of a fluorescent complex, the fluorescence of which is reduced by adding a drug, since this displaces ethidium from the DNA itself. The concentration of drug leading to 50% reduction in the fluorescence intensity of DNA saturated with ethidium bromide is defined as the C_{50} value and is inversely proportional to the apparent binding constant of the drug [8]. This approach offers reliable comparisons of DNA-binding ability of different compounds, since a lowering in the concentration leading to 50% fluorescence indicates an increased DNA-affinity (greater K_a value). Both assayed substances **3** and **4** gave a very small decrease in the fluorescence, which did not differ from that due to the solvent, indicating no affinity for the DNA (Fig. 1). Ellipticine as a typical intercalator was used as a reference compound.

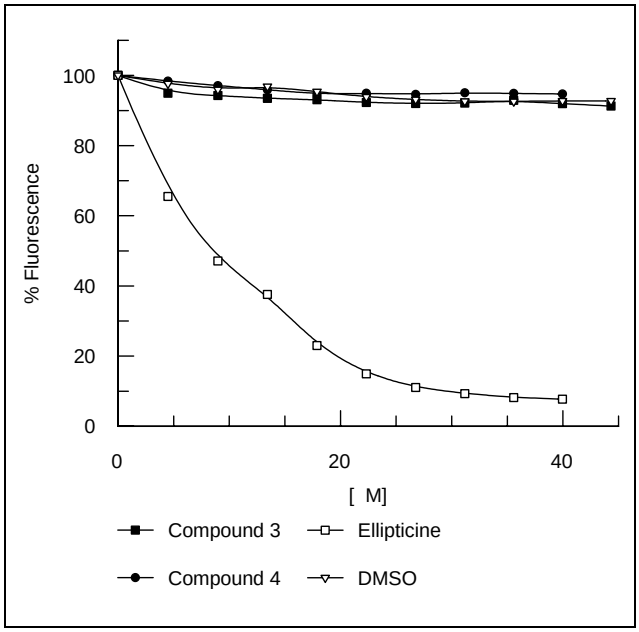

Fig. 1: Ethidium bromide displacement assay revealing decreasing fluorescence by successive additions of the test compounds **3** and **4**; ellipticine and pure DMSO are reported as reference compounds

2.3.3. Unwinding assay

To determine the ability of compounds **3** and **4** to intercalate into DNA we used the topoisomerase I-unwinding assay, because unwinding of the double strand of the DNA helix is a typical feature of intercalating drugs such as ellipticine [11, 14, 15]. After incubation with topoisomerase I the DNA is fully relaxed. Upon intercalation of the test compound the twisting number (the arrangement in space of one strand twisting about the other) is reduced, but in the presence of topoisomerase the equilibrium distribution of topoisomers is adjusted by the enzyme itself. Upon terminating the reaction and extracting the DNA so that protein and drug are removed, the DNA shrinks. This can be revealed by agarose gel electrophoresis. Comparison of the DNA unwinding with well known intercalators gives some idea of the potency of DNA binding. It is important to notice that if the test drug is inhibiting topoisomerase I activity, the distribution of topoisomers during the incubation with the test drug cannot be adjusted by the enzyme, so no unwinding will be detectable. However, in this assay both substances showed no intercalation into the DNA (Fig. 2a) and therefore were then tested for topoisomerase I inhibition.

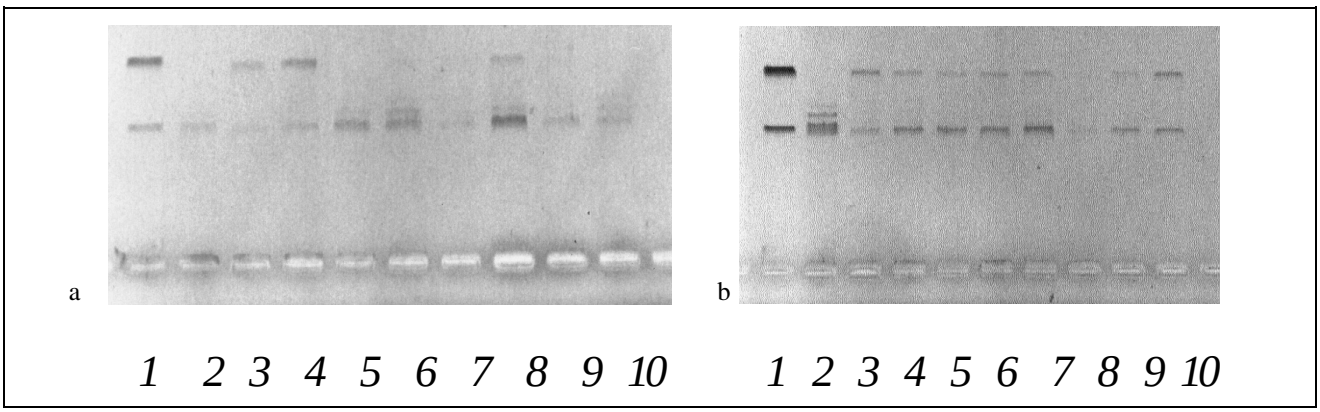

Fig. 2: Agarose gel electrophoretograms of the unwinding assay (a) and of the topoisomerase I inhibition assay (b). a): line 1: scDNA (Form I); line 2: relaxed DNA (after incubation with topo I, Form IV); lines 3 and 4: ellipticine 100 and 200 μM respectively; lines 5, 6, 7: compound (**4**) 50, 100 and 200 μM respectively; lines 8, 9, 10: compound (**3**) 50, 100 and 200 μM respectively. b): line 1: sc DNA (Form I); line 2: relaxed DNA (after incubation with topo I, Form IV); lines 3 and 4: camptothecin 100 and 200 μM respectively; lines 5, 6, 7: compound (**4**) 50, 100 and 200 μM respectively; lines 8, 9, 10: compound (**3**) 50, 100 and 200 μM respectively

2.4. Topoisomerase I inhibition assay

Incubation of the DNA topoisomerase I mixture in the absence of and in the presence of the test drug produces different population distributions of topoisomers which can be revealed by electrophoresis [11, 14]. Comparison of the obtained plasmid DNA forms (supercoiled or relaxed) will indicate the ability of the tested drug to inhibit the enzyme. Both compounds **3** and **4** inhibit the topoisomerase I, so that the determination of DNA intercalation with the unwinding test (see paragraph 2.3) was impossible (Fig. 2b).

2.5. Molecular modelling of interaction with protein kinase C or DNA

The possibility of the two compounds interacting with protein kinase C was additionally studied *via* molecular modelling (Fig. 3). GRIN-GRID calculations of the hydrogen bond interaction fields of ATP, staurosporine and compound **4** with the protein kinase A as a reliable model for protein kinase C [16–21] showed overlapping regions where ATP and staurosporine form hydrogen bonds to the aminoacids Glu122 and Val124 of protein kinase A [3, 4, 18, 19]. At the time the calculations were made, no X ray-geometry of the protein kinase C was known. Meanwhile, an X-ray structure of protein kinase C complexed with staurosporine at the ATP binding site is known [22] which complements our theoretical model. Molecular modeling (SYBYL; TRIPOS force field [19–22] studies suggested also the possibility that the two compounds can intercalate geometrically into the DNA, since no distortion of the DNA conformation is induced by substitution of an intercalator such as ellipticine or proflavine with compound **3** or **4** in the intercalation model (Fig. 4) [3, 4, 18].

In conclusion, compounds **3** and **4** seem to be extremely weak DNA-binding ligands but they are able to inhibit topoisomerase I and protein kinase C_α. The PKC inhibition could also be rationalized and anticipated with preliminary molecular modeling techniques. The possibility of DNA intercalation of **3** and **4** due to the planar chromophore was also predicted by molecular modelling studies on the basis of molecular mechanics calculations. However, a confirmation of these theoretical findings was neither possible by thermal denaturation experiments nor by the unwinding assay. In some other cases we have succeeded in predicting intercalation complexes by similar molecular modeling methods, which correlated well with the experimental findings [20–23]. Thus, in the present case the frontiers of the applications of molecular modeling techniques are outlined for this type of carbazoles.

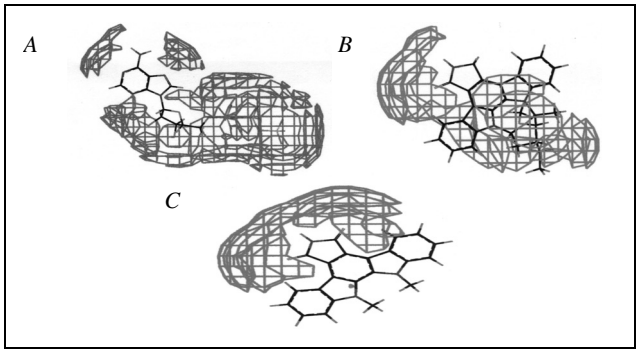

Fig. 3: GRID hydrogen bond interaction fields of ATP (A), staurosporine (B) and compound **4** (C) to PKA [18]. According to the interaction model of reference [16] ATP forms hydrogen bonds with the aminoacids Glu122, Val124 and Thr183 of the enzyme. Staurosporine and compound **4** partially reveal interactions with the same aminoacids.

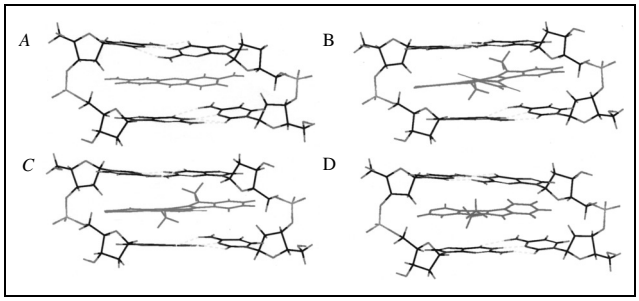

Fig. 4: Molecular models of the energy minimized intercalation complexes of proflavine (A), compound **3** (B), compound **4** (C) and ellipticine (D) in the dinucleotide $d(CG)_2$ [Molecular mechanics calculations; SYBYL]. The interaction energies of the complexes are in the range of 30–35 kcal/mol [19–23].

3. Experimental

3.1. Thermal denaturation experiments with DNA

Melting curves were measured using an Hitachi U-3200-spectrophotometer coupled to a Julabo hermostat. The measurements were performed in BPE buffer, pH 7 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) with a drug : DNA ratio of 1 : 1, using [Poly(dAdT) · Poly(dAdT)] as DNA. The temperature inside the cuvette was increased over the range 25–90 °C with a heating rate of 1.5 °C/min. The absorption data were registered and plotted against the temperature. The melting curve was visualized using the program ORIGIN for data analysis and technical graphics. The melting temperature (T_m) was taken as the midpoint of the hyperchromic transition [7].

3.2. Ethidium bromide displacement assay

All fluorescence measurements were conducted on an Hitachi F-2000 spectrofluorometer. Calf thymus DNA (Sigma, Type I) (1.0×10^{-5} M in base pairs) was added in small aliquots to ethidium bromide (GIBCO BRL, CAUTION) (5.0×10^{-6} M) resulting in a 2 : 1 ratio of base pair/ethidium in 2 ml of a 10 mM Tris-HCl (pH 7.4), 75 mM NaCl buffer solution. The fluorescence of the DNA-ethidium buffer solution was calibrated at room temperature to 100% fluorescence and that of the ethidium buffer solution to 0% fluorescence, respectively. The premixed DNA-ethidium solution was titrated with 3 µl aliquots of the stock solution of the test substances or of ellipticine (Fluka) (3 mM drug in DMSO) and stirred at room temperature for 30 min prior to each fluorescence measurement. The fluorescence was measured with 545 nm excitation and 595 nm emission with a slit width of 10 nm. Results were calculated with the Data Analysis and Graphics Program GraFit.

3.3. Unwinding assay

Plasmid DNA (pUC 19, GIBCO; 0.033 µg/µl) was incubated for 30 min at 37 °C with topoisomerase I (GIBCO; 0.16 U/µl) in 1x topoisomerase I reaction buffer (50 mM Tris HCl pH 7.5, 50 mM KCl, 10 mM MgCl, 0.5 mM DTT, 0.1 mM EDTA, 30 µg/ml bovine serum albumine). The mixture containing relaxed plasmid DNA was added in 10 µl aliquots to the solutions of different concentrations of the drugs (10 and 100 µM). After 1 h at 37 °C, the mixtures were treated with TE-Buffer (pH 7.9) and extracted twice with phenol/CIA solution (chloroform : isoamyl alcohol 24 : 1) 1 : 1 and once with CIA-solution. Sodium acetate and absolute ethanol were then added to the upper solution which was incubated for 1 h at −20 °C and spun briefly in a microfuge. The obtained DNA pellet was then dried, dissolved in 20 µl loading dye and loaded onto a 1% agarose gel (Tris-Glycine-Buffer) which was run for 4 h at 55 V.

3.4. Topoisomerase I inhibition assay

Plasmid DNA (pUC 19, GIBCO; 0.033 µg/µl) was incubated for 30 min at 37 °C with topoisomerase I (GIBCO; 0.16 U/µl) in 1x topoisomerase I reaction buffer (50 mM Tris HCl pH 7.5, 50 mM KCl, 10 mM MgCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 30 µg/ml BSA) in the presence of different concentrations of the tested drugs (10 µM and 100 µM). After extracting DNA by the same procedure as above, a gel was run under the same conditions.

3.5. Protein kinase C_α inhibition assay

After incubation of the protein kinase C_α with the test compound and the cosubstrate ATP, the remaining activity of the protein kinase was measured at NCI as the amount of radioactive phosphor which was transferred from [γ-^{32}P]ATP onto H1-Histone [23, 24]. The inhibition achieved by 1 μM staurosporine was defined as 100% [3].

3.6. Molecular modelling

The molecular modelling studies were performed by quantum chemistry and molecular mechanics methods. The program packets MOPAC 6.0 (AM1 hamiltonian for geometry and charge calculations), SYBYL 5.5 (build up of intercalation complexes, molecular mechanics and graphical presentation) and GRIN-GRID (interaction analysis) were used. Details of the procedures are described elsewhere [15, 19–21].

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References

1 Bush, J. A.; Long, B. H.; Catino, J. J.; Bradner, W. T.; Tomita, K.: J. Antibiot. Tokyo **40**, 668 (1987)
2 Tamaoki, T.; Nomoto, H.; Takahashi, I.; Kato, Y.; Morimoto, M.; Tomita, F.: Biochem. Biophys. Res. Commun. **135**, 397 (1986)
3 Kim, Y. S.: Thesis, University of Mainz 1997
4 Pindur, U.; Kim, Y. S.; Mehrabani, F.: Curr. Med. Chem. **6**, 29 (1999)
5 Pindur, U.; Lemster, T.: Recent Res. Devel. in Org. Bioorg. Chem. **1**, 53 (1997)
6 Wilson, W. D.; Tanious, F. A.; Fernandez-Saiz, M.; Rigl, C. T. in: Fox, K. R. (Ed.) Drug-DNA Interactions Protocols, Humana Press, Totowa 1997
7 Bailly, C.; Gu, X.; Anizon, F.; Prudhomme, M.; Riou, J. F.; Chaires, J. B.: Molec. Pharm. **55**, 377 (1999)
8 Morgan, A. R.; Lee, J. S.; Pulleyblank, D. E.; Murray, N. L.: Nucleic Acids Res. **7**, 547 (1979)
9 Boger, D. L.; Chen, J-H.; Saionz, K. W.: J. Am. Chem. Soc. **118**, 7 (1979)
10 Wellmann, S. E.: Biopolymers **39**, 491 (1996)
11 Sattler, K.: Thesis, University of Mainz 1994
12 Bonnard, I.; Bontemos, N.; Lahmy, S.; Banaigs, B.; Combaut, G.; Francisco, C.; Colson, P.; Houssier, C.; Waring, M. J.; Bailly, C.: Anti-Cancer Drug Des. **10**, 333 (1995)
13 Keepers, Y. P.; Pizao, P. E.; Peters, G. J.; van Ark Otte, J.; Winograd, B.; Pinedo, H. M.: Eur. J. Cancer. **27**, 897 (1991)
14 Pindur, U.; Marotto, A.; Schulze, E.; Fischer, G.: Pharmazie **55**, 727 (2000)
15 Kim, J. S.; Sun, Q.; Gatto, B.; Yu, C.; Liu, A.; Liu, L. F.; La Voie, E. J.: Bioorg. Med. Chem. **4**, 622 (1996)
16 Furet, P.; Caravatti, G.; Lydon, N. B.; Priestle, J. P.; Sowadski, J. M.; Trinks, U.; Traxler, P.: J. Comp.-Aid. Molec. Design **6**, 465 (1995)
17 Toledo, L. M.; Lydon, N. B.; Elbaum, D.: Curr. Med. Chem. **6**, 775 (1999)
18 Fischer, G.; Pindur, U.: Pharmazie **54**, 83 (1999)
19 Rehn, C.; Pindur, U.: Monatsh. Chem. **127**, 631 (1996)
20 Rehn, C.; Pindur, U.: Monatst. Chem. **127**, 645 (1996)
21 Rehn, C.: Thesis, University of Mainz 1995
22 Toledo, L. M.; Lydon, N. B.: Structure **5**, 1551
23 Teng, K.; Marquez, V. E.; Milne, G. W. A.; Barchi, J. J.; Kazanietz, M. G.; Lewin, N. E.; Blumberg, P. M.; Abushan, E.: J. Am. Chem. Soc. **114**, 1059 (1992)
24 Kozikowski, A. P.; Ma, D.; Du, N.; Lewina, N. E.; Blumberg, P. M.: J. Am. Chem. Soc. **117**, 6666 (1995)

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