Symmetrically substituted carbazole derivatives exert antiproliferative effects through catalytic inhibition of topoisomerase II and apoptosis induction

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Abstract

Human DNA topoisomerases are vital enzymes for DNA replication, transcription, chromatin condensation, and maintenance of their structure. Due to this fact inhibition of topoisomerase II is a common approach used in cancer treatment. Carbazole scaffold has a wide range of biological activities and appears as a core in many active compounds. It also plays important role in anticancer research. The present study shows the in vitro biological evaluation of three symmetric carbazole derivatives, substituted with furan or thiophene, as potential antitumor agents. Compounds efficiently inhibited the proliferation of all tested cancer cell lines mostly at nanomolar concentrations. They were further characterized for their effect on cell cycle progression, mitochondria disruption, DNA damage induction, and type of cellular death. Moreover, analysis of their mode of action indicates, that investigated carbazole derivatives inhibit topoisomerase II. Among them, compound 36a exhibited the strongest catalytic inhibitory activity against topoisomerase IIα and could be a potential lead compound for developing novel promising anticancer compounds.

Introduction

Cancer is a multifaceted disease that progresses through various stages, with its incidence linked to global population growth, aging, and advances in economic development. As per the World Health Organization, cancer is the second most frequent cause of mortality in developing countries, next to cardiovascular diseases. The contemporary management of cancer relies on various therapeutic modalities such as surgery, radiotherapy, immunotherapy, and the widely employed chemotherapy. In light of the escalating incidence of cancer and the rise of resistance to anticancer drug regimens, the exploration and innovation of novel approaches to cancer therapy will be crucial in the ensuing decades. However, chemotherapy still is the most typical approach to combat cancer.

The carbazole scaffold, characterized by a tricyclic structure with two benzene rings fused to a five-membered ring with a nitrogen substitute, represents a vital structural element in various biologically active compounds, including natural and synthetic anticancer agents. The anticancer mechanism of action of some carbazole derivatives comprises cell cycle arrest, intercalation into DNA, inhibition of human topoisomerases, mitochondria disruption, and apoptosis induction. Alectinib and midostaurin are examples of carbazole-based anticancer agents approved by the US Food and Drug Administration (FDA) for use in chemotherapy, demonstrating the potential of carbazole scaffolds as anticancer candidates. Based on the inhibition of topoisomerase II and intercalation into DNA, elliptinium (celiptium) is an efficient antineoplastic drug to treat metastatic breast cancer.

The human genome encodes for six topoisomerases (Topo 1, Topo1mt, Topo IIα, Topo IIβ, Topo IIIα, and Topo IIIβ), which catalyze topological changes of nuclear and mitochondrial DNA. Generally, their mechanism of action involves removing unwanted tensions during the unwinding of the DNA helix in the process of DNA replication or transcription. The tension release is caused by the formation of transitional
single-stranded breaks by type I topoisomerases or double-stranded breaks in the DNA double helix by type II topoisomerases \textsuperscript{16}. Noteworthy, both modes of action rely on forming a transient enzyme-DNA adduct called the Topo cleavage complex (Topo cc). Due to the unlimited proliferation of cancer cells, topoisomerases have become an important target for many anticancer drugs \textsuperscript{17}.

Topo II\textsubscript{α} and Topo II\textsubscript{β} are the two isoforms of Topo II, that require the presence of Mg\textsuperscript{2+} ions and ATP hydrolysis to function. The mechanism of action of drugs that target Topo II can be classified into two broad groups based on their action type. Compounds termed Topo II poisons stabilize the covalent complex Topo II/DNA and cause increased levels of DNA damage in cells leading to apoptosis \textsuperscript{18}. Topo II poisons can be classified into two types based on their ability to bind to DNA: those that do not intercalate with DNA (such as etoposide and teniposide) and those that do intercalate (such as doxorubicin, m-AMSA, and mitoxantrone) \textsuperscript{19}. The second category of Topo II targeting agents describes catalytic inhibitors that inhibit the crucial enzymatic activity of Topo II. These inhibitors induce cytotoxic effects without generating increased levels of DNA damage mediated through the stabilizing of the Topo II/DNA complex. Catalytic inhibitors can target Topo II by different mechanisms, including prevention of ATP hydrolysis (e.g., ICRF-187, ICRF-193), competition for the ATP binding site (e.g., novobiocin), or prevention of the cleavage of DNA (e.g., merbarone) \textsuperscript{20,21}. Overexpression of Topo II\textsubscript{α} in fast-proliferating cancer cells is considered to be responsible for the antitumor effect of Topo II inhibitors. Taken together, the disruption functions of Topo II through inducing DNA damage or blocking catalytic activity is a systematic approach in oncology therapy \textsuperscript{22}.

Regarding the literature on carbazole’s biological activity, we present the evaluation at the molecular pharmacology level of three symmetrically substituted carbazole derivatives for their anticancer properties against various cancer cell lines. The compounds: 2,7-Di(furan-2-yl)-9H-carbazole (27a), 3,6-Di(furan-2-yl)-9H-carbazole (36a), and 3,6-Di(thiophen-2-yl)-9H-carbazole (36b) (Fig. 1) have been obtained, purified, physiochemically characterized, and tested for cytotoxic, antiproliferative, DNA damage induction, and proapoptotic properties. Overall, topoisomerase assays confirmed the inhibitory activity of carbazole derivatives toward human Topo II.

**Synthesis Of Compounds**

The two carbazole derivatives, 27a and 36a, were synthesized according to the protocol described by Öğuztürk \textit{et al.} \textsuperscript{23}, and the third one 36b – described by Damit \textit{et al.} \textsuperscript{24} (Fig. 1). Compounds 36a and 36b contain the 3,6- locants in the carbazole skeleton that differ by heteroatoms in the substituent’s rings. In 36a, the oxygen atoms in the heterocyclic substituents (furanyle) were replaced by sulfur atoms (thiophenyle) to create the 36b carbazole derivative. Interestingly, the 27a and 36a compounds obtained are constitutional isomers with substituents located in 2,7 and 3,6 positions of the carbazole skeleton, respectively. Due to the above, the complete comparison analyses of the 27a, 36a, and 36b of biological activities corresponded directly to the mentioned structural similarities or differences were made herein.
The selected compounds studied are the three models that acts in different modes, which correspond directly to their structures.

**Biological Studies**

*Carbazoles display a potent cytotoxic effect and inhibit the capability to form colonies*

**Table 1** In vitro growth inhibitory activity (IC$_{50}$ ± SD, µM) of 27a, 36a, and 36b.$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>A549</th>
<th>HCT-116</th>
<th>MCF-7</th>
<th>U-2 OS</th>
<th>U-87 MG</th>
<th>HEK293</th>
</tr>
</thead>
<tbody>
<tr>
<td>27a</td>
<td>0.26±0.12</td>
<td>0.22±0.04</td>
<td>0.79±0.21</td>
<td>0.37±0.05</td>
<td>0.45±0.15</td>
<td>0.19±0.07</td>
</tr>
<tr>
<td>36a</td>
<td>0.93±0.15</td>
<td>0.48±0.06</td>
<td>1.39±0.29</td>
<td>0.99±0.18</td>
<td>2.19±0.30</td>
<td>1.65±0.13</td>
</tr>
<tr>
<td>36b</td>
<td>0.60±0.10</td>
<td>0.27±0.11</td>
<td>0.83±0.21</td>
<td>0.71±0.06</td>
<td>1.40±0.24</td>
<td>0.32±0.11</td>
</tr>
<tr>
<td>Etoposide</td>
<td>0.54±0.21</td>
<td>0.39±0.01</td>
<td>0.83±0.15</td>
<td>0.61±0.04</td>
<td>11.86±1.31</td>
<td>1.91±0.97</td>
</tr>
</tbody>
</table>

$^a$ Values represent a concentration that inhibits 50% of cell growth and are means of three independent experiments. Etoposide was used as a reference.

The antiproliferative activity of studied carbazole derivatives was examined. The compounds were tested at different concentrations for 72 hours using five different human cancer cell lines, namely A549 (non-small lung cancer), HCT-116 (colon cancer), MCF-7 (breast cancer), U-2 OS (bone cancer), U-87 MG (brain cancer), and a nonmalignant human embryonic kidney cell line, HEK293. The MTT assay was used to determine the IC$_{50}$ concentration for each compound compared to cells treated with 1% v/v DMSO. The carbazole derivatives showed high antiproliferative efficacy against all tested cancer cell lines (Table 1). Compound 27a had the most increased activity, with an IC$_{50}$ below 1 µM for all investigated cell lines, including HEK293. Compound 36b had similar growth inhibition activity to most of the studied cell lines as compound 27a, except for the U-87 MG line with an IC$_{50}$ of 1.40±0.24 µM. Compound 36a showed the highest and lowest cytotoxicity against the HCT-116 and U87-MG cell lines, with IC$_{50}$ values of 0.48±0.06 µM and 2.19±0.30 µM, respectively. The effect observed for 36a against HEK293 cells was similar to the IC$_{50}$ value of the etoposide, used as a reference.

A549 and HCT-116 cells were used in the clonogenic assay to investigate further the impact of carbazole derivatives on cancer cell growth. The results showed that treatment with carbazole treatment significantly reduced the number of cell-forming colonies compared to control cells treated with DMSO (Figure 2). Specifically, treatment with 27a and 36a reduced the colony number in both tested cell lines in a concentration-dependent manner, while 36b showed lower inhibitory activity against the A549 cell line (p>0.01).

**Carbazole derivatives inhibit DNA synthesis**
The *in vitro* antiproliferative effect of carbazole derivatives was further substantiated by evaluating the bromodeoxyuridine (BrdU) incorporation assay on selected cancer cells. The treatment of equitoxic concentrations of compounds led to a time-dependent reduction in DNA synthesis in almost all investigated cell lines (Figure S1). The U2OS cell line exhibited the least anti-proliferative effect (Figure S1), while the HCT-116 cells showed the most significant effect (Figure S1). The BrdU incorporation assay showed that all tested carbazoles inhibited BrdU incorporation in the A549, HCT-116, and MCF-7 cell lines, with 36b being the most potent. This compound displayed its most prominent antiproliferative properties on A549 and HCT-116 lines, leading to an approximately 4-fold and 3.5-fold decrease in BrdU-positive cells, respectively (Figure S1). Taken together, these signify that carbazole derivatives have the potential to inhibit the proliferation of cancer cells *in vitro*. The observed differences in antiproliferative effects among the tested cell lines could be attributed to their genetic heterogeneity and variability in response to carbazole treatment.

**Carbazole derivatives disrupt cell cycle progression**

Carbazole derivatives were evaluated for their effect on cell cycle progression by monitoring the phases of A549, HCT-116, MCF7, and U2OS cells after exposure to equitoxic concentrations of compounds for 24 and 48 h (Figure 3). Topoisomerases are essential for DNA replication and are vital in mitotic chromosome condensation and separation. Their inhibition can result in cell cycle arrest and apoptosis. The cell-cycle profiles depicted in Figure 3 indicate that all carbazole derivatives caused a significant G0/G1 arrest (p<0.0001) in A549 cells associated with a reduced G2/M phase. Among all the compounds tested, only 36a displayed a time-dependent increase in the number of cells in the G0/G1 phase in HCT-116 cells (Figure 3). In MCF-7 cells, all carbazole derivatives resulted in a significant, time-dependent increase in the G0/G1 phase with a concomitant reduction of the S phase compared to DMSO-treated cells. The most potent blockage of the cell cycle was observed after 48 h exposure to 36b, with the G0/G1 phase showing a substantial increase (59.85±2.15%; p<0.00001) (Figure 3). The time-dependent treatment of U-2 OS cells resulted in a ~1.7-fold increase in the number of cells in the G2/M phase compared to DMSO-treated cells, which was associated with compensation in the G0/G1 phase (Figure 3). The observed effect of 36a on the cell-cycle accumulation in the G1 phase correlated well with its cytotoxic and topoisomerase IIα catalytic inhibitory activities. These findings suggest that carbazole derivatives can potentially cause G0/G1 arrest in A549 and MCF-7 cells, as well as can increase the number of cells in the G2/M phase in U-2 OS cells. The differences in the response of these cells to carbazole treatment could be attributed to their unique genetic profiles and variability in their sensitivity to these compounds. As an alternative scenario, the carbazole derivatives have differential preferred biological targets in the two types of cell lines. Further investigation is necessary to elucidate the molecular mechanisms underlying the observed cell cycle effects and their relationship to carbazole derivative's cytotoxic and topoisomerase inhibitory activities.

**Carbazoles induced a lower level of DNA double-strand breaks than etoposide in A549 and HCT-116 cells**
Carbazole derivatives have been shown to induce DNA damage by causing DNA double-strand breaks (DSBs) \(^{27}\). To evaluate the potential of these compounds to cause DNA damage, the level of phosphorylated histone H2AX at Ser 139 (\(\gamma\)-H2AX) was investigated. A549 and HCT-116 cells, the most sensitive to carbazole analogs, were selected for this study and treated with equitoxic compound concentrations for 24 and 48 h. \textbf{Figures 4a} and \textbf{4b} show that both cell lines exhibited a time-dependent accumulation of \(\gamma\)-H2AX-positive cells after carbazole treatment, suggesting that the compounds induced DNA damage. The potency of DNA damage induction varied among the tested compounds and depended on the cell line. After 24 h of treatment, \textbf{27a}, \textbf{36a}, and \textbf{36b} induced less than a 20% increase in the number of \(\gamma\)-H2AX-positive cells in A549 and HCT-116 cells. This increase was 2.7- and 3.2-fold times less, respectively, compared to etoposide. However, after 48 h of treatment with these compounds, A549 cells showed a significant 4.9-fold (**p < 0.001), 5.2-fold (**p < 0.001), and 8.1-fold (****p < 0.00001) increase in DNA damage, respectively. Exposure of HCT-116 cells to these compounds led to a significant 6.5-fold (***p < 0.0001), 6.2-fold (***p < 0.0001), and 5.1-fold (**p < 0.001) increase in \(\gamma\)-H2AX compared to vehicle control-treated cells. Etoposide, a Topo II poison, rapidly increased the number of \(\gamma\)-H2AX foci compared to DMSO in both tested cell lines. These results indicate that all tested carbazole derivatives strongly induce DNA damage only after 48 h of treatment. They have varying potency in inducing DNA damage for two tested cell lines, suggesting their mode of action differs from etoposide.

**Carbazole derivatives inhibit human topoisomerase II**

To confirm that carbazole derivatives function as topoisomerase II inhibitors, a pBR322 DNA relaxation assay was performed in the presence of Topo II\(\alpha\)/II\(\beta\). The results depicted in \textbf{Figures 5a} and \textbf{5b} demonstrate that the tested compounds exhibit concentration-dependent activity toward Topo II\(\alpha\) and Topo II\(\beta\). Compound \textbf{36a} entirely inhibits DNA relaxation in the presence of Topo II\(\alpha\) at the highest concentrations (20-100 \(\mu\)M) while not affecting Topo II\(\beta\) activity. The present study demonstrates that compounds \textbf{36b} and \textbf{27a} moderately impact the functionality of Topo II\(\alpha\) and Topo II\(\beta\), leading to the formation of supercoiled DNA at the highest tested concentrations. Conversely, etoposide and ICRF-187 demonstrated complete inhibition of relaxation activity toward Topo II\(\alpha\), and comparatively lower inhibitory activity against Topo II\(\beta\), relative to the negative control. These results suggest that the anticancer mechanism of action of \textbf{36a} is based on the inhibition of Topo II\(\alpha\). To investigate the intercalating properties of the tested compounds, a DNA unwinding assay was performed using Topo I and relaxed pBR322 DNA as a substrate. Etoposide (non-intercalating agent) and doxorubicin (intercalator) served as controls. The outcomes shown in \textbf{Figure 5c} indicate that carbazole derivatives cannot convert the relaxed plasmid into a supercoiled DNA form in the presence of Topo I. These results suggest that \textbf{36a}, \textbf{36b}, and \textbf{27a} are non-intercalating topoisomerase inhibitors. Additionally, to confirm that Topo II is a target for \textbf{36a}, Nalm-6 cells with heterozygous Topo II\(\alpha\) deletion (Top2\(\alpha\)+/-) and homozygous Topo II\(\beta\) deletion (Top2\(\beta\)-/-) \(^{28,29}\) were treated with \textbf{36a} and m-AMSA followed by a cell viability assay. The results illustrated in \textbf{Figure 5d} show that \textbf{36a} has approximately 3.5 times less cytotoxicity on Nalm-6 (Top2\(\alpha\)+/-) cells than on wild-type (Nalm-6 WT) cells. According to the relaxation assay in the presence of Topo II\(\beta\), \textbf{36a} displays similar activity on Nalm-6 (Top2\(\beta\)-/-) cells as on the wild-
type cell line, further validating its selectivity for the Topo IIα isoform. Conversely, Nalm-6 (Top2α+/-) and (Top2β-/-) cell lines were 5.7 and 2.4 times more resistant to the reference compound m-AMSA than wild-type cells.

**Carbazole derivatives are not topoisomerase IIα poisons**

The catalytic inhibitors act by blocking a step in the catalytic cycle before DNA double-strand cleavage, thereby decreasing cell viability by reducing critical enzymatic activity. Topo IIα plays a significant role in decatenation, a process required for separating catenated DNA duplexes at the end of replication. The ability of carbazole derivatives to inhibit the catalytic decatenation activity of Topo IIα was tested using electrophoretic separation in the presence of highly knotted circular kDNA and Topo IIα (Figure 6a). The absence of Topo IIα in the negative control (DMSO Top IIα (-)) resulted in high molecular weight kDNA not moving from the well. Compound 36a presented efficient and concentration-dependent inhibition of decatenation. At the same time, 36b and 27a reduced the decatenation process entirely only in the highest concentration, or partially for higher-order catenates containing two, three, four, or more minicircles that moved slower on the gel than the decatenated kDNA. Catalytic inhibitor ICRF-187 significantly decreased the decatenating activity of the enzyme compared to etoposide, which stabilizes the cleavage complex of DNA/Topo II.

To understand that carbazole derivatives do not qualify as Topo II poisons, we additionally performed a DNA cleavage assay using Topo IIα and pBR322 plasmid. As shown in Figure 6b, only etoposide induced a detectable level of linear plasmid on the gel, indicating that 36a, 36b, and 27a did not stabilize the Topo II/DNA covalent cleavage complex like etoposide. To further confirm this, the cleavage assay again in the presence of etoposide was performed to determine whether 36a could inhibit Topo IIa like ICRF-187 by stabilizing the non-covalent Topo II/DNA complex after inducing the Topo II/DNA cleavage complex by treatment with etoposide (Figure 6c). Co-treatment of 36a reduced the level of linear plasmid compared to etoposide and ICRF-187, indicating that 36a prevented the formation of etoposide-induced DNA cleavage in reaction with Topo IIa. We concluded that 36a could inhibit Topo IIa activity by preventing the establishment of the Topo II/DNA cleavage complex. Further investigation is needed to identify the particular step or stages of the Topo II catalytic cycle that are influenced by 36a.

**Carbazoles induce apoptotic cell death via a mitochondrial pathway**

Apoptosis evasion is one of the hallmarks of tumor transformation. Commonly used chemotherapeutics aim to promote cancer cell death through apoptosis activation and especially topoisomerase inhibitors among others are the most efficient inducers of apoptosis. The confocal imaging with Annexin V-FITC and Hoechst33342 staining was performed to observe changes in nuclei morphology of A549 and HCT-116 after 24 h treatment with compounds (Figure S8). As depicted in Figures 7a and 7b, compounds 27a, 36a, and 36b showed typical features of cells undergoing apoptosis as evidenced by multiple shrunken cells, fragmented nuclei, and apoptotic bodies. The apoptotic potential of the carbazoles was measured by flow cytometry using double staining with Annexin V-FITC and 7-AAD,
which allows for determining viable (Annexin V-FITC(-)/7-AAD(-)), early apoptotic (EA) (Annexin V-FITC(+)/7-AAD(-)), late apoptotic (LA) (Annexin V-FITC(+)/7-AAD(+)), and necrotic (Annexin V-FITC(-)/7-AAD(+)) cells. As shown in Figure 7c, after 24 h of A549 cells treatment, tested compounds increased the proportion of apoptotic cells (EA + LA) to above 40%, compared to the control group (8.7 ± 2.3%). Later exposure (48 h) to carbazoles demonstrated a potent increase in the late apoptotic phase, from approximately 30% to 60%, depending on the compound (Figure 7c). Like A549, the treatment of HCT-116 cells with investigated compounds lead to a time-dependent increase of apoptotic cells, whereas the apoptotic potential of carbazoles was similar in both cell lines (Figure 7c and 7d). The most substantial pro-apoptotic properties exhibit compound 27a, which on both tested cancer lines caused an 8-fold increase in the fraction of apoptotic cells compared to the control. Moreover, in both tested cell lines, carbazoles also slightly activated necrosis, and this effect was only statistically significant for 27a and 36b against HCT-116 (**p<0.001) (Figures 7c, 7d and Figure S9). Importantly, all tested compounds induced apoptosis approximately 2.5 times more than the reference compound etoposide, a well-known topoisomerase II inhibitor used in chemotherapies for treating numerous tumors, such as lung cancer 35.

One of the characteristics of cells undergoing apoptosis is the activation of cysteine proteases known as caspases, Executioner caspases 3 and 7 are known as central players during apoptotic cell death 36. To determine, whether carbazoles triggered caspase-dependent cell death, A549 and HCT-116 cells were treated with equitoxic concentrations of compounds and analyzed by flow cytometry. As depicted in Figure 7e, after 24 h of treatment A549 cells, carbazoles activate caspase-3/7, as revealed by a 3.4-fold, 5.7-fold, and 2.5-fold increase in apoptosis for 27a, 36a, and 36b respectively, as compared to control. Later treatment A549 cells (48 h) showed a slight increase in apoptotic cells. In comparison, exposure to HCT-116 cells led to a comparable increase in subpopulation cells with activated caspase 3/7 for all carbazole derivatives, as revealed by approximately five times higher than in control (DMSO) (Figure 7f). After 48 h of treatment, HCT-116 cell compounds enhanced 1.5-2-fold caspase-3/7 activity compared to the vehicle (Figure 7f). Moreover, ETP also caused a significant increase in caspase-3/7 activity in both cell lines; however, this effect was more pronounced than with carbazoles (Figures 7e, 7f, and Figure S10).

Mitochondria play a significant role in cell death, and loss of mitochondrial outer membrane potential (MOMP) is essential to initiate mitochondrial apoptosis. The change in JC-1 fluorescence demonstrated a loss of ΔΨm from red (aggregates) to green (monomers). As shown in Figures 7g and 7h, treatment of A549 and HCT-116 cells with carbazoles for 24 h led to a significant rise in the percentage of cells with dissipated MOMP, as revealed by ~5-fold increase in JC1-monomers by all carbazoles in comparison to a vehicle (DMSO). On the other hand, the reference compound FCCP led to an 11-fold and 6.3-fold increase in JC-1-monomers for A549 and HCT-116 cells, respectively, compared to the control (Figure 7g and Figures S11-S13).

DNA fragmentation is the hallmark of the last step of apoptosis, achieved by endonucleases activation, which cleavages genomic DNA in dying cells into internucleosomal DNA fragments 37. To confirm the proapoptotic properties of investigated carbazoles, terminal deoxynucleotidyl transferase dUTP nick end
labeling (TUNEL) was assessed after treatment A549 and HCT-116 cells for 24, and 48 h. As depicted in Figure S14, all compounds led to a time-dependent, significant increase in the rates of TUNEL-positive cells on both cancer cell lines; however, observed DNA fragmentation was more pronounced on HCT-116 than on A549 cells, where the most massive effect induced compound 27a which is consistent with results obtained from Annexin V-FITC/7-AAD assay (Figures 7c and 7d).

Discussion

The incidence and mortality rates of cancer have been on the rise in recent years. According to data from the Globocan 2020 report, the number of diagnosed cancer cases was 19.3 million, while the number of deaths was 10 million 38. Lung cancer (18% of cases) and colon cancer (9.4% of cases) were found to be the most deadly types of cancer. Moreover, lung and colon cancers were the most common types of cancer among men, while breast, colon, and lung cancers were the leading types of cancer among women 38. The incidence of lung cancer and colorectal cancer is likely to increase considerably each year. This increase is associated with genetic mutations, aging, and the resistance of these types of cancers to conventional pharmacological treatments 39,40. In light of this, searching for new and effective cancer treatments, especially lung, colon, and breast cancer is crucial. Many studies have shown that compounds containing a carbazole scaffold exhibit potent biological activity in cancer treatment 41. In this study, we evaluated at the molecular level the growth inhibition and cell death properties based on human topoisomerase type II inhibition of three symmetric carbazole derivatives substituted with furan or thiophene. Initially, all compounds were tested for their antiproliferative activity in selected cancer cell lines (bone, breast, colon, and lung). For most of the tested cancer cell lines, 36a, 36b, and 27a exhibited nanomolar IC$_{50}$ values, effectively reducing colony-forming ability. Since Topo IIa activity is highly expressed primarily in replication, we found that carbazole derivatives effectively inhibited DNA synthesis in all tested cancer cell lines. In light of the cytotoxicity results, compound 36a exhibited a comparatively weaker impact on the HEK293 cell line in contrast to the majority of other cancer cell lines that were evaluated during this study, and its effect was equivalent to the reference compound, etoposide.

Cell cycle regulation plays a crucial role in malignant transformation and developing resistance to chemotherapy 42. Several existing catalytic topoisomerase IIa inhibitors exhibit a different mechanism of cell cycle suppression than Topo II poisons 43. Studies on cell cycle progression showed that among all compounds, 36a, along with significantly reduced cell proliferation induced G1 phase cell cycle arrest in almost all cancer cell lines tested, except for the U-2-OS, where we observed a time-dependent block in the G2/M. Similarly, Perdih's group discovered a new chemical class of Topo IIa catalytic inhibitors inducing the G1-phase block of the cell cycle in MCF-7 and Hep-G2 cells 44. Kang et al. reported a different effect on treatment with the catalytic inhibitor of Topo IIa, which suppressed the viability of ovarian cancer cells by arresting the cell cycle in the S phase 45. On the other hand, Chen et al. described that fibrosarcoma
cells treated with ICRF-193 lead to a G2/M delay \(^{46}\). These studies confirm that the cellular response to
treatment with catalytic inhibitors differs from Topo II poisons at the cellular level.

Anticancer drugs targeting Topo II is a systematic approach in clinical oncology. Commonly used
chemotherapeutic agents such as doxorubicin and etoposide, initially selected for their high potential to
induce cell death, have been found to target Topo II \(^{47}\). These compounds referred to as Topo II poisons
are characterized by the ability to stabilize the cleavable complex between Topo II and DNA, causing the
accumulation of DNA double-strand breaks in the cell. On the contrary, catalytic inhibitors reduce the
activity of Topo II without directly inducing DNA damage. In this study, we evaluated three carbazole
derivatives for their ability to inhibit human Topo II as a potential anticancer mechanism of action. Out of
the three carbazole derivatives that were tested, we did not notice any significant variations in their
cytotoxic activity when the compounds were substituted with furanyl at positions 2,7 (\(^{27a}\)) and 3,6 (\(^{36a}\));
however, we did observe that the substitution played a significant role in the inhibitory activity of
topoisomerase II. We assessed that \(^{36a}\) blocks Topo II activity with high selectivity for the Topo IIa
isoform, while most human Topo II inhibitors act on both \(\alpha\) and \(\beta\) isoforms \(^{48}\). Recently, there has been an
increasing interest in the search and development of isoform-selective poisons and catalytic inhibitors of
topo II, because treatment with Topo II poisons is associated with several side effects, including
cardiomyopathy and secondary malignancies \(^{49}\). Furthermore, recent work established that targeting
Topo II\(\beta\) by chemotherapeutics is correlated with side effects \(^{50}\). Few compounds have been described so
far preferentially inhibit one isoform \(^{28,51,52}\).

Numerous drugs that block the catalytic activity of Topo II have been identified. Current research
indicates that catalytic inhibitors act through various mechanisms. Depending on which particular agent
targets the step or steps of the Topo II catalytic cycle, the cellular effects of that drug can be significantly
different \(^{53}\). We determined that the tested carbazoles did not stabilize the cleavable DNA/Topo II
complex. The results of kDNA decatenation indicate that among all tested compounds, \(^{36a}\) has the most
promising properties to inhibit the catalytic activity of Topo IIa. Furthermore, co-treatment with etoposide
showed that its mechanism of action is based on inhibiting the step in the catalytic cycle of the enzyme
before the formation of the Topo II/DNA cleavable complex. In addition, the possibility that tested
carbazole derivatives could disrupt the Topo II-DNA interaction through distortion of the DNA helical
structure was investigated. Several studies report that the structure of the carbazole moiety has the
potential to intercalate with DNA \(^{54-56}\); however, none of the investigated compounds did bind to DNA,
which explains that the inhibitory effect of Topo II was direct.

Currently, many pharmacological therapies exert anticancer effects by inducing a decrease in \(\Delta \Psi_m\) and
the permeabilization of the mitochondrial membrane leading to the release of apoptotic factors.
Subsequently inducing activation of caspases causes cell degradation through limited proteolysis of
many cellular proteins. \(^{57}\). Topoisomerases are important biological targets whose inhibition initiates
multiple signaling pathways, including apoptosis. In the present study, our research revealed that
carbazole derivatives inhibit Topo II function, initiating the mitochondria-dependent apoptosis pathway.
by activating effector caspases 3/7 and DNA fragmentation of lung and colon cancer cells. Many Topo II poisons are well-known apoptosis inducers including etoposide, doxorubicin, or mitoxantrone. Similarly, several reports show that Topo II catalytic inhibitors such as ICRF-187 and ICRF-193, induce apoptosis by activating the activation of caspases and internucleosomal DNA fragmentation. Topo II catalytic inhibitors should not induce increased levels of DNA damage in the cell. Studies of carbazole derivatives for A549 and HCT-116 cell lines showed that carbazole derivatives caused the most significant increase in the level of γ-H2AX-positive cells after the most extended treatment time point, correlated with the occurrence of massive apoptosis in the cells at the same time. This finding may be related to the accumulation of γ-H2AX to the outer part of the nucleus and the formation of a characteristic apoptotic ring during cell death, which may be caused by DNA damage by activation of caspase 3.

This study demonstrates that symmetric carbazole derivatives reduce the viability of A549 and HCT-116 cells in vitro, as well as their ability to form colonies. Specifically, we found that 36a, which contains a furanyl moiety, effectively inhibits DNA synthesis and induces G1 phase arrest in cancer cells. Furthermore, we discovered that compound 36a is a novel and promising non-intercalating catalytic inhibitor of Topo II, exhibiting highly selective inhibition toward the Topo IIα isoform and inducing apoptosis through the intrinsic pathway with DNA fragmentation. This compound represents a unique example of symmetrically substituted carbazoles with potent anticancer properties. However, further comprehensive studies are necessary to fully elucidate the mechanism by which 36a inhibits the catalytic activity of Topo IIα.

**Methods And Experimental**

**Compounds synthesis**

The MALDI-TOF mass spectra of 27a, 36a, and 36b compounds studied were recorded on an autoflex TOF/TOF maX instrument (Brüker Daltonics) with DHB matrix in the range of m/z 100 to 1000. Additionally, the ¹H (500 Hz) and ¹³C NMR (125 Hz) spectra of the 27a, 36a, and 36b compounds studied d₆-DMSO solutions were obtained with a Brücker AVANCE III 700 MHz spectrometer. UV absorption spectra of 27a, 36a, and 36b acetonitrile solutions were recorded on an Evolution 300 (ThermoScientific) spectrophotometer with a data interval of 1.0 nm, a slit width of 1.0 nm and a scan speed of 240 nm/min. The fluorescence emission spectra (λₑₓc = 300 nm for 36a and λₑₓc = 340 nm for 27a and 36b) of all compounds studied in acetonitrile were recorded using FL 6500 Fluorescence spectrophotometer (Perkin Elmer) in the wavelength area between 350 and 550 nm. The results obtained for all samples studied 27a, 36a, and 36b by the fluorometric method are in excellent agreement with those reported previously.

**General procedure for the Stille cross-coupling reaction**

Pathways and the consecutive steps of the three syntheses were in Figure 1a.
The first synthesis step: 3,6-Dibromo-9H-carbazole or 2,7-dibromo-9H-carbazole derivatives (0.61 mmol, 1 eqv.) and Pd(PPh₃)₄ (0.015 mmol, 0.025 eqv.) were dissolved in 7 mL of dry toluene. The toluene solution was intensely stirred under Ar atmosphere at room temperature for 20 min. Then, 2-(tributylstannyl)thiophene (precursor of 36b) or 2-(tributylstannyl)furan (precursor of 27a or 36a) (1.52 mmol, 2.5 eqv.) was added and the reaction mixture was heated at 90 °C for 24 h.

The second synthesis step: After the reactions are complete (TLC confirmation), toluene solutions are cooled to room temperature. The solvent was removed under reduced pressure and crude products were extracted with EtOAc (3 x 20 mL), washed twice with water (first fraction), saturated KF (second fraction), and DCM (third fraction) solvents and all fractions dried over Na₂SO₄.

The third synthesis step: After evaporation, the oily (27a) and powders (36a, 36b) crude products were purified by column chromatography. In the case of 27a derivative 1:10 (v/v) of the ethyl acetate:petroleum, ether mixture was used as an eluent, and in the case of 36a and 36b derivatives, the different, specific and accordant mixture of toluene:petroleum ether (v/v = 1:1) was applied. The structures of the objects studied and their numbering style were introduced in Figure 1b, the equipment used as well as TLC monitoring made during syntheses were presented in Figure S15.

2,7-Di(furan-2-yl)-9H-carbazole (27a)

MALDI-TOF spectrum registered for 27a, DHB matrix [m/z signal; found (calc.)]: [M] 299.101 (299.05) (Figure S16). ATR signals [cm⁻¹]: 3398 n(N-H); 2956-2854 n(C-H)arom.; 1599 n(C=C); 1454, 1422 and 1377 n(C=N); 1326 (C-C)interring; 1240 n(C-N); 1155, 1075 and 1012 n(C-C)interring. (Figure S17). Electronic absorption and fluorescence spectra obtained for 27a were included in the SI file (Figure S18) and agreed with the data reported in 23, 24. The images of the colorful carbazole derivatives solutions in d₆-DMSO were included in Figure S19.

¹H NMR chemical shifts (500 Hz; d₆-DMSO); d [ppm]: 11.46 (carbazole-NH); 8.18-8.09 (m, 4H, carbazole H-4, H-4a, H-5 and H-5a); 7.66-7.56 (m, 4H, furan H-10 and H-10a, carbazole H-2 and H-2a); 7.39-7.28 (m, 2H, carbazole H-8 and H-8a); 7.01 (t, 1H, J₉,₁₀ = 3.38; J₈,₉ = 9.39; furan H-9); 6.63 (dd, 1H, J₉₉,₁₀₉ = 3.4; J₈₉₈,₉ = 1.79; furan H-9a) (Figure S20).

¹³C NMR chemical shifts (125 Hz, d₆-DMSO); d [ppm]: 155.3; 143.1; 141.4; 132.1; 130.1; 122.7; 117.1; 114.5; 112.7; 106.1 (Figure S21). Additionally, the 2D COSY (Figure S22) and 2D HSQC (Figure S23) of 27a were registered.

3,6-Di(furan-2-yl)-9H-carbazole (36a)

MALDI-TOF spectrum registered for 36a, DHB matrix [m/z signal; found (calc.)]: [M+2H⁺] 301.045 (301.0) (Figure S24). ATR signals [cm⁻¹]: 3420 n(N-H); 3252 n(C-H) in furan ring; 2957-2854 n(C-H)arom.; 1635 and 1609 n(C=C); 1467 and 1379 n(C-N); 1325 (C-C)interring; 1290 n(C-N); 1237 and 1153 n(C-N); 1073 n_sym(-
C=C); 1011n(C-C)\textsubscript{ring}. (Figure S25). Electronic absorption and fluorescence spectra obtained for 36a were included in the SI file (Figure S26) and agreed with the data presented in\textsuperscript{23,24}.

\textsuperscript{1}H NMR chemical shifts (500 Hz; \textit{d}\textsubscript{6}-DMSO); d [ppm]: 11.53 (carbazole-N\textsubscript{H}); 8.53 (s, 1H, carbazole H-5); 8.44 (s, 1H, carbazole H-5a); 7.78 (d, 1H, J\textsubscript{2,3} = 8.22 Hz, carbazole H-3); 7.74 (s, 1H, carbazole H-3a); 7.55-7.47 (m, 4H, carbazole H-2 and H-2a, furan H-10 and H-10a); 6.87 (d, 2H, J\textsubscript{8,9} = 3.16, furan H-8 and H-8a); 6.61 (t, 2H, J\textsubscript{9,10} = 1.6 Hz, furan H-9 and H-9a) (Figure S27).

\textsuperscript{13}C NMR chemical shifts (125 Hz, \textit{d}\textsubscript{6}-DMSO); d [ppm]: 154.7; 142.4; 139.9; 124.9; 123.5; 123.1; 116.3; 113.6; 111.3; 104.3 (Figure S28).

The 2D COSY (Figure S29) and 2D HSQC (Figure S30) of 36a were registered.

### 3,6-Di(thiophen-2-yl)-9H-carbazole (36b)

MALDI-TOF spectrum registered for 36b, DHB matrix [m/z signal; found (calc.)]: [M] 331.03 (331.05) (Figure S31). ATR signals [cm\textsuperscript{-1}]: 3411 n(N-H); 3259-3100 n(C-H) in thiophene ring; 2955-2844 n(C-H)\textsubscript{arom.}; 1629; 1602 n(C=C); 1468; 1378 n(C-N); 1288, 1234, 1176 and 1158 n(C-N); 1070 and 1050 n\textsubscript{sym}(C=C); 1017 n(C-C)\textsubscript{ring} (Figure S32). Electronic absorption and fluorescence spectra obtained for 36b were included in the SI file (Figure S33) and correlated with the data presented elsewhere\textsuperscript{64}.

\textsuperscript{1}H NMR chemical shifts (500 Hz; \textit{d}\textsubscript{6}-DMSO); d [ppm]: 11.54 (carbazole-N\textsubscript{H}); 8.52 (s, 2H, carbazole H-5 and H-5a); 8.48 (s, 1H, carbazole H-3); 8.44 (s, 1H, carbazole H-3a); 7.74 (s, 1H, thiophen H-10); 7.72 (s, 1H, thiophen H-10a); 7.53 (m, 2H, thiophen H-9 and H-9a); 7.49 (m, 2H, carbazole H-2 and H-2a); 7.15 (m, 2H, thiophen H-8 and H-8a) (Figure S34).

\textsuperscript{13}C NMR chemical shifts (125 Hz, \textit{d}\textsubscript{6}-DMSO); d [ppm]: 145.2; 139.2; 129.2; 128.8; 124.8; 123.8; 122.8; 118.1; 113.7; 112.2 (Figures S35).

Moreover, the 2D COSY (Figure S36) and 2D HSQC (Figure S37) of 36b were registered.

The structural characterization of the three carbazole derivatives studied made based on data obtained are in good agreement with those reported in\textsuperscript{23,24,64}.

### Cell culture

A549 (CCL-185), HCT-116 (CVCL-427), MCF-7 (HB-8065), U-2 OS (HTB-96), U-87 MG (HTB-14), and HEK-293 (CRL-1573) cells used in this study were acquired from ATCC. Nalm-6 (CVCL-0092) and Nalm-6 topoisomerase knock-out cells, originally obtained by Noritaka Adachi (Yokohama City University, Japan) and was kind gifted by Caroline Austin and Ian Cowell (Newcastle University, UK). The non-small lung cancer cell line A-549, breast cancer cell line MCF-7, leukemia cell line Nalm-6, and derived cells (Nalm-6 Top2α+/−, and Nalm-6 Top2β−/−), colon cancer cell line HCT-116, and bone cancer cell line U-2 OS were
cultured in RPMI-1640 medium and McCoy's 5A medium, respectively. The brain cancer cell line U-87 MG was cultured in MEM medium, while the nonmalignant embryonic kidney cell line HEK293 was cultured in DMEM. All culture media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics (penicillin 62.6 µg/ml and streptomycin 40 µg/ml). The cells were cultured in a humidified atmosphere containing 5% CO$_2$ at 37 °C and were routinely screened for *Mycoplasma* contamination. All reagents used in this study were purchased from Corning unless otherwise stated.

**Drug sensitivity assay**

Cell viability was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay. The cells were seeded into 96-well plates and treated with the investigated compounds at concentrations ranging from 0 to 50 µM for 72 h. Etoposide (Sigma Aldrich) or m-AMSA (Cayman Chemical) were used as references. After treatment, the cells were incubated with the MTT solution (0.4 mg/ml PBSx1) for three hours at 37°C. The medium was removed, and the formazan crystals were dissolved in 100 µl of DMSO. The absorbance was measured at 540 nm using an ASYS UVM340 microplate reader (Biochrom Ltd.) and the experiment was conducted in triplicate independently.

**Clonogenic assay**

At a density of 400 cells/well, A549 and HCT-116 cells were seeded into 6-well plates. The cells were treated with various concentrations of investigated carbazole derivatives for 24 h followed by a wash and cultured for 8 days. The methanol was used to fix the cells and then followed by 0.5% crystal violet staining. The visible colonies were counted by ImageJ software and then viability was calculated concerning the control. The colony visualization and counting were achieved through ImageJ 1.53n software.

**Flow cytometry**

For each flow cytometry experiment, A549, HCT-116, MCF-7, and U-2 OS cells were seeded onto tissue culture plates and allowed to attach for 24 h. Cells were incubated with investigated compounds at IC$_{50}$ concentration for the appropriate time for each experiment. 10,000 events were analyzed using Guava easyCyte 8 cell sorter (Merck Millipore) and FlowJo v10 software. Each experiment was repeated independently three times.

**Cell cycle analysis**

A549, HCT-116, MCF-7, and U-2 OS cells were treated with carbazole derivatives for 24 and 48 h. After that, the cells were harvested, fixed in ice-cold 75% ethanol, and stored overnight at −20°C. In the final step, after centrifugation, the cells were rinsed with PBS and stained with 20 µg/µl PI (Sigma-Aldrich) and 100 µg/µl RNaseA (Thermo Fisher Scientific) in PBS for 20 min at RT.

**BrdU incorporation**
To detect DNA synthesis, A549, HCT-116, MCF-7, and U-2 OS cells were incubated with 20 μM BrdU (5-bromo-2'-deoxyuridine) (Sigma Aldrich) for 1 h before the end of treatment. Next, samples were harvested with trypsin solution and fixed in 75% ethanol (overnight or longer, -20°C). Following 10 min rehydration with PBS, samples were denatured with 2 M HCl (45 minutes, RT), and the suspension was neutralized with 0.1 M sodium tetraborate, pH 8.5 (10 min, RT). 1% w/v bovine serum albumin (BSA) in PBS was used for blocking (30 min, RT). After that, samples were incubated with rat anti-BrdU antibody (1 h, 37°C; 1:100 dilution; Abcam, #ab6326) and then with anti-rat conjugated goat antibody (30 min, 37°C; 1:200 dilution; Abcam, #ab150157). 20 μg/μl PI and 100 μg/μl RNaseA in PBS (20 min, RT) were used to stain the DNA.

**DNA damage**

Briefly, treated cells were harvested, fixed in 75% ethanol, and stored overnight or longer at −20°C. Cells were rehydrated with PBS and permeabilized in 0.2% Triton X-100 in PBS for 15 min at RT to detect DNA damage. Then samples were blocked with 2% BSA in PBS and incubated with Alexa488-conjugated mouse anti-γH2AX (Ser139) antibody (1:100 dilution; BioLegend, #613406). The DNA was stained with 7-AAD (Sigma Aldrich) and 100 μg/μl RNaseA in PBS (20 min, RT). Etoposide was used as a reference.

**JC-1 staining**

After time treatment ended, the culture medium of the A549 and HCT-116 cells was replaced with fresh medium containing 5 μg/ml JC-1 dye (Sigma-Aldrich) and incubated in the dark for an additional 20 min at 37°C. Then, the cells were washed two times with PBS and measured. As a reference, 50 μM FCCP (Sigma-Aldrich) was added 15 min before the end of drug treatment incubation.

**Apoptosis and caspase 3,7 activation**

Briefly, after incubation with tested compounds and etoposide, A549, and HCT-116 cells were harvested by trypsinization, rinsed twice with PBS, and stained with Annexin V FITC conjugate (Thermo Fisher Scientific, #A13199) for apoptosis assay and with CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Thermo Fisher Scientific, #C10427) for caspase-3/7 activation according to the manufacturer’s protocols. Etoposide was used as a reference.

**DNA fragmentation analysis**

A549 and HCT-116 cells were tested using TUNEL assay Kit – FITC (Abcam, #ab66108). After drug treatment, samples were harvested using trypsin solution, washed with PBS, and fixed with 1% formaldehyde. The experiment was performed according to the manufacturer’s protocol. Etoposide was used as a reference.

**Relaxation/Decatenation of Human Topoisomerase IIα/IIβ**
Determination of the inhibitory activity of investigated compounds was performed according to the manufacturer's protocol (Inspiralis; #HT205). In brief, a mixture containing 250 ng supercoiled pBR322 (Thermo Fisher; #SD0041), tested compounds, and a reaction buffer was prepared. Then, the reaction was initiated by adding diluted assay buffer human topoisomerase IIα or IIβ, and samples were incubated for 30 min at 37°C. The reaction was terminated by adding a loading buffer (New England BioLabs; #B7024S). Samples were loaded onto 1% (w/v) agarose gel and subjected to electrophoresis in 1xTBE at 20 V for 18 h. The gel was stained with ethidium bromide, destained in H2O, and photographed by ChemiDoc Imaging System (Biorad). 250 ng of kDNA (Inspiralis, #K1002) was used for the decatenation assay. Etoposide and ICRF-187 (Cayman Chemical) were used as references.

**Formation of cleavable complexes**

The composition of the mixture was the same as for the relaxation of human topoisomerase IIα assay, except for the amount of enzyme (5 times more was used). The reaction was initiated by adding the enzyme to the samples, and incubation for 10 min at 37°C. Then, 0.35% SDS and 0.3 mg/ml proteinase K (A&A Biotechnology) were added, and the probes were additionally incubated at 56°C for 1 h, before adding loading buffer (New England BioLabs, #B7024S) and chloroform:isoamyl alcohol (24:1 v/v). Etoposide and ICRF-187 were used as references. The electrophoresis was run in the presence of EtBr (1 µg/ml) in TBEx1.

**Intercalation into DNA**

The unwinding assay was performed according to the manufacturer's instructions (Inspiralis, #DUKSR002) to determine the intercalating ability of the tested carbazole derivatives. Etoposide and doxorubicin (Sigma Aldrich) were used as references. In brief, diluted in assay buffer wheat germ topoisomerase I is added to a mixture containing assay buffer, tested compounds, and relaxed pBR322. After incubation (30 min, 37°C), the reaction was terminated by 50 µl of butanol and 20 µL of H2O. Next, samples were vortexed and centrifuged, and the aqueous layer was mixed with chloroform/isoamyl alcohol (24:1 v/v) and loading buffer (New England BioLabs, #B7024S).

**Live-cell imaging**

For live-cell imaging of A549 and HCT-116 cells, JC-1 and Annexin V-FITC staining were performed by seeding them into glass-bottom 24-well plates. The drug-treated samples were then stained in the same way as flow cytometry. The Hoechst33342 (Sigma-Aldrich) was used to visualize the cell nuclei under the microscope. Images were obtained using an LSM 800 inverted laser-scanning confocal microscope (Carl Zeiss), equipped with a ×63 1.4-NA Plan Apochromat objective (Carl Zeiss) and an airyscan detector for high-resolution confocal scanning. The incubation chamber was maintained at 37°C with 5% CO2 during the analysis. Etoposide was used as a reference.

**Immunofluorescence**
The cells were seeded on coverslips and allowed to attach overnight. Following this, cells treated with tested compounds or etoposide were rinsed with PBS, fixed for 10 minutes at room temperature using 4% paraformaldehyde (Sigma-Aldrich) in PBS, and permeabilized for 15 minutes with 0.2% Triton X-100 (Sigma-Aldrich) in PBS. Subsequently, the cells were washed twice with PBS, blocked using 3% BSA in PBS for 1 hour at RT, and incubated for 1 hour at 37°C with the primary antibodies, which were diluted in 3% BSA in PBS-T (PBS containing 0.1% (v/v) Tween-20 (Sigma-Aldrich)). The primary antibodies used were mouse anti-Tubulin (Sigma-Aldrich, T8328) at a dilution of 1:200 and Alexa488-conjugated mouse anti-γH2AX (Ser139) antibody (1:250 dilution; BioLegend, #613406). The slides were washed twice with PBS-T and then incubated for 1 hour at 37°C with the secondary antibody in 3% BSA in PBS-T. The secondary antibody was a goat anti-mouse IgG antibody conjugated to Alexa Fluor 594 (1:200 dilution, Jackson ImmunoResearch). After the secondary incubation, the slides were washed and stained with 0.5 µg/ml DAPI (Sigma-Aldrich). The images were acquired using an LSM 800 inverted laser scanning confocal microscope (Carl Zeiss) equipped with an Airyscan detector and a × 63 1.4 NA Plan Apochromat objective (Carl Zeiss).

Statistical analyses
Statistical analysis was performed using GraphPad Prism 9 software and uniform significance levels were used throughout the entire manuscript: ns= $p > 0.01$; *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$; ****$p < 0.00001$. Statistical significance was calculated in comparison to the DMSO-treated control (1% v/v) using two-way ANOVA.

Declarations

Data availability
The datasets presented in the current study are available from the corresponding author upon reasonable request.

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Contributions
conceptualization: M.O.; methodology: M.O., N.M., A.C, A.M.D., M.Bi.; validation: M.O., A.C., A.M.D.; formal analysis: M.O.; writing—original draft preparation: M.O., N.M.; graphical conceptualization: M.O., N.M.; writing—review and editing: M.O., N.M., A.K., J.M.P., M.M., M.B.

Competing interests
The authors declare no competing interests.
References


**Figures**
Figure 1

(a) The synthetic routes of the three carbazole derivatives (27a, 36a, 36b). (b) The structures of compounds 27a, 36a, and 36b, together with the numbering of the atoms, were adapted to the analysis of the NMR spectra (not the chemical compound names).
Figure 2

Colony-forming ability of HCT-116 and A549 cells after treatment with 27a, 36a, and 36b. Representative images of the clonogenic assay for HCT-116 and A549 cell lines (a,b) and its quantification (c,d). Data represent the mean ± SD of three independent experiments.
Figure 3

Cell cycle profiles of A549, HCT-116, MCF-7, and U-2 OS cells after treatment with 36a, 36b, and 27a. Representative histograms after PI staining and their quantification are presented on graph bars. Error bars represent the mean ± SD of data obtained in three independent events.
Figure 4

Induction of DNA double-strand breaks. (a) The photomicrographs indicate the formation of γ-H2AX levels and changes in the structure of the microtubule after incubation for 48 h. The arrows indicate the cells with an accumulation of γ-H2AX foci forming apoptotic rings. Etoposide served as a reference. The microtubule is shown in red, γ-H2AX in green, and the nucleus in blue. (b) Dot-plots diagrams of γ-H2AX
staining and quantification [%] of γ-H2AX-positive cells plotted as a bar graph. Error bars represent the mean ± SD of data obtained in three independent events.

**Figure 5**

Inhibition of topoisomerase IIα (a) and IIβ (b) mediated pBR322 relaxation. The experiment was carried out either with topoisomerase IIα/IIβ in the presence of solvent (DMSO IIα/IIβ (+)) or with a different
concentration range of carbazole derivatives. Etoposide (100 µM), and ICRF-187 (100 µM) were used as references. (c) Unwinding assay. Determination of the ability of carbazole derivatives to intercalate into DNA in the presence of topoisomerase I (Top I). Etoposide and doxorubicin were used as negative and positive controls, respectively. (d) Cytotoxic activity of 36a toward Nalm-6 cells with heterozygous deletion of topoisomerase IIα (TOP2α+/-) or homozygous deletion of topoisomerase IIβ (TOP2β-/−) compared to the wild-type (Nalm-6) cells. m-AMSA was used as a reference. Data (mean ± SD) present IC$_{50}$ value obtained from three independent experiments. These gels have been cropped and full length gels are presented in supplementary Figures S2-S4.
Figure 6

(a) Inhibition of topoisomerase IIα mediated kDNA decatenation by carbazole derivatives Figure 5 (b, c). DNA cleavage assay in the presence of 36a, 36b, and 27a, respectively. Etoposide (100 µM) and ICRF-187 (100 µM) were used as references. These gels have been cropped and full length gels are presented in supplementary Figures S5-S7.
Analyses of proapoptotic activities of carbazole derivatives. Confocal imaging of A549 (a) and HCT-116 (b) cells at 24 h of treatment with 27a, 36a, and 36b. The cells were stained with Hoechst33342, scale bar=10 µm. The arrows indicate cell shrinkage (white), fragmented nuclei (red), and apoptotic bodies (yellow). Quantification of flow cytometry analysis of A549 (c) and HCT-116 (d) cell lines (24, 48 h) using Annexin V/7-AAD. Modulation of caspase 3/7 in A549 (e) and HCT-116 (f) cells (24, 48 h) after treatment
with 27a, 36a, and 36b. Analysis of changes in mitochondrial potential using JC-1 staining. Bar charts (g) with statistical quantification and confocal images of A549 and HCT-116 cells (h) acquired after 24 h treatment with compounds. Error bars represent the mean ± SD of data obtained in three independent events. Etoposide or FCCP served as references.

**Supplementary Files**

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- Supplementaryinformation.pdf