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Highlights

- Analysis of the most relevant literature of the last ten years about dual Topoisomerases I and II inhibitors
- Overview of the new strategy regarding the development of multiple Topoisomerases and Tyrosyl-DNA Phosphodiesterases inhibitors
- Some of the reviewed compounds exert tumor growth inhibition *in vivo*
- Structural requirements necessary for developing potent multiple inhibitors as new efficient and safe anticancer agents.

Multiple Topoisomerase I (TopoI), Topoisomerase II (TopoII) and Tyrosyl-DNA Phosphodiesterase (TDP) inhibitors in the development of anticancer drugs

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Abstract

DNA Topoisomerases (Topos) are ubiquitous nuclear enzymes involved in regulating the topological state of DNA and, in eukaryotic organisms, Topos can be classified into two structurally and functionally different main classes: TopoI and TopoII.

Both these enzymes proved to be excellent targets of clinically significant classes of anticancer drugs. Actually, TopoI or II inhibitors show considerable wide spectrum antitumor activities, an important feature to be included in many chemotherapeutic protocols.

Despite their clinical efficacy, the use of inhibitors targeting only one of the two enzymes can increase the levels of the other one, favouring the onset of unwanted phenomena such as drug resistance. Therefore, targeting both TopoI and TopoII can reduce the probability of developing resistance, as well as side effects thanks to the use of lower doses, given the synergistic effect of the dual activity.

Moreover, since drug resistance is also due to DNA repair systems such as tyrosyl-DNA phosphodiesterases I and II, inhibiting Topoisomerases concomitantly to Tyrosyl-DNA phosphodiesterase enzymes could allow more efficient and safe drugs.

This review represents an update of previous works reporting about dual TopoI and TopoII inhibitors, but also an overview of the new strategy regarding the development of derivatives able to simultaneously inhibit Topo and TDP enzymes, with particular attention to structure-affinity relationship studies. The newly collected derivatives are described focusing attention on their chemical structures and their biological profiles. The final aim is to highlight the structural requirements necessary for the development of potent multiple modulators of these targets, thus providing new potential antitumor agents for the clinical usage.

Keywords

Cancer; Topoisomerase; Tyrosyl-DNA Phosphodiesterase; Multitarget drug; Poison; Catalytic inhibitor.

Abbreviations

ALL MOLT-4, acute lymphoblastic leukemia; A2780, human ovarian carcinoma; A431, human squamous carcinoma; A549, human lung carcinoma; ACHN, human renal adenocarcinoma; AGS, human stomach adenocarcinoma; BE2-C, neuroblastoma; BEL-7402, human hepatocellular carcinoma; BT483, breast cancer; CCRF-CEM, human acute lymphoblastic leukemia; CL141T, human lung adenocarcinoma; DU-145, human prostate cancer; DWD, oral cancer; H460, human lung non small carcinoma; HCT116, human colon carcinoma; HCT15, human colorectal adenocarcinoma; HEK293, human embryo kidney; HeLa, epithelial cervix adenocarcinoma; Hep3B, hepatocellular carcinoma; HepG2, human hepatocellular liver carcinoma; HL60, human

promyelocytic leukemia; Hop62, human lung cancer; HT-29, human colon carcinoma; Huh7, human hepatocarcinoma; K562, human chronic myelogenous leukemia; LO2, human normal liver; MCF-10A, non-malignant breast cell line; MCF-7, epithelial breast adenocarcinoma; MDA-MB231, human breast adenocarcinoma; MIA, human pancreatic carcinoma; MRC-5, human fetal lung fibroblast; MX-1, human breast cancer; NCIH23, human lung non-small cell carcinoma; NCIH520, human lung squamous cell carcinoma; NUCG-3, gastric cancer; OECM1, human oral epidermoid carcinoma; P388, murine leukemia; PC-3, prostate cancer; SAR, structure–activity relationship; SJ-G2, glioblastoma; SKHep, hepatocelluar carcinoma; SK-OV‑3, ovarian adenocarcinoma; SMA, glioblastoma; SW480, human colon adenocarcinoma; T47D, human breast tumour; U87, human glioblastoma astrocytoma; Zr-75-1, human breast carcinoma.

1. Introduction

Although significant progresses against cancer were achieved in recent years, thanks to an improvement in the prevention, detection and treatment of this pathology, in 2040 it is expected to arrive at 29.4 million of new cases/year due to the aging and growth of the population (compared to 18.1 million in 2018). Moreover, with the decrease in mortality for cardiovascular diseases, cancer remains the leading cause of death of this century. For this reason, in a view to increasing patients' survival and quality of life, the search for anticancer drugs is still one of the main goal of the Medicinal Chemistry (Jemal, 2019).

The most important features searched in the development of a novel anticancer drug, suitable for the clinical use, are a better balance between toxic and pharmacological effects and the ability to overcome the problem of primary or acquired resistance to the tumor.

This review deals with cancer chemotherapeutic agents targeting DNA and enzymes involved in DNA metabolism, including DNA Topoisomerases and Tyrosyl−DNA phosphodiesterases.

DNA Topoisomerases (Topos) are ubiquitous nuclear enzymes involved in regulating the topological state, as well as many other functions (i.e. transcription and replication) of DNA, as they have the ability to relax positive or negatively supercoiled DNA (Wang, 2002). In eukaryotic organisms, Topos can be classified into two structurally and functionally different main classes: type I (TopoI) and type II (TopoII) topoisomerases.

TopoI are monomeric proteins that do not require cofactors for their activity, but exploit the intrinsic energy of the supercoiled DNA itself. TopoI regulate the topological state of DNA through the passage of a single strand of DNA through the breakage which takes place when a tyrosine residue in its active site forms a covalent bond with the 3'- or 5'-DNA phosphate (Capranico et al., 2017). A further subdivision based on the DNA-protein bond (3'- or 5'-DNA phosphate), mechanism of action (strand passage or rotation) and dependence on Mg^{2+} as a requirement for catalytic activity, classifies TopoI into TopoIA and TopoIB (Hevenern et al., 2018). TopoIA need the presence of Mg^{2+} for their catalitic activity and make use of a "strand-passage" mechanism in which a single strand of DNA is cleaved with the formation of a 5'- phosphodiester bond between the enzyme and DNA (Garnier et al., 2018). Topo IB, represented by eukaryotic TopoI, are independent from Mg^{2+} for their catalytic activity; they have a "hindered rotation mechanism", in which the enzyme binds to DNA and cleaves a single strand through a 3'-phosphodiester bond; then the 5'-end rotates around the second DNA strand, relaxing the DNA.

Eukaryotic TopoII (TopoIIA, represented by TopoIIα and TopoIIβ isoforms) are homodimer proteins which require ATP and Mg^{2+} for their catalytic activity. Each monomer is made up of four domains, namely the N gate, the DNA gate and the C gate, which are involved in the TopoII catalytic cycle, and the C-terminal domain, responsible for DNA recognition (Wendorff et al., 2012; Bollimpelli et al. 2017). TopoII exert their activity through a multistep catalytic cycle, where the main steps are: (1) recognition and binding to the first double-stranded DNA chain; (2) trapping of the second double-stranded DNA chain through ATPase domain dimerization; (3) double-stranded break, which results in the formation of a 5′-phosphodiester bonds between DNA and TopoII; (4)

passing of an unbroken strand through the transient break, mediated by hydrolysis of ATP; (5) religation of the DNA strand break in which ADP product is released; (6) restoring the enzyme and DNA integrity (Roca and Wang, 1994; Nittis, 2009a; Chen et al., 2018).

Under physiological conditions, DNA cleavage mediated by TopoI or TopoII is transient since the religation phase is faster than the breaking one and therefore it is well tolerated by the cells. If the breakage amount or duration become too high, DNA undergoes permanent changes, which prevent the progression of the cell cycle and become of fundamental importance in cancer cells, that are in continuous proliferation.

The ability to interfere with Topo activity is an effective strategy for cancer therapy; indeed, the various types of DNA Topos have been the target of marketed drugs for several decades (Holden et al., 2001; Nitiss, 2009b; Wilstermann and Osheroff, 2003). Topo inhibition can occur according to one of the mainly two reported and proven molecular mechanisms (Pommier, 2013; Wendorff et al., 2012; Leelaram et al., 2013; Pommier et al., 2010).

Following the first mechanism, there is the formation of a locked ternary Topo-DNA-inhibitor cleveable complex, whose accumulation prevents the enzyme from completing its catalytic cycle, stabilizing the DNA breaks and leading to a cytotoxic effect (Pommier et al., 2010; Lindsey et al., 2014). These compounds are termed “Topo poisons” because they convert functional Topos into lethal lesions of the DNA with consequent triggering of apoptosis. Based on the different DNA binding patterns, Topo poisons can be further divided into DNA intercalating agents and non-intercalating agents. Intercalating agents have planar structures able to fit between the base pairs of DNA in the enzyme-DNA cleavage complex, preventing DNA religation, transcription and replication (Pommier et al., 2010). Non-intercalating agents bind to a site distal from the DNA active site and perform their functions forming a covalent drug-enzyme complex. In this way they trap the Topo-DNA cleavage complex resulting in a similar build-up and then cell death (Gibson and Deweese, 2013).

The second highly relevant Topo inhibition mechanism consists in the competitive binding of small molecules (catalytic inhibitors) to the ATP binding site of TopoII, which results in the catalytic inhibition of TopoII, halting DNA transcription and replication, and finally cell death (Maxwell and Lawson, 2003; Pommier, 2013).

Compounds that can bind to the DNA-protein complex and prevent cleavage, represent yet another mechanism of catalytic inhibition (Larsen et al., 2003).

Moreover, all the other topoisomerase I and II inhibitors acting with a mechanism other than poisons and prevent the formation of the complex are however defined as catalytic inhibitors.

Actually, marketed Topo inhibitors used for the treatment of cancer include anthracycline agents (doxorubicin, epirubicin, valrubicin, daunorubicin, idarubicin), anthracenediones (mitoxantrone, pixantrone), epipodophyllotoxines (etoposide, teniposide), amsacrine, which are all TopoIIA poisons, and the TopoIB poisons camptothecines (topotecan, irinotecan, belotecan) (Hevenern et al., 2018). Despite their clinical efficacy, the use of these inhibitors is limited by some important negative consequences. Indeed, their own mechanism of action appears to be responsible for additional toxicities, including secondary leukemia (Mistry et al., 2005; Azarova et al., 2007). In addition: anthracyclines are known for their cardiotoxic properties (Sawyer, 2013); antracenediones are similar to the anthracyclines with less toxicities, but they cause long term cell damages (Beeharry et al., 2015); also, camptothecines show dose-limiting toxicities and drug resistance (Pizzolato and Saltz, 2003).

Drug resistance in anticancer therapy involving TopoI or TopoII inhibitors is mainly due to DNA repair systems. In particular, the DNA damage caused by these inhibitors can be recovered by various repair systems, including pathways dependent from the recently disclosed tyrosyl-DNA phosphodiesterases I and II (TDP1 and TDP2) (Pommier et al., 2006; Maede et al., 2014; Pommier et al., 2014).

TDP1 is a member of the phospholipase D superfamily (Interthal et al. 2001). It catalytically hydrolyzes mainly the 3′-phosphotyrosyl bond associated with TopoI cleveable complex, but also

the 5′-phosphotyrosyl bond involved in TopoII-mediated DNA damage, playing a main role in the maintenance of genomic stability (Murai et al., 2012; Ashour et al., 2015; Nitiss et al., 2006). Considering that TDP1-deficient cells are hypersensitive to TopoI poisons, it has been suggested that overexpression of TDP1 will confer resistance to TopoI-mediated DNA damage, and therefore TDP1 inhibitors would have the ability to potentiate the effects of TopoI poisons or act synergistically with them. All these considerations make TDP1 an effective target for the development of anticancer drugs (Huang et al., 2011; Dexheimer et al., 2008).

TDP2 is a recently discovered member of the metal-dependent phosphodiesterases, with repair function linked to TopoII-mediated DNA damage (Zeng et al., 2011; Ledesma et al., 2009). Its main activity is to excise TopoII from 5′-phosphotyrosyl DNA adducts catalyzing the hydrolysis of the covalent bond between the DNA phosphate and TopoII (Gao et al., 2012; Pommier et al., 2014). In addition, experimental evidences are reported indicating that TDP2 causes cellular resistance to TopoII poisons; *in vitro* and *in vivo* studies showed that the lack of TDP2 has led to greater cellular sensitivity to DNA breaks induced by TopoII poisons (Gómez-Herreros et al., 2013; Gómez-Herreros et al., 2014; Ledesma et al., 2009; Zeng et al., 2011). For all these reasons inhibition of TDP2 may represent a suitable approach to overcome intrinsic or acquired resistance to TopoII-targeted drug therapy (Zeng et al., 2011; Ledesma et al., 2009).

In severe and complex pathologies like cancer, in which many pathogenic factors are involved, single-target treatment may result inadequate due to the activation of compensatory mechanisms and alternative pathways (Bishop and Sham, 2000; Morphy and Rankovic, 2005; Nussinov et al., 2014). Accordingly, the use of a drug targeting two or more different pathways involved in tumor insurgence and/or progression can lead to a synergistic effect, as well as a lower probability of developing resistance. Moreover, the enhanced therapeutic effects of a multitarget drug require lower doses, with the potential to reduce side effects respect to individual drug treatments (Bayat Mokhtari, et al., 2017; Anighoro et al., 2014; Blagosklonny, 2005; Albain et al., 2008). The multitarget approach in cancer therapy allowed the discovery of dual TopoI and TopoII inhibitors:

these enzymes share overlapping functions in the cell cycle progression, so targeting them simultaneously might lead to synergistic anticancer effects. Furthermore, drug resistance against TopoI or TopoII inhibitors is often a consequence of downregulation of the target enzyme and compensatory upregulation of the other Topo isoform, making the cancer cell hypersensitive to this upregulated enzyme. It has been reported that resistance to inhibition of one class of topoisomerase enzymes is accompanied with an elevated sensitivity to the other class of topoisomerase inhibitors *in vitro* (Tan et al, 1989; Sugimoto et al, 1990). In this view, targeting both TopoI and TopoII simultaneously should reduce the potential for the development of resistance against such inhibitors (van Gijn et al., 2000), although only a few experimental studies were reported supporting this hypothesis which deserves to be deepened. As an example, intoplicine (dual TopoI and TopoII inhibitor) circumvents TopoI- and TopoII-mediated resistance by poisoning both enzymes simultaneously, since it showed to be cytotoxic in cells that are resistant to either TopoI or TopoII inhibitors (Poddevin et al, 1993).

In the last decades, several classes of dual TopoI and II inhibitors were developed, both natural and synthetic compounds, and many of them, have reached phase I and/or II of clinical trials (Denny, 1997; Denny and Baguley, 2003; Salerno et al., 2010). In a previous work, we reviewed dual TopoI and TopoII inhibitors published until 2010, and classified them according to their mechanism of action and structural features (Salerno et al., 2010).

More recently, a new strategy regarding the development of multiple Topo/TDP inhibitors have emerged with the goal to develop compounds able to produce a synergistic anticancer effect (Nguyen et al., 2012).

The present review analyzes the most relevant literature of the last ten years about dual TopoI and II inhibitors, but also about inhibitors able to simultaneously inhibit Topo and TDP enzymes, with particular attention to structure-affinity relationship studies. The final aim is to highlight the structural requirements necessary for the development of potent multiple modulators of these

targets, thus providing new perspectives on existing data that will result useful for the scientific community involved in the development of potent, efficient and safe anticancer agents.

2. Dual DNA Topoisomerases I and II inhibitors

As we have discussed above, many studies in tumor models have shown that compounds inhibiting both TopoI and TopoII have an improved efficacy compared to that of single-target drugs, also showing potentially reduced toxicity compared to that of the combination therapy (Denny, 1997; Denny and Baguley, 2003; Salerno et al., 2010). Consequently, dual TopoI/TopoII inhibitors have been suggested as a promising alternative to the combinational approaches (Denny and Baguley, 2003).

In the past decade, various new compounds targeting both TopoI and TopoII have been identified, including natural compounds and their derivatives, metal complexes as well as many classes of small molecules.

2.1. Natural compounds

Natural compounds represent an important source for drug development; indeed, most of the lead compounds used for the design of novel therapeutic agents are of natural origin. Notably, natural products and their derivatives constitute more than 50% of the drugs used as anticancer agents (Newman et al., 2012; Newman et al., 2007; Balamurugan et al., 2003).

2.1.1. Antraquinones from Rubia Cordifolia

In 2012, Jeong S.Y. *et al*. (Jeong et al., 2012) isolated different compounds characterized by an antraquinone system from *Rubia Cordifolia*, and among them compounds **1-3** (Chart 1) showed to be dual TopoI/TopoII inhibitors. When biologically evaluated at 100 μM, **1-3** showed inhibition properties (%, inhibition ratio of relaxation) ranging from 25% to 48% in the DNA TopoI assay, and values of 64-84% in the DNA TopoII assay. In addition, compounds **1** and **2** showed good cytotoxic activity toward different cell lines (A549, SK-OV-3, HepG2, HT-29), whereas compound

3 presented only a weak cytotoxicity, indicating the lack of a strict correlation between the biological effect and the TopoI/II inhibitory activity (Jeong et al., 2012).

2.1.2. Simocyclinone Streptomyces antibioticus Tü 6040

Simocyclinone D8 (SD8), **4**, (Chart 1) is an antibiotic obtained from *Streptomyces antibioticus* Tü 6040. In 2010, Sadiq A.A. *et al*., demonstrated that **4** shows anticancer activity due to its ability to inhibit TopoII (Sadiq et al., 2010). Two years later, Oppegard L.M. *et al.*, further studied the compound effects on human Topos, and found that it is also a TopoI catalytic inhibitor (Oppegard, et al., 2012), demonstrating the dual catalytic human TopoI/TopoII inhibitory activity of **4**.

2.1.3. Gossypol from Gossypium

Gossypol (GSP), **5** (Chart 1) is a natural polyphenol isolated from cotton plant, *Gossypium* (Malvaceae). In 2013, Senarisoy M. *et al*., (Senarisoy et al., 2013) reported that **5** is able to inhibit both TopoI and TopoII enzymes, and in particular this compound belongs to the class of the catalytic inhibitors.

2.1.4.1. Rutaecarpine from Evodia rutaecarpa and its derivatives

Rutaecarpine, **6**, (Chart 1) is an indoloquinazoline alkaloid obtained from Rutaceous plants (Asahina, et al. 1915) such as *Evodia rutaecarpa*. Although its significant cytotoxicity on many cancer cell lines, **6** does not present Topos' inhibitory activity (Xu et al., 2006); however, among its derivatives, compounds **7** (Lee et al., 2005) and **8** (Kim et al., 2012), showed to be dual TopoI/TopoII inhibitors, with a stronger activity than the positive controls, camptothecin, and etoposide.

2.1.4.2. Evodiamine from Evodia rutaecarpa and its derivatives

A further *Evodia rutaecarpa* derivative is evodiamine, **9** (Chart 1), a quinazolinocarboline alkaloid, which has been reported to induce cell death in several types of cancer cell thanks to its dual TopoI/TopoII catalytic inhibitory activity (IC_{50} of 60.74 and 78.81 μM, respectively) (Pan et al., 2012). SAR studies underlined the relevance of its indole NH group, which establishes a hydrogen bond with an arginine residue at the TopoI DNA cleavage site (Dong et al., 2010). Starting from this observation, in 2012 a preliminary optimization of **9** was performed by Dong G. *et al*., (Dong et al., 2012) which developed several series of derivatives. Among them, compounds **10-12** (Chart 1**)** showed the ability to inhibit both TopoI and TopoII at concentration of 50 μM. Additional SAR studies were performed to evaluate how substitutions on the various rings of **9** influenced the anticancer activity of the obtained derivatives. Specifically, the introduction of different groups in C1, C3, C10, and C12 positions are favourable. In particular, the hydroxyl group leads to compounds with a significant antiproliferative activity, with a usefully improved water solubility. In anchoring to TopoI, the hydroxyl group forms a hydrogen bond with Arg364, that is the same residue by which the indole NH of **9** interacts, while the carbonyl moiety establishes a hydrogen bond with Asn352. As regards the interaction with TopoII, molecular docking studies showed that the hydroxyl group at 10-position (R_1=OH) of the quinazolinocarboline scaffold establishes a hydrogen bond with the backbone amide of Ile88.

It was evaluated also the mechanism of TopoI and TopoII inhibition and the results indicated that derivatives **10-12** are TopoI poisons, but TopoII catalytic inhibitors. Moreover, these compounds showed a significant antiproliferative activity (GI_{50} < 3 nM), a good *in vivo* anticancer efficacy and low toxicity at the dose of 2 mg/kg (Dong et al., 2012).

Continuing the research in this field, in 2015, Wang *et al*. (Wang et al, 2015) designed several compounds based on **9**-inspired new scaffold, where C-, D-, or E-ring of **12** were substituted with different heterocycles. Among all the synthesized compounds, only **13-16**, featuring a modified D-ring (X = O, S, SO_2) (Chart 1) were able to concomitantly inhibit TopoI and TopoII. Interestingly,

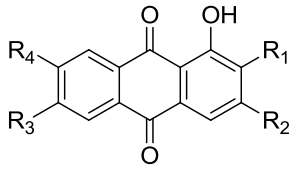
compound **13**, showed the ability to inhibit tubulin too, so it was recognized as the first triple TopoI/TopoII/tubulin inhibitor.

2.1.5. 2-methoxycinnamaldehyde from Cinnamonum verum

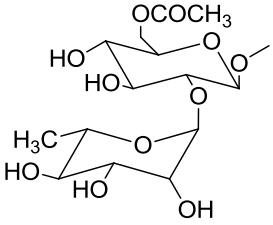
2-Methoxycinnamaldehyde (2-MCA), **17**, (Chart 1), is a constituent of the *Cinnamomum verum* bark (*Lauraceae* family), a Chinese herbal medicine.

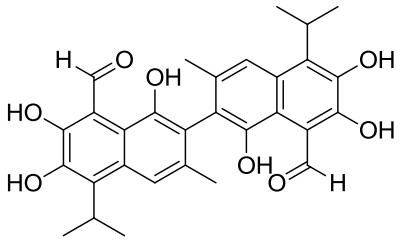
In 2016, Perng *et al*. (Perng et al., 2016) demonstrated that **17** shows anticancer effect in hepatocellular carcinoma Hep 3B cells, and is able to inhibit both TopoI and TopoII in a concentration-dependent manner (using camptothecin and etoposide as positive controls), which partially, could be the mechanism driving the cells to apoptosis.

Later on, in 2017, Liu. *et al*. (Liu et al., 2017) investigated the activity of compound **17** relatively to cell growth, cytotoxicity, apoptosis, and dual TopoI/TopoII inhibition in human lung squamous cell carcinoma NCIH520 cells, both *in vitro* and *in vivo*. The obtained results revealed that **17** has an antiproliferative effect also *versus* NCI-H520 cells in a concentration dependent and time-dependent manner. *In vitro* data allowed to correlate this activity with the downregulation of both TopoI and TopoII, and upregulation of proapoptotic molecules (caspase 3 and caspase 9). Moreover, in a *in vivo* tumor xenograft study (in nude mice), **17** reduced the tumor size, resulting in a significant clinical impact (Liu et al., 2017).

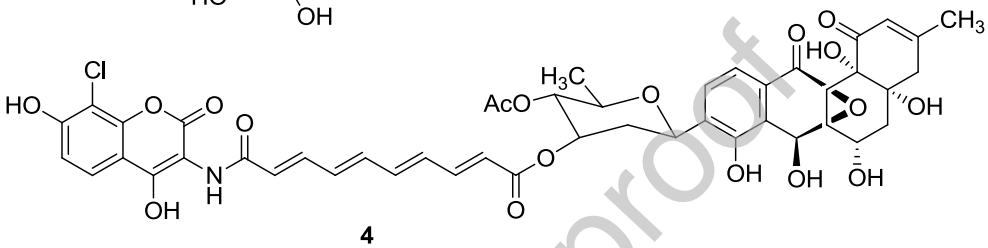


1-3

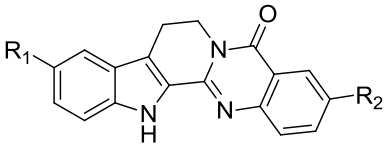
	R_1	R_2	R_3	R_4
1	CH_3	OH	OH	H
2	CH_3	OH	H	OH
3	CH_2OH		OH	H



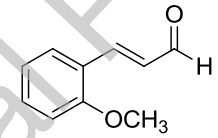
5



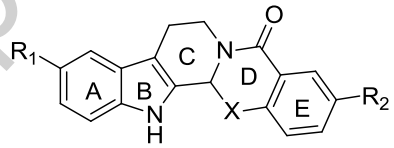
4



6 $R_1 = R_2 = H$
7 $R_1 = Br, R_2 = H$
8 $R_1 = H, R_2 = Cl$



17



9 $X = NCH_3, R_1 = R_2 = H$
10 $X = NCH_3, R_1 = OH, R_2 = H$
11 $X = NCH_3, R_1 = OH, R_2 = Cl$
12 $X = NCH_3, R_1 = OH, R_2 = NH_2$
13 $X = O, R_1 = R_2 = OH$
14 $X = SO_2, R_1 = H, R_2 = OCH_3$
15 $X = S, R_1 = OH, R_2 = Cl$
16 $X = S, R_1 = R_2 = OH$

Chart 1. Structures of natural compounds and their derivatives **1-17** as dual TopoI and TopoII inhibitors.

2.2. Metal Complexes

The advantages of obtaining metal-complexes as altenative to organic molecules consist in their stronger environmental stability and their higher variability of tunable electronic properties thanks to the coordination metal center.

2.2.1. Ruthenium(II) complexes

Ruthenium(II) complexes are reported in literature as potent antitumor agents (Liu et al., 2010) Several studies focused on Ruthenium(II)polypyridyl complexes, because of their simply constructed rigid chiral structure covering all three spatial dimensions, prominent DNA binding properties and encouraging biological effects (Liu et al., 2010; Xu et al., 2014; Boer et al., 2014). Moreover, numerous data evidenced that Ru-complexes can inhibit TopoII interfering with DNA religation and directly binding TopoII (Gao et al., 2007; Gao et al., 2008a; Gao et al., 2008b; Chen et al., 2010).

Considering that molecules bearing a benzofuran group show a significant anticancer activity (such as bufuralol or amiodarone) (Zhou et al., 2003; Abdel-Wahab, 2008), in 2011 Du K.J. *et al*. developed two Ruthenium(II)polypyridyl complexes, **18** and **19** (Chart 2). Spectroscopic and viscosity measurements suggested that **18** and **19** bind to DNA in an intercalative mode. The ability of the complexes to exert a dual TopoI/TopoII inhibition was tested and the obtained results demonstrated that compounds **18** and **19** are TopoI inhibitors (IC_{50} of ∼17 μM and ∼8 μM, respectively), and TopoIIα poisons (IC_{50} of ∼ 18 μM and 13 μM, respectively) (Du et al., 2011). MTT experiments performed on three tumour cell lines (BEL-7402, MCF-7, HeLa) higlighted that these Ru-complexes possess higher *in vitro* cytotoxicity with respect to 5-fluorouracil, but lower activity than cisplatin. Moreover, the apoptosis assays-acridine orange/ethidium bromide (AO/EB) staining evidenced that **18** and **19** can induce apoptosis in HeLa cells. (Du et al., 2011). This assay takes advantage of the fact that AO can stain both live and dead cells, while EB stains only cells that have lost their membrane integrity, so detecting the difference between necrotic and apoptotic

cells. Under the fluorescence microscope, live cells appear green while necrotic cells stain red. Apoptotic cells appear green but the formation of apoptotic bodies is visible (Amarante-Mendes et al., 1998).

One year on, Kou J.F. *et al.*, developed two chiral Ru-complexes analogues to **18**, where the benzofuran moiety was replaced by an anthraquinone group giving Δ/Λ $[Ru(bpy)2(ipad)]^{2+}$ (**Δ/Λ-20**, Chart 2) (Kou et al., 2012). Compounds **Δ-20** and **Λ-20** turned out to be DNA intercalators and dual TopoI/TopoII inhibitors, with IC_{50} of ~4 μM and ~2 μM toward TopoI, respectively, and IC_{50} ≤ 3 μM toward TopoII. The two enantiomers were evaluated for their cytotoxicity *in vitro* in several cancer cell lines (HeLa, HepG2, MCF-7, and BEL-7402) through MTT experiment. Results indicated that both the complexes are more active respect to 5-fluorouracil, and weaker than cisplatin, with HeLa cells that proved to be the most susceptible cell line among those tested. Finally, apoptosis assays performed with AO/EB-stained HeLa showed that complexes **Δ-20** and **Λ-20** induce apoptosis (Kou, 2012). The two enantiomers present only few differences in their cytotoxicity activity, which could be ascribed to slight differences in their Topo inhibition profile, probably deriving from little variation in the DNA binding mode. (Kou, 2012).

To further study chiral complexes, in 2013, Zhang *et al.*, developed novel chiral ruthenium(II)complexes **21-24** characterized by phenolic hydroxyl groups of series **I** and **II** (Chart 2) (Zhang et al., 2013). These compounds turned out to be dual Topos inhibitors, with an IC_{50} range of 3-15 μM. Of note, the most potent complexes, **Δ-21** and **Λ-21**, (Chart 2) bearing a greater number of hydroxyl groups, showed to be Topo poisons, with IC_{50} values of 3 μM toward TopoI and 5 μM toward TopoII, more active with respect to the used standards (IC_{50} = 17 μM for camptothecin and IC_{50} = 35 μM for etoposide). DNA binding assays demonstrated that all the complexes of series **I** and **II** are DNA intercalators, and rationalized the strongest interaction of **Δ-21** and **Λ-21** with the presence of the many hydroxyl groups on the phenyl ring of the complexes.

Moreover, comet assay, a cell gel electrophoresis assay, in which DNA strand breaks were detected in Hep-G2 cells upon treatment with the complexes, (and camptothecin and etoposide as controls) demonstrated that **Δ-21** and **Λ-21** stabilize the cleavable complexes similarly to the reference compounds (Zhang et al., 2013).

MTT experiment was performed to evaluate cytotoxicity of all compounds of series **I** and **II** toward several cancer cell lines and a health cell line, using cisplatin and NAMI-A (Clarke, et al., 2006) as standards. Results showed a higher *in vitro* cytotoxicity respect NAMI-A, but a weaker cytotoxicity than cisplatin. Of note, it was furthermore underlined the importance of hydroxyl groups on the phenyl ring, as compounds **Δ-21** and **Λ-21** were validated as the most potent against the cell lines tested. Interestingly, the complexes exhibited higher cytotoxicity against cancer cells with respect to health cells, suggesting a certain selectivity among the two types of cells (Zhang et al., 2013). The AO/EB staining assay suggested also that both **Δ-21** and **Λ-21** induced apoptosis of the Hep-G2 cells.

Pursuing the study on the chiral Ru(II)complexes to achieve a greater topoisomerase inhibition, in 2014, Wang *et al.*, developed two novel chiral complexes, introducing a thiophene group, indeed, many compounds bearing this moiety show the capacity to interact with DNA and to bind to several receptors, so it was assumed that this moiety could led to compounds endowed with a higher DNA affinity and Topos inhibition activity (Wilson et al., 2007; Ye et al., 2010; Thapa et al., 2010). In line with these considerations, two Ru(II)complexes, Δ/Λ-[Ru(bpy)2(pscl)]$^{2+}$ (**Δ/Λ-25**) and Δ/Λ-[Ru(bpy)2(psbr)]$^{2+}$ (**Δ/Λ-26**), were synthetized (Chart 2), (Wang et al., 2014). DNA binding studies evidenced that the two complexes interact with DNA through an intercalative manner, and it was not observed a DNA-binding selectivity among the enantiomers. Complexes **Δ/Λ-25** and **Δ/Λ-26** showed to be dual TopoI/TopoII poisons with an activity higher or comparable to that of classical TopoI and TopoII inhibitors. MTT experiments demonstrated the high *in vitro* cytotoxicity of the complexes against the selected cancer cell lines (HeLa, HepG2, A549, BEL-7402) being HeLa cell line the most sensitive one. Interestingly, the two enantiomers displayed different potencies, Δ-

enantiomers resulting moderately more active (Wang et al., 2014). Finally, annexin V-Affinity assay (Van Engeland et al., 1998) evidenced that **Δ-25** and **Δ-26** induced HeLa cell death mainly through apoptosis.

All the complexes analysed above were characterized by a bidentate system, whereas tridentate ligands had aroused less interest; only, in 2014, Du K. et *al.* developed a series of Ru(II)complexes characterized by tridentate system (Du et al., 2014). Between all the obtained complexes, **27-29** (Chart 2) proved to be dual inhibitors and, in particular, they were defined as TopoI inhibitor and TopoIIα poisons. Complex **27** showed a moderate dual inhibition, while complexes **28** and **29** exhibited a significant inhibition toward both the enzymes. Moreover, compound **29** turned out to be the strongest TopoI inhibitor (IC_{50} = 0.7 μM), (Du et al., 2014), compared with all complexes previously described (Du et al., 2011; Zhang et al., 2013; Wang et al., 2014). MTT assay was performed on three different cell lines (Hep G2, HeLa, BEL-7402) and on a normal cell line (LO2), using cisplatin and 5-fluorouracil as standards to test the antiproliferative activity of compounds **27-29**. The complexes were more active than 5-fluorouracil, but weaker than cisplatin, with HeLa cell line more responsive to them with respect to the other cell lines tested. Interestingly, the complexes did not result cytotoxic toward the normal cell line, being mainly active on cancer cells (Du et al., 2014).

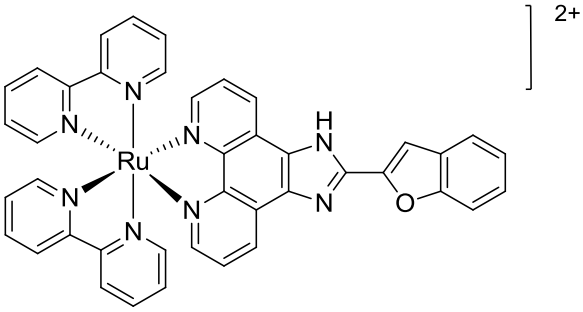
In 2015 X. He *et al*. developed two Ru(II)complexes, $[Ru(dppz)_2dppz\text{-}11\text{-}CO_2Me](ClO_4)_2$ (**30**) and $[Ru(dppz)_3](ClO_4)_2$ (**31**), (Chart 2) (He et al., 2015). When evaluated for their effects on the TopoI-mediated relaxation of supercoiled DNA (He et al., 2015), both complexes inhibited TopoI with IC_{50} values of 5.3 μM and 1.9 μM for **30** and **31**, respectively, lower than that of other claimed TopoI inhibitors, like camptothecin (IC_{50} = 17 μM) (Hsiang et al., 1988). Furthermore, to evaluate the TopoII inhibition, a concentration-depended TopoII inhibition assay of **30** and **31** was performed (IC_{50} values of 25.0 μM and 4.0 μM, respectively). Finally, **30** and **31** were tested for their cytotoxicity *in vitro* through MTT experiments on HeLa and HepG2 cell lines, using cisplatin as reference standard (Du et al., 2011). The results demonstrated that HeLa cells are more affected

by the two Ru complexes as compared to the other cell line tested, however the cytotoxicity of **30** and **31** against the two cell lines is rather lower than that of the standard (He et al., 2015).

In a successive study, in 2016, two Ru-complexes characterized by a polypyridyl system and a 1,8-naphtalimide moiety (**32** and **33,** Chart 2) were developed and tested through DNA-binding studies to evaluate their capacity to inhibit both TopoI and TopoII. The IC_{50} values were 4.5 μM and 22 μM against TopoI and 4 μM and 8 μM against TopoII, confirming compounds' activity as dual inhibitors (Sun et al., 2016).

During the same year, De Camargo *et al*., described three Ru-complexes [Ru(pySH)(bipy)(dppb)]PF_6 (**34**), [Ru(HSpym)(bipy)(dppb)]PF_6 (**35**), and [Ru(SpymMe2)(bipy)(dppb)]PF_6 (**36**) (Chart 2) which were tested for their TopoI activity by a plasmid relaxation assay. The obtained results showed that **36** is the most potent TopoI inhibitor, inducing a full TopoI inhibition at 25 μM; **35** showed a full inhibition at 50 μM, whereas **34** was inactive. Compounds **34-36** also showed good antiproliferative activities in a MTT assay on HepG2, MDA-MB-231 and CHO cell lines, sometimes higher than that of doxorubicin and cisplatin, used as positive controls (De Camargo et al., 2016).

In 2019, the same research group, tested complexes **34-36** for their TopoII inhibition activity by a plasmid relaxation assay, showing that compounds **35** and **36** are also TopoII inhibitors, demonstrating their dual TopoI/TopoII inhibitory activity (De Camargo et al., 2019).



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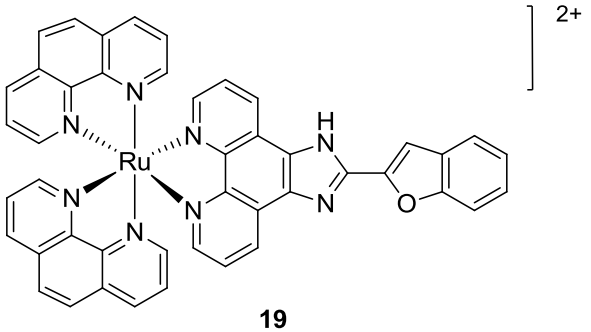

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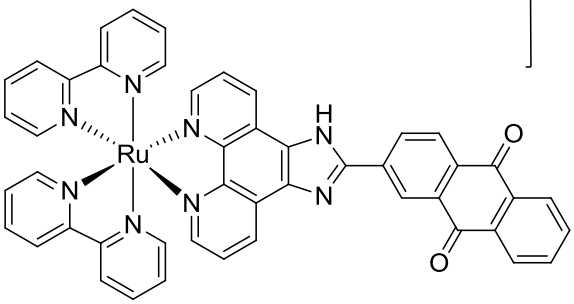
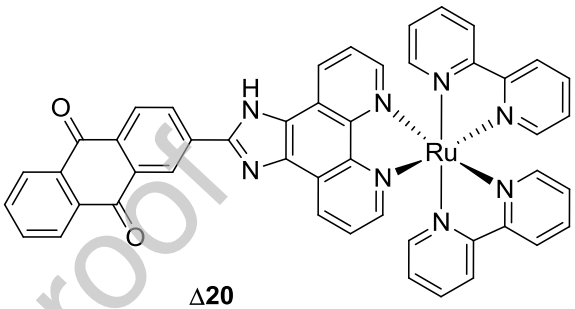
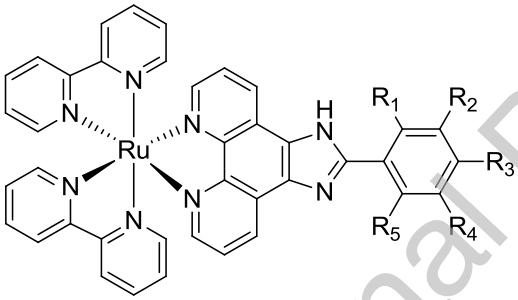


Λ20



Δ20



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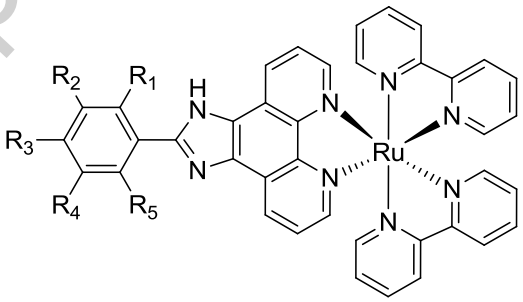
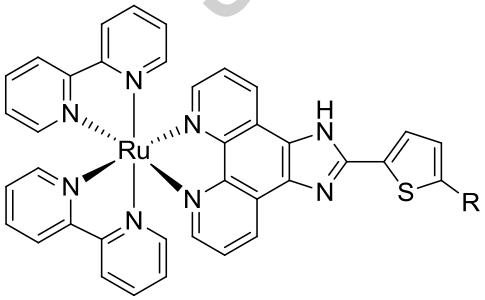


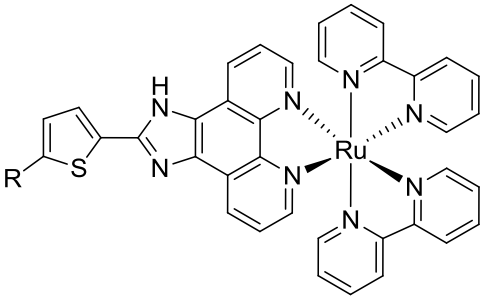
II

Cmp	R_1	R_2	R_3	R_4	R_5
Δ/Λ-**21**	OH	H	OH	H	OH
Δ/Λ-**22**	OH	OH	OH	H	H
Δ/Λ-**23**	OH	H	H	OH	H
Δ/Λ-**24**	H	OH	H	OH	H

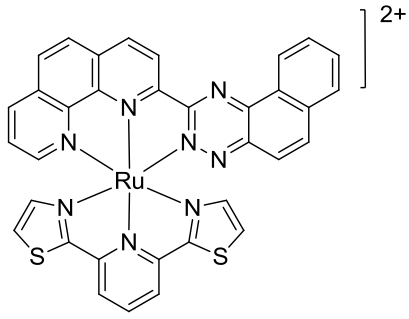


Δ-25/Δ-26

25 R = Br
26 R = Cl



Λ-25/Λ-26



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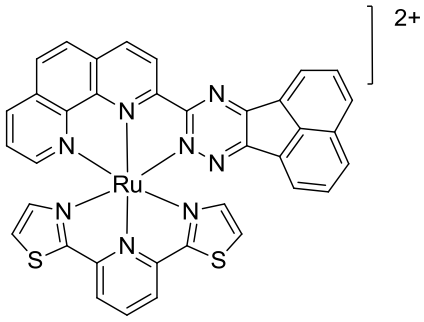

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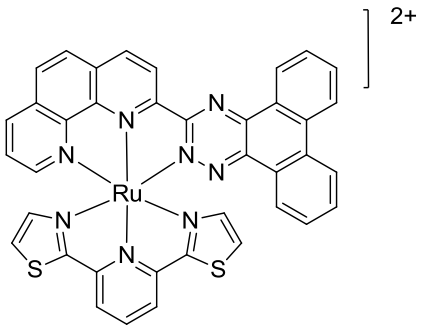

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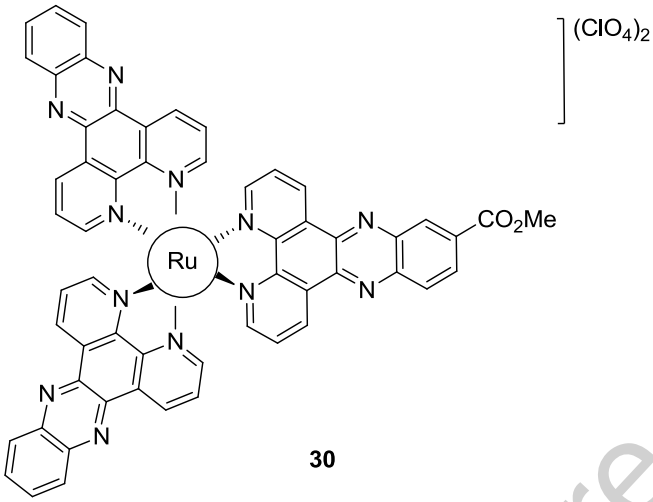

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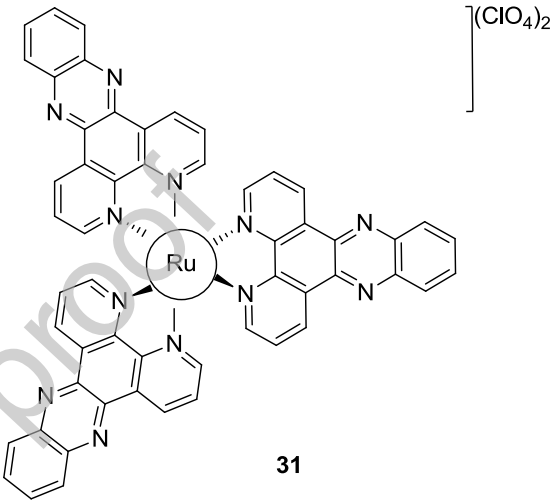

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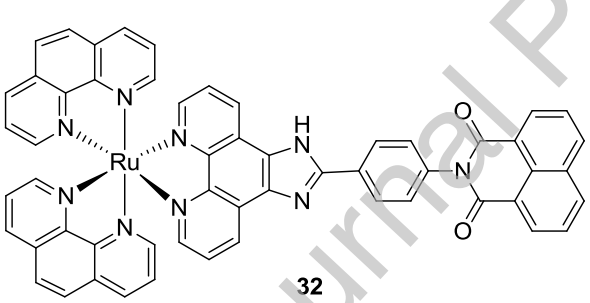

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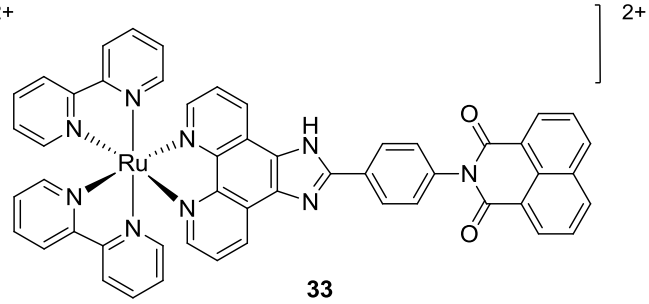

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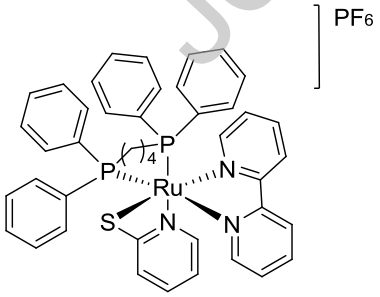
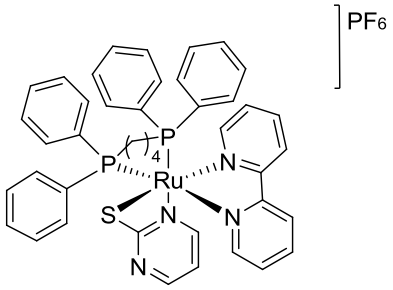


34



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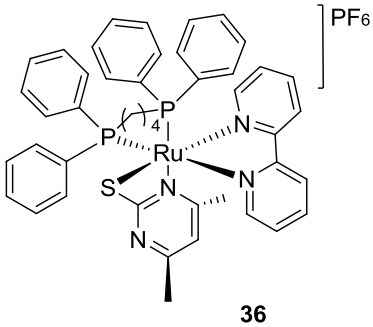


36

Chart 2. Structures of Ru(II)-complexes **18-36** as dual TopoI and TopoII inhibitors**.**

2.2.2. Other metal complexes

2.2.2.1. Copper-complexes

The hydroxy-9,10-anthraquinone moiety in anthracyclines is reported to be the seat of the oxygen species (ROS) generation, which is the mainly responsible for the cardiotoxicity associated with this class of drugs (Minotti et al., 2004). Of note, in anthracyclines complexed with metal ions the cardiotoxicity considerably decreases (Fialo and Suillerot, 1985; Beraldo et al., 1985). Since ROS are essential for the anticancer activity, it is necessary to find a right balance in the ROS generation to retain the biological activity without toxic effects in normal cells (Moreno-Aspitia and Perez, 2009; Pecere et al., 2000). In particular copper(II)-complexes of anthracyclines are deemed interesting because they produce less ROS, maintaining anyway the anticancer activity (Fialo and Suillerot, 1985; Beraldo et al., 1985; Santini et al., 2014; Kheirolomoom et al., 2010)

Taking into account these considerations, in 2014, Das *et al*., synthetized a Cu(II) complex of purpurin (an anthracycline derivative), [Cu(II)-$(LH_2)_2$] **37** (Chart 3), as analogue of metal-anthracycline complexes (Das et al., 2014). *In vitro* DNA Topo relaxation assays showed that complex **37** is a dual Topo inhibitor, establishing enzyme-DNA covalent complexes within the cells, and specifically it results a TopoI poison and TopoII inhibitor. Moreover, MTT experiments showed that **37** was most potent in killing ALL MOLT-4 cells (IC_{50} 18.59±0.77 μM) with respect to its parent compound purpurin (26.35±0.31 μM).

2.2.2.2. Gold-complexes

In 2013, Wilson C.R. and co-workers, developed a class of metal-complexes bearing gold(III), (Wilson et al., 2013); among them, compound **38** (Chart 3) turned out to be cytotoxic in a five-dose NCI-60 screen (IC_{50}~38 μM). The cytotoxic activity of **38** was ascribed to its ability to inhibit both TopoI and TopoII. In particular, hierarchical cluster analysis methods showed that **38** is mechanistically equivalent to topotecan and similar to zorubicin (TopoI and TopoII poisons, respectively). Moreover, TopoII inhibition assay showed that this Au-complex is a TopoII poison at

low concentration, but a TopoII catalytic inhibitor at higher concentrations and it proved to be a catalytic inhibitor of TopoI, in accordance with its capability to intercalate DNA.

2.2.2.3. Silver-complexes

Metal complexes bearing N-heterocyclic carbene (NHC) ligands could exert anticancer and antimicrobial activity (Oehninger et al., 2013). Moreover, silver-NHC-complexes show some advantages than gold-NHC-complexes, because their potentially lesser toxicity and higher complex stability.

Considering these observations, in 2017, Allison *et al*., developed an Ag-complex with pleurotin, a known inhibitor of thioredoxin reductase 1 (Trx-R) (Welsh et al., 2003), **39** (Chart 3) (Allison et al., 2017). In TopoI and TopoII relaxation assays, compound **39** was the first Ag-complex able to inhibit both Topos, determining DNA damage. The complex showed to be mainly a TopoI inhibitor (0.16 μM), but a moderate activity toward TopoII was observed. Compound **39** shows low micromolar activity toward various kind of cancer cell line, moreover it presents a major or comparative selectivity *in vitro* against the cancer cell lines tested *vs* the normal type, with respect to cisplatin. **39** showed also the ability to increase ROS levels through the Trx-R inhibition, determining apoptosis which contributes to an increasing of the anticancer activity.

2.2.2.4. Titanium-complexes

Literature data widely reported the cytotoxic activity of titanium(IV)-complexes toward solid tumor (Harding et al., 2000; Guo et al., 2000). In this view, Chimento *et al.* developed different Ti(IV)-complexes (Chimento et al., 2015), and among them compounds **40-43** (Chart 3) showed to inhibit both TopoI and TopoII at the tested concentration of 50 μM. These complexes proved to be also antiproliferative agents against MCF-7 cell line (assessed by MTT experiment, using cisplatin as positive control). In particular, compound **43** presents a dose-dependent antiproliferative activity, and the most active compound is **41**, with an antiproliferative activity analogue to cisplatin; these

data highlighted that the presence of lipophilic groups can achieve higher antiproliferative effects. Moreover complexes **40-43** were also tested on a non-malignant breast cell line (MCF-10A), to evaluate how they behave with normal cell; outcomes from these experiments underline that complexes are higher selective toward cancer cells than normal one, in comparison with cisplatin.

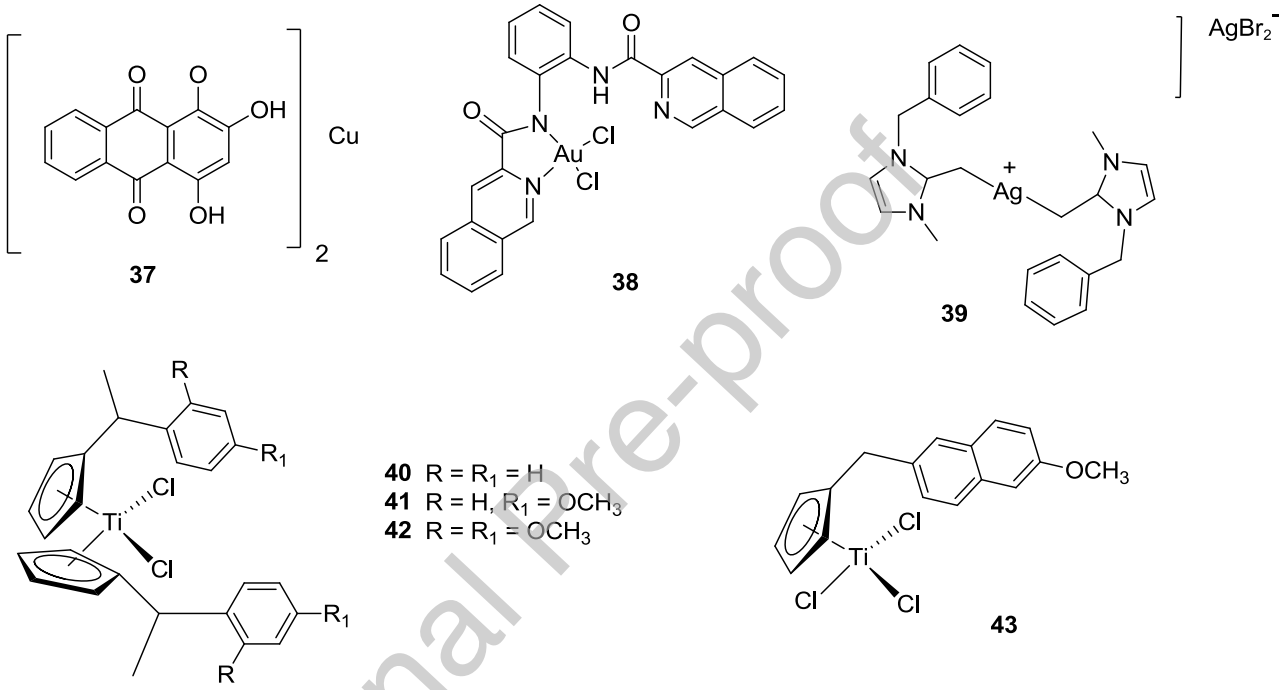


Chart 3. Structures of other metal complexes **37-43** as dual TopoI and TopoII inhibitors.

2.3. Arylpyridine derivatives

α-Terpyridine derivatives, as well as compounds featuring a 2-thyenyl-4-furyl-pyridine scaffold present cytotoxicity toward many human cancer cell lines and significant dual TopoI/TopoII inhibitory activity (Zhao et al., 2001; Zhao et al., 2004; Zhao et al., 2006; Basnet et al., 2007).

Starting from these evaluations, in 2010, Thapa *et al.* (Thapa et al., 2010a, 2010b) developed 2-thienyl-4-furyl-6-aryl pyridine derivatives (series **III,** Chart 4) with chlorine or methyl substituents on the different aryl moieties. Most compounds were TopoII inhibitors, whereas **44**-**51** are able to inhibit both the enzymes with % inhibition values range of 25.8-85.9 for TopoI and 21.8-75.9 for TopoII, assessed at 100 μM.

SAR studies highlighted that 2-(5-chlorothiophen-2-yl)-4-(furan-3-yl) moiety discloses an important role to achieve a gain in activity against TopoII, whereas the presence of 4-chlorophenyl substituent (R_2 = 4-chlorophenyl) improves the TopoI inhibitory activity (Thapa et al., 2010a); moreover, a 4-(5-chlorofuran-2-yl)-2-(thiophen-2-yl) group seems to promote the dual inhibitory activity (Thapa et al., 2010b).

When tested for their antiproliferative activity against several human cancer cell lines (MCF-7, DU-145, HeLa, HCT15, and K562), almost all compounds from series **III** display strong cytotoxicity against HCT15 cell line, but a moderate effect against the other cell lines (Thapa et al., 2010a, 2010b).

The same year, Basnet *et al.* (Basnet et al., 2010) developed 2,6-dithienyl-4-furyl pyridine derivatives **IV** (Chart 4) as dual TopoI/TopoII inhibitors. Few of the new compounds inhibit TopoI or TopoII, since, among them, only **52** and **53** (Chart 4) showed to be dual inhibitors with % inhibition values of 50 and 52 for TopoI and 55 and 58 for TopoII at 100 μM, respectively. SAR studies underlined that the introduction of a chlorine on the 4-furyl moiety or of a methyl group on the 6-thienyl one, enhances the TopoI/TopoII inhibitory activity. Compounds with some TopoI or TopoII inhibition activity were selected to be tested for their cytotoxic activity toward different cancer cell lines (DU-145, MCF-7, MDA-MB231, HeLa, HCT116) and they were moderately

cytotoxic against HCT 116, MDA-MB231, and HeLa cell lines. Anyway, there was no direct correlation between antitumor cytotoxicity and Topo inhibitory activity (Basnet et al., 2010).

Molecular modeling studies of clinically used drugs, including camptothecin and etoposide, evidenced the essential role of the hydroxyl group in 20- and in 4′-position, respectively, in stabilizing the ternary cleavable complex by establishing hydrogen bond (Fan et al., 1998; Wang et al., 1999). Taking into account these findings in 2010, Karki *et al*., (Karki et al., 2010) developed derivatives of series **V** (Chart 4), characterized by a 2,4-diphenyl-6-arylpyridine system bearing hydroxyl groups at different positions (*ortho, meta, para*) of the phenyl rings linked in the 2-, 4- and 6-positions of the central pyridine. Compounds **54**-**58** (Chart 4) turned out to be potent dual Topo inhibitors with % inhibition values ranging of 48.8-75.8 for TopoI and 42.8-86.5 for TopoII, at 100 μM, respectively. The obtained data proved that the introduction of phenol rings on the pyridine core could enhance the dual inhibitory activity, with compound **56** (R = 3-OH, R_1 = H, R_2 = *meta*-hydroxyphenyl) resulting the most potent dual TopoI/TopoII inhibitor of the series. Further SAR studies evidenced that the presence of the hydroxyl group on the phenyl rings improves the dual inhibitory activity in the order of *meta* > *para* > *ortho* position (Karki et al., 2010). The best performing compounds of these series were evaluated also for their antiproliferative activity and they generally showed moderate cytotoxicity toward the cell lines tested (MDA-MB231, HeLa, DU145, HCT15, HL60), slightly weaker respect to the positive controls (camptothecin, etoposide, adriamycin). Of note, compounds bearing a hydroxyl group either in *ortho* or *meta* did not show a significant cytotoxicity, despite to their potency as dual inhibitors, whereas their analogues bearing a *para*-hydroxyphenyl group (R or R_1 = 4-OH) displayed good cytotoxicity, which was positively correlated to Topos inhibition (Karki et al., 2010).

Based on these outcomes, the same research group, in 2012, synthetized a series of compounds **VI** (Chart 4) characterized by the introduction of two hydroxyl groups at different positions (*ortho*, *meta*, *para*) on the 2-phenyl and on 6- or 4-phenyl ring linked to the central pyridine (Karki et al., 2012). The evaluation on how this further hydroxyl group could influence the activity on TopoI and

TopoII, and the relative cytotoxicity, proved that all the compounds are weaker TopoI inhibitors respect their monohydroxylated analogues, while they display a significant TopoII inhibitory activity. Only compound **59** (Chart 4) results able to inhibit TopoII (92.2 % inhibition) and, moderately, also TopoI (11.0 % inhibition) at 100 μM. These outcomes show that, generally, dihydroxylation enhance the TopoII selectivity and SAR studies evidenced that *meta*- or *para*-hydroxy-substitution on the 2-phenyl ring is preferable to the *ortho*- one to reach TopoII inhibition. (Karki et al., 2012). All the compounds of series **VI** were tested for their cytotoxicity on different cancer cell lines (HCT15, K562, MCF-7, HeLa), resulting at least active against K562 and HCT15. Furthermore, it was observed that dihydroxylated-2,4,6-triphenyl pyridines exhibited a gain in cytotoxicity respect their monohydroxylated and/or nonsubstituted analogues (Karki et al., 2012).

Considering that many natural and synthetic products bearing a chlorine often exhibit both Topos inhibitory activities and anticancer effects (Bailly, 2012), Thapa *et al.*, (Thapa et al., 2012a) developed 2,4,6-triaryl pyridine derivatives of series **VII** (Chart 4) featuring a *ortho*, *m*eta, or *para* chlorophenyl moiety at the 2-position of the central pyridine ring, with the purpose to optimize some molecule characteristics, such as the potency, or the ability to pass the blood brain barrier.

Compounds **60**-**65** (Chart 4) were able to inhibit both TopoI and TopoII, (% inhibition range: 38.8-68.0 for TopoI and 67.5-83.6 for TopoII at 100 μM, respectively), and SAR studies highlighted that the introduction of a *para*-chlorophenyl at 2-position of the pyridine leads to stronger TopoII inhibitors. Furthermore, compounds characterized by *para*-phenolic moiety (R_1 = 4-OH) at the 4-position are more active toward TopoI than their analogues bearing *meta*-phenolic group (R_1 = 3-OH) at the same position. The most potent dual inhibitor results compound **63** (R = 3-chlorophenyl, R_1 = 4-OH, R_2= phenyl), (Chart 4), that shows TopoI and TopoII inhibitory activities of 68.0 % and 80.9 % at 100 μM and of 55.2% and 44.6% at 20 μM respectively, thus resulting more active than the controls (camptothecin and etoposide) (Thapa et al., 2012a).

In virtue of its high activity, compound **63** was selected for molecular docking studies which evidenced that the molecule binding pattern with TopoI and TopoII partially overlapped to that of

topotecan and etoposide, respectively. In particular, **63** establishes hydrogen interactions with the Asp533 in the side chain of TopoI and Asp 479 and Gly 478 of TopoII, and it forms π-π interactions with DNA base pairs of the TopoII active site. All these findings explain the dual Topo I/TopoII inhibitory activity of **63** (Thapa et al., 2012a). Finally, compounds of series **VII** with some inhibitory activity on TopoI or II were tested for their cytotoxicity toward four different cancer cell lines (HEK293, DU145, HCT15, T47D), and most of the compounds presented moderate cytotoxicity (IC_{50} values range 1.48 μM–39 μM) (Thapa et al., 2012a).

Later on, Karki R. *et al*., (Karki et al., 2015) developed several compounds bearing a 2,6-diphenyl-4-aryl pyridine skeleton substituted with *ortho*, *meta*, or *para* hydroxyl groups on the phenyl moiety, inserted at either the 2- or 6- position of the central pyridine (**VIII**, Chart 4). Among the compounds of this series, most of the 2-thienyl and 2-furyl derivatives proved to be dual TopoI/TopoII inhibitors with % inhibition values range of 61.7-100 for Topo I and 52.2-92.8 for TopoII, at 100 μM concentrations. Compounds **66 (**R = 3-OH, R_2 = 3-OH) and **70 (**R = 3-OH, R_2 = 4-OH) (Chart 4) belonging to the 2-thienyl subclass and **71 (**R = 3-OH, R_2 = 4-OH) and **72 (**R = 4-OH, R_2 = 4-OH) (Chart 4) belonging to the 2-furyl subclass, turned out to be the most potent dual inhibitors of the series.

The antiproliferative effects of the synthesized compounds were tested against four different cell lines (HCT15, DU145, T47D, HeLa); despite their strong dual TopoI /TopoII inhibitory activity, most of 2-thienyl and 2-furyl derivatives of series **VIII** showed a low cytotoxicity toward all the tested cell lines, only few of them exhibited a significant cytotoxicity at least toward DU145 and T47D (< 2 μM). (Karki et al., 2015).

One year later, the same research group developed 2-phenol-4-aryl-6-chlorophenyl pyridine derivatives **IX** (Chart 4), most of them were validated as moderate to strong dual TopoI/TopoII inhibitors at 100 μM, in particular those bearing a phenyl, 2-thienyl or 2-furyl moiety at the 4-position of the pyridine. (Karki et al., 2016). SAR studies evidenced that the presence of an *ortho*, *meta*, or *para* chlorophenyl moiety at the 6-position and a *para*- or *meta*-phenolic substituent at the

2-position of the pyridine leads to dual Topo inhibitors and determines a gain in cytotoxicity. Compound **70** (*meta*-OH-phenyl, *ortho*-Cl-phenyl, R_1 = phenyl) results the most interesting dual TopoI/TopoII inhibitor, as well as cytotoxic compound against the tested cell lines with low IC_{50} values ($\leq$ 2 μM); thus, it was used as the template to perform 3D-QSAR study. This analysis underlined that the addition of a chlorine atom on the phenyl ring at 6-position do not determine any essential effect on the inhibitory activity of the compound. Instead, the phenolic moiety, which is able to form H-bond, at the 2-position of the pyridine ring, results a significant element for compounds' biological activity.

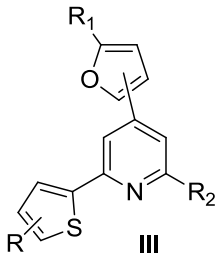
Subsequently, compounds characterized by a 2-hydroxyphenyl-4-chlorophenyl)-6-(substitutedphenyl)pyridine skeleton (**X**, Chart 4) were further developed (Shrestha et al., 2018) (Bist et al., 2017).

In particular, dihydroxylated compounds (6-(hydroxyphenyl)pyridines **72-79**, Chart 4), characterized by a *meta*- or *para*-phenol at 2- and 6-position of the central pyridine, presented a significant dual TopoI/TopoII inhibitory activity, and strong cytotoxicity toward the tested human cancer cell lines. Of note, their analogues bearing an *ortho* phenol at 2- or 6-position showed either a very weak or none TopoI/TopoII inhibitory activity. SAR studies were performed and highlighted that most compounds show an increasing order of TopoI and TopoII inhibitory activities with respect to the *ortho*, *meta*, and *para* chlorophenyl substituents, respectively. Indeed, it was evidenced that the *para*-chlorophenyl at the 4-position of the pyridine ring discloses an essential role for exhibiting the most considerable TopoI/TopoII inhibition. It was underlined that removing the chlorine atom from the 4-phenyl moiety significantly improves the TopoII inhibitory potency, but at the same time it does not affect the TopoI inhibitory activity (Bist et al., 2017). Compound **78** (R = 4-OH, R_1 = 3-Cl, R_2 = 4-OH) results the most potent dual inhibitor of this series, showing a Topo % inhibition at 100 μM of 82.7 toward TopoI and 100.0 toward TopoII. Therefore, it was selected to better study its mechanism of action. In particular, different experiments were performed, such as band depletion assay, cleavage complex stabilization assay, and ethidium

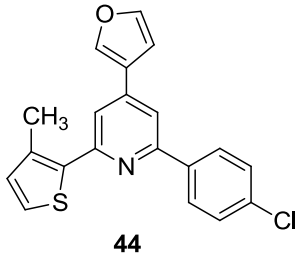
bromide displacement assay, to investigate the mode of action of **78**, that results a potent DNA non-intercalative catalytic dual TopoI/TopoII inhibitor (Bist et al., 2017).

On the contrary, among the mono hydroxylated compounds (6-chlorophenylpyridines, Chart 4), those bearing a *para*- or a *meta*-phenolic group displayed a potent TopoII inhibition, but none of the *meta*-phenolic compounds showed TopoI inhibition activity, and among the *para*-phenolic ones only few compounds showed to be weak TopoI inhibitors. Only *ortho*-phenolic compounds **80-88** (Chart 4) showed a potent dual inhibitory activity at 100 μM (% inhibition values range: 54.9%-100.0% toward TopoI and 62.9%-90.9% toward TopoII), (Shrestha et al., 2018).

All derivatives of series **X** were tested for their cytotoxicity against three different cell lines (HCT15, T47D, HeLa) and the most interesting dihydroxylated compounds **71-79** displayed submicromolar antiproliferative activity toward T47D cell line (Bist et al., 2017). Moreover, the mono *meta*- or *para*-phenolic compounds exhibited a significant anticancer activity, with a better antiproliferative effect respect to the positive control (etoposide) against HCT-15 and T47D cell lines. On the other hand, *ortho*-phenolic compounds were weakly cytotoxic (IC_{50} > 9.37 μM) with the sole exception of compound **81** (Chart 4); this aspect could be ascribed to the hindrance of compound to interact with Topo enzyme inside the cell line. (Shrestha et al., 2018).

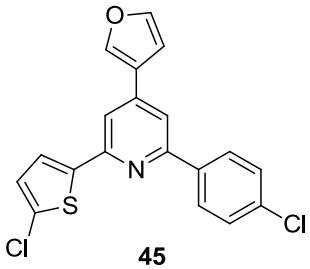


III



44

R = H, 5-chloro-, 3-methyl; R_1 = H, 5-chloro-
R_2 = 2-furyl, 2-(5-methylfuryl), 2-(5-chlorofuryl), 2-pyridyl, 3-pyridyl, 2-tolyl, 3-tolyl, 4-tolyl, 2-chlorophenyl, 3-chlorophenyl, 4-chlorophenyl,



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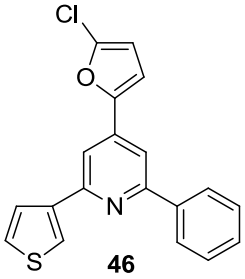

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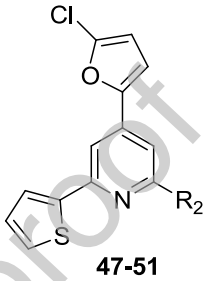
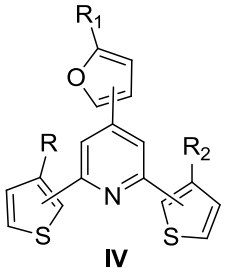
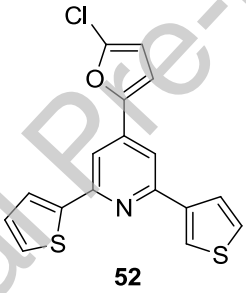


47-51

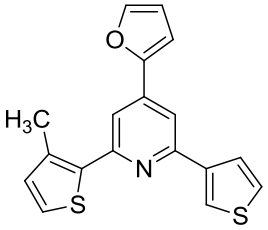
47 R_2 = 4-tolyl
48 R_2 = 2-chlorophenyl
49 R_2 = **3**-chlorophenyl
50 R_2 = 4-chlorophenyl
51 R_2 = **3**-pyridyl



IV

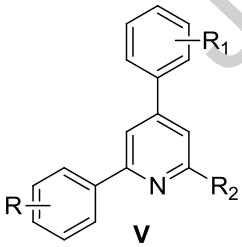


52



53

R = H, 3-methyl, 2,5-dimethyl
R_1 = H, 5-chloro-, 5-methyl
R_2 = H, 3-methyl,

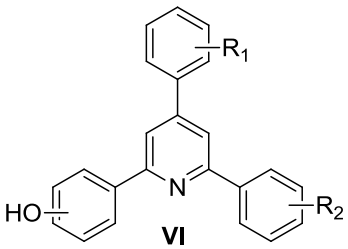


V

54 R = 3-OH, R_1 = H, R_2 = 2-thienyl
55 R = 3-OH, R_1 = H, R_2 = 3-thienyl
56 R = 3-OH, R_1 = H, R_2 = *m*-hydroxyphenyl
57 R = H, R_1 = 3-OH, R_2 = 2-furyl
58 R = H, R_1 = 3-OH, R_2 = -pyridyl

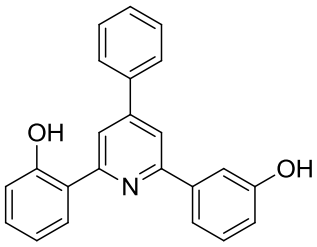
R, R_1 = H, 2-OH, 3-OH, 4-OH

R_2 = phenyl, *o*-hydroxyphenyl, *m*-hydroxyphenyl, *p*-hydroxyphenyl, 2-furyl, 2-thienyl, 3-thienyl, 2-pyridyl,3-pyridyl, 4-pyridyl

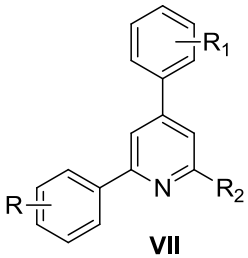


VI

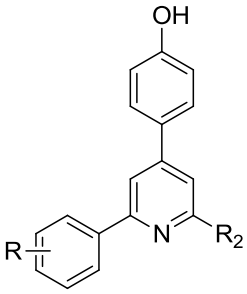
R_1, R_2 = H, 2-OH, 3-OH, 4-OH



59



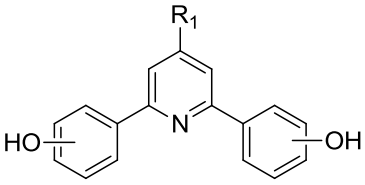
VII



60-65

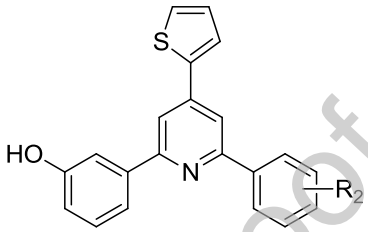
60 R = 3-Cl, R_2 = 2-thienyl
61 R = 3-Cl, R_2 = 2-pyridyl
62 R = 3-Cl, R_2 = 3-pyridyl
63 R = 4-Cl, R_2 = phenyl
64 R = 4-Cl, R_2 = 2-thienyl
65 R = 4-Cl, R_2 = 3-thienyl

R = 3-Cl, 4-Cl ; R_1 = 3-OH, 4-OH
R_2 = phenyl, 2-furyl, 2-thienyl, 3-thienyl, 2-pyridyl,3-pyridyl, 4-pyridyl

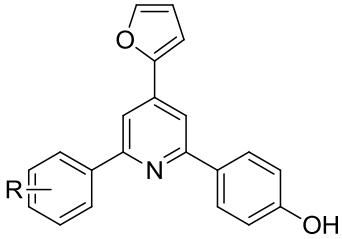


VIII

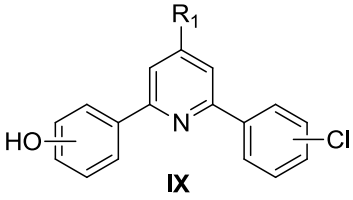
R_1 = 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl,3-pyridyl,



66 R_2 = 3-OH
67 R_2 = 4-OH

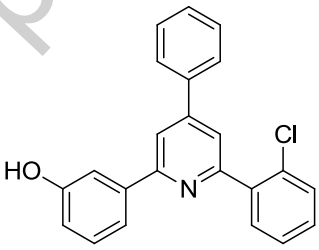


68 R = 3-OH
69 R = 4-OH

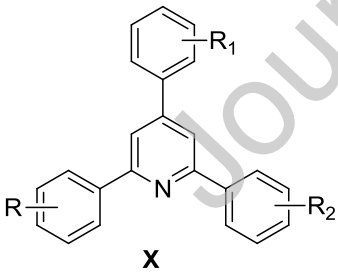


IX

R_1 = phenyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl,



70



X

R = OH; R_1 = Cl; R_2 = OH, Cl

71 R = 3-OH, R_1 = 2-Cl, R_2 = 3-OH
72 R = 3-OH, R_1 = 3-Cl, R_2 = 3-OH
73 R = 3-OH, R_1 = 4-Cl, R_2 = 3-OH
74 R = 3-OH, R_1 = 2-Cl, R_2 = 4-OH
75 R = 3-OH, R_1 = 3-Cl, R_2 = 4-OH
76 R = 3-OH, R_1 = 4-Cl, R_2 = 4-OH
77 R = 4-OH, R_1 = 2-Cl, R_2 = 4-OH
78 R = 4-OH, R_1 = 3-Cl, R_2 = 4-OH
79 R = 4-OH, R_1 = 4-Cl, R_2 = 4-OH

80 R = 2-OH, R_1 = 2-Cl, R_2 = 2-Cl
81 R = 2-OH, R_1 = 3-Cl, R_2 = 2-Cl
82 R = 2-OH, R_1 = 4-Cl, R_2 = 2-Cl
83 R = 2-OH, R_1 = 2-Cl, R_2 = 3-Cl
84 R = 2-OH, R_1 = 3-Cl, R_2 = 3-Cl
85 R = 2-OH, R_1 = 4-Cl, R_2 = 3-Cl
86 R = 2-OH, R_1 = 2-Cl, R_2 = 4-Cl
87 R = 2-OH, R_1 = 3-Cl, R_2 = 4-Cl
88 R = 2-OH, R_1 = 4-Cl, R_2 = 4-Cl

Chart 4. Structures of arylpyridine derivatives of series **III-X** as Dual TopoI and II inhibitors.

2.4. Costrained arylpyridine derivatives

It is widely reported that rigid structures having planar configurations and low conformational entropies facilitate compounds to fit more effectively into the active site of the Topo enzyme (Xiao and Cushman, 2005).

2.4.1. 2,4-diaryl chromenopyridines

2,4-diArylchromenopyridines could be considered a constrained rigid structure derived from terpyridine skeleton. The stiffening of the molecule makes the scaffold more planar, suitable for intercalating DNA in the Topo-DNA ternary complex (Xiao and Cushman, 2005).

In 2011 a library of 2,4-diaryl-chromenopyridine derivatives **XI** were synthesized (Chart 5) (Thapa et al., 2011) and evaluated for their TopoI/TopoII inhibitory activity, by examination of the conversion of supercoiled plasmid DNA to relaxed DNA by TopoI and TopoII in their presence. The obtained data showed that **89 (**R_1 = 2-thienyl, R_2 = 2-pyridyl) was the most interesting compound with both TopoI (48.2%) and TopoII (19.4%) inhibitory activity at 100 μM concentration. SAR studies on these 2,4-diaryl chromenopyridines confirmed the hypothesis that a 2-furyl or 2-thienyl group at 2- or 4-position of the pyridine ring are important for TopoI/TopoII inhibitory activity (Zhao et al., 2004; Basnet et al., 2007, 2010; Thapa et al., 2010a, 2010b; Karki et al., 2010).

Compounds possessing a certain TopoI or TopoII inhibitory activity were further evaluated for their cytotoxicity *in vitro* on 5 different human cancer cell lines, (MDA-MB231, HeLa, DU145, HCT15, HL60). IC_{50} values of the compounds range from 0.1 to 25 μM, indicating a general moderate cytotoxic activity for all the tested compounds.

In a subsequent study, in 2012, 2,4-diaryl-chromeno pyridine derivatives, with a 3-furyl, 3-pyridyl and 3-thienyl group at the 2-position of the scaffold, were investigated (Thapa and Lee, 2012a). As for the previous analogues (Thapa et al., 2011), the new derivatives were evaluated for their TopoI and TopoII inhibitory activities. Outcomes revealed that compounds **90-94** (Chart 5) showed strong TopoI (61.2-96.0%) and TopoII (62.3-100%) inhibitory activity at 100 μM concentration, and

moderate (3.6-15.5%) TopoII inhibitory activity at 20 μM concentration. Based on these results compounds **90-94** were reported as dual TopoI/TopoII inhibitors (Thapa and Lee, 2012a). Unfortunately, when **90-94** were evaluated for their cytotoxicity *in vitro* against four different human cancer cell lines (HEK293, DU145, HCT15, T47D), no direct correlation between cytotoxicity and TopoI/TopoII inhibitory activity was found.

2.4.2. Indenopyridines

Several studies reported that compounds with the indenopyridine system show various biological effects including anticancer activity (Utsugi et al., 1997; Manpadi et al., 2007; Ghorab and Al-Said, 2012).

In 2015 Kadayat T.M. *et al*. synthetized 2,4-diaryl-5*H*-indeno[1,2-*b*]pyridine derivatives X**II** (Chart 5) characterized by different aryl substituents (phenyl, 2- or 3- pyridyl, 2- or 3- thienyl, and 2- or 3-furyl) at 2- (R_2) and 4-positions (R_1) of the scaffold (Kadayat et al., 2015a). All the new compounds were evaluated for their ability to inhibit both TopoI and TopoII, and for their cytotoxicity toward different human cancer cell lines.

Most of the obtained compounds did not show a considerable inhibitory activity toward TopoI, with two exceptions: the compound bearing a 2-thienyl group at 2-position and a 2-furyl moiety at 4-position (R_2 = 2-thienyl, R_1 = 2-furyl), and the compound substituted with 3-thienyl groups in both 2- and 4- positions (R_1 = R_2 = 3-thienyl), (TopoI inhibitory activity, 76.3% and 90.9% at 100 μM, respectively).

As regard TopoII inhibition, the most active molecule is compound **95**, (R_2 = 2-furyl, R_1 = 2-pyridyl), (Chart 5) which displayed TopoII inhibitory activity of 100% at 100 μM, and also a moderate inhibitory activity against TopoI (40.7% at 100 μM). SAR studies evidenced the importance of the 2-furyl, 2-thienyl or 3-thienyl moieties at the C-2 or C-4-positions for both TopoI

and TopoII inhibitory activity; whereas, in general, compounds bearing a pyridyl or a phenyl group did not show any activity against both the enzymes, (Kadayat et al., 2015a).

Data concerning the cytotoxicity evaluation indicated that there is no correlation among cytotoxicity and Topos inhibitory activity; however, molecules with a considerable cytotoxicity showed moderate to strong TopoI and/or TopoII inhibitory activities (Kadayat et al., 2015a).

The same year, Kadayat *et al*., synthetized two series of compounds (**XIII**-X**IV**, Chart 5) bearing thienyl, furyl or pyridyl group linked to the 4-position of the pyridine (R_1), and an unsubstituted phenyl at 2-position (**XIII**), or a phenyl ring substituted with a hydroxyl group at different positions (*para*, *meta* or *ortho*) (**XIV**) (Kadayat et al., 2015b). In particular compounds of series **XIII** derived from a previous observation that the introduction of a hydroxyl group on the molecules could increase the Topo inhibitory activity (Bandele and Osheroff, 2007, 2008).

The results from biological evaluation indicated that none compound of series **XIII** showed dual TopoI/TopoII inhibitory activity, with the exception of a sole compound (R_1 = phenyl, R_2 = pyridyl) showing a % inhibition value of 73.0 for TopoI and 44.5 for TopoII at100 μM concentration.

Within the series **XIV**, compounds with a dual inhibitory activity toward the enzymes are characterized by a *meta*-hydroxyphenyl in C-2 with a furyl or a thienyl moiety at 4-position (R_1 = 2-furyl, 3-furyl, 2-thienyl or 3-thienyl) and by a *para*-hydroxyphenyl at C-2 with a 3-furyl moiety at C-4 (R_1 = 3-furyl), (% inhibition values range 55.6-76.9 for TopoI and 56.2-58.2 for TopoII at 100 μM concentration).

All compounds were also evaluated *in vitro* for their cytotoxicity toward two different cell lines: HeLa and DU145. The outcomes indicate that compounds able to inhibit both TopoI and TopoII showed also a relevant cytotoxicity in the cancer cell lines tested (Kadayat et al., 2015b). SAR studies highlight that non-hydroxylated compounds presented weaker activity, whereas their hydroxylated counterparts showed moderate to high activity, underlining that the insertion of a hydroxyl group on the phenyl ring could increase the dual TopoI/TopoII inhibitory activity.

Furthermore, within the hydroxylated compounds, derivatives featuring a thienyl or furyl substituent at the 4-position (R_1) were the best performing ones.

Considering these results, to expand the chemical diversity in the field of potent dual inhibitors, Park S. and co-workers decided to reverse the R_1 and R_2 substituents on the indenopyridine system of series **XIII** and **XIV**, obtaining compounds of series **XV**-**XVI** (Chart 5) (Park et al., 2017a). Only products with R_2 = 2-furyl and R_1 = *ortho*-hydroxyphenyl or R_1 = *para*-hydroxyphenyl showed to be dual TopoI/TopoII inhibitors, with the latter giving the best results (69.7% inhibition for TopoI and 95.1% inhibition for TopoII at 100μM). SAR studies pointed out that non-hydroxylated compounds are weaker inhibitors with respect to the hydroxylated ones; moreover, compounds with 2- or 3-furyl moiety and 2- or 3-thienyl moiety at the 2 position (R_2) and the 4-phenolic ring (R_1) are potent TopoII inhibitors, whereas compounds bearing a pyridyl in R_2 are almost inactive.

Biological evaluation highlighted **96** (R_2 = 2-furyl, R_1 = *para*-hydroxyphenyl, Chart 5) as the strongest TopoIIα/TopoI catalytic inhibitor of the series. Molecular docking studies were therefore performed with **96** to evaluate its mechanism of action with both the enzymes. These studies underlined the significant role of the furyl oxygen that establishes a hydrogen bond with Arg364 (the same residue interacting with camptothecin) of TopoI. In addition, **96** binds TopoII DNA cleavage site through the interaction between the hydroxyl moiety and Asp479 residue (this interaction is also observed with etoposide). Taken together these observations lead to the conclusion that the binding site of **96** partly overlaps with those of campthotecin and etoposide (Park et al., 2017a).

All these studies have highlighted the importance of some structural requirements that indenopyridines must possess to obtain potent dual TopoI/TopoII inhibitors.

2.4.4. Benzofuro[3,2-b]pyridines

Since various benzofuro[3,2-b]pyridines have been patented as Topo inhibitors (Deady et al., 1998) in 2013 a series of 2,4-diaryl-benzofuro[3,2-b]pyridine derivatives **XVIII** was synthesized as conformationally constrained analogues of 2,4,6-trisbustituted pyridines (Thapa et al., 2013).

These compounds proved to be dual inhibitors, from moderate (34.5-45.9 %) to good (65.5%) *versus* TopoI and significant *versus* TopoII in the range of 81.1-90.4% at 100 μM. In particular, compound **97** (R_1 = R_2 = 2-furyl) was the most interesting dual TopoI/Topo II inhibitor. The best performing compounds were also tested for cytotoxicity against five different human cancer cell lines (MCF-7, DU145, HeLa, K562, and HCT15) and most of them showed moderate cytotoxicity, (IC_{50} range: 4.35-78.01 μM) which did not directly correlate with Topos inhibitory activity.

SAR revealed that the presence of 2-furyl, 2-thienyl or 2-phenyl moiety in combination with the interesting 4-(furan-2-yl)benzofuro[3,2-b]pyridine skeleton is more essential respect to pyridyl moiety for the Topo inhibitory activity (Thapa et al., 2013).

In 2015 two series of benzofuropyridine-containing or chromenopyridine-based compounds **XIX** and **XX** (Chart 5) (Kwon *et al*., 2015), bearing thienyl, furyl, phenyl, chlorofuryl, and hydroxyphenyl moieties were investigated (Chart 5). **XIX** and **XX** were designed in accordance with previous studies reporting the importance of the chlorine and the hydroxyl group in one of the aryl ring at 2- or 4- position of the pyridine nucleus (Thapa et al., 2010a, Karki et al., 2010, 2012). The compounds' effects on human DNA TopoI and TopoIIα were evaluated with the relaxation assay and the obtained results showed that most of the benzofuropyridines **XIX** inhibited both TopoI and IIα with a sole exception (R_1 = chlorofuryl, R_2 = phenyl), whereas the majority of chromenopyridines **XX** inhibited only TopoIIα.

In general, compounds decorated with *ortho*, *meta*, *para* hydroxyphenyl at R_2 and each of phenyl, 2-furyl, or 3-furyl at R_1 of both series **XIX** and **XX** lost TopoI inhibitory activity, with the exception of few compounds, but retained potent TopoIIα inhibitory activity. Compound **98** (series **XIX,** R_1 = phenyl, R_2 = *meta*-hydroxyphenyl) showed the strongest TopoI/TopoIIα dual inhibitory activity of 63.3% and 81.7% at 100 μM, respectively, while **99** (series **XX**, R_1 = phenyl, R_2 = *meta*-

hydroxyphenyl) had higher inhibitory activity *versus* TopoIIα (84.0% at 100 μM) than *versus* TopoI (6.3% at 100 μM). Moreover, the IC_{50} values for compound **98** were 65.2 ± 5.7 μM and 13.4 ± 1.2 μM against recombinant TopoI and TopoIIα, respectively, while that of compound **99** against TopoIIα was 60.2 ± 9.5 μM (Kwon et al., 2015).

The ability of all compounds to inhibit cell proliferation in three human cancer cells (HCT15, T47D, DU145) and HEK293 was tested, most compounds of series **XIX** and **XX** displayed potent antiproliferative activity with IC_{50} micromolar values, relatively lower respect to those in HEK293 cell line. In particular, the most interesting compounds **98** and **99** have recorded IC_{50} values of 0.83 ± 0.04 μM and 0.54 ± 0.01 μM for T47D cells, 0.15 ± 0.01 μM and 0.005 ± 0.00004 μM for HCT15 cells, and 4.93 ± 0.07 μM and 5.69 ± 0.21 μM for HEK293 cells, respectively.

The modes of action of **98** and **99** were analized with various assays, and the results showed that **98** was a non intercalative TopoI/TopoII dual catalytic inhibitor, while **99** was a non intercalative TopoIIα specific catalytic inhibitor (Kwon et al., 2015). Furthermore, compounds **98** and **99** induced apoptosis in addition to G1 arrest in T47D cells and significantly inhibited tumor growth in HCT15 subcutaneously implanted xenograft mice. Finally, the derivatization of the hydroxyl group of these two compounds with a methyl substituent enhanced Topo inhibitory activity, metabolic stability and improved pharmacokinetic parameters in rat plasma, thus increasing their bioavailability (Kwon et al., 2015).

Based on these results, in 2017 new benzofuro[3,2-b]pyridines of series **XX** bearing *ortho*, *meta*, or *para*-chlorophenyl and *ortho*, *meta*, or *para*-phenol moiety at 2- and 4-positions of the pyridine were synthesized (Park et al., 2017b). Their biological assays indicated a significant dual TopoI/Topo II inhibitory activity with % inhibition range of 62.3-95.6 and 82-100 at 100 μM concentration, respectively, for compounds featuring 2-chlorophenyl (R_2 = *ortho*-, *meta*-, or *para*-chlorophenyl) and 4-hydroxyphenyl (R_1 = *ortho*-, *meta*-, or *para* –hydroxyphenyl) groups. In particular, **100** (R_1 = *para*-hydroxyphenyl, R_2 = *para*-chlorophenyl) proved to be the most

interesting dual TopoI (95.6% at 100 μM, 47.9% at 20 μM) and TopoII (100% at 100 μM and 59.8% at 20 μM) inhibitor.

Most of the compounds of this series exhibited significant antiproliferative activity on the three cancer cell lines HCT15, T47D and HeLa, with HCT15, with good sensitivity values. In particular, compounds bearing phenol at 4-position of the pyridine (R_1 = *ortho*-, *meta*-, or *para*-hydroxyphenyl) showed stronger antiproliferative activity than those bearing phenol at 2-position. In general, a direct correlation between dual TopoI/TopoII inhibitory activity and antiproliferative effect was observed. Moreover, the benzofuro[3,2-b]pyridines showed a higher dual TopoI/II inhibitory activity as well as antiproliferative effect with respect to the corresponding analougsly substituted indenopyridines previously described (Kadayat et al., 2015a, 2015b).

Finally, compound **100** (R_1 = *para*-hydroxyphenyl, R_2 = *para*-chlorophenyl) was selected to study its mode of action, showing to be a potent non-intercalative catalytic TopoI/TopoII dual inhibitor, and it was also able to induce apoptosis in HCT15 cell (Park et al., 2017b).

*2.4.5. Phenanthroline*s

In 2012 two series of 2,4-diaryl-phenanthrolines **XXI** (Chart 6) were synthesized and studied as dual TopoI/TopoII inhibitors (Thapa et al., 2012b; Thapa and Lee., 2012b). Most of the described compounds were TopoI inhibitors (% inhibition range 60.9%-66.5% at 100 μM concentrations) while were devoid of any TopoII inhibitory activity. However, studies on the phenanthroline system led to the synthesis of an interesting antitumor phenanthrolinic compound, P8-D6 (**101**) (Chart 6) (Steinhauer et al., 2016). One year later compound **101**, evaluated on 60 human tumor cell lines (NCI-60 DTP human tumor cell line screening), recorded a broad-spectrum effect at nM concentrations (Meier et al., 2017) and also anti-leukemic effects. This activity has been ascribed to **101**'s ability to induce apoptosis caused by a dual TopoI/TopoII inhibition. More recently, the mechanism of action of **101** was fully elucidated and the compound proved to be a dual TopoI/TopoII poison at a concentration ≥ 1 μM, not only under cell-free conditions, but also in

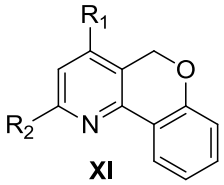
human tumor cells (Aichinger et al., 2020). P8-D6 (**101**) physicochemical drug-like profile encourages its development as promising candidate in antineoplastic chemotherapy in alternative to existing dual TopoI/TopoII inhibitors in clinical use.

2.4.6. Benzochromenonaphthyridines

Up to here it has been widely described that fused angular heterocyclic compounds targeting TopoI and Topo II can adapt properly in the active site of the enzymes and/or better intercalate in the enzyme-DNA complex, due to their conformational rigidity.

Based on this, in 2018 angular pentacyclic fused benzochromeno-naphthyridines **XXII** and benzochromeno-naphthyridin-5-ium chlorides **XXIII** were synthesized (Chart 6) (Arepalli et al., 2018).

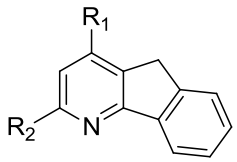
All the compounds of the two series were tested for their growth inhibitory activity against six different human cancer cell lines: NCIH23, HCT15, NUCG-3, ACHN, PC-3, and MDA-MB-231. Some compounds, showing interesting cytotoxicity profiles, were also evaluated for their inhibitory activity against human TopoI and TopoIIα. Compound **102** (Chart 6) was a dual TopoI/TopoIIα inhibitor with percentage inhibition values of 57.2 % and 69.8% for TopoI and TopoII at 100 μM, respectively; interestingly, at 20 μM concentrations, compound **102** exhibited 1.25 times more potent TopoIIα inhibitory activity than etoposide. Docking studies of **102** with TopoI revealed that it intercalated the single-stranded cleavage site engaging van der Waals interactions with Arg364, Pro431, and Asn722 residues. Docking studies of compound **102** with TopoIIα revealed that it binds to the cleavage site of TopoIIα similarly to etoposide. Moreover, compound **102** had π-π interactions with the stacked DNA bases of both TopoI and II cleavage sites.



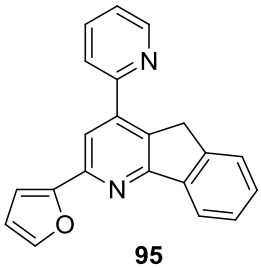
XI

89 R_1 = 2-thienyl; R_2 = 2-pyridyl
90 R_1 = 3-furyl; R_2 = 2- furyl
91 R_1 = 3- furyl; R_2 = 3- thienyl
92 R_1 = 3-thienyl; R_2 = 3-thienyl
93 R_1 = 3-thienyl; R_2 = 2- thienyl
94 R_1 = phenyl; R_2 = 3-thienyl

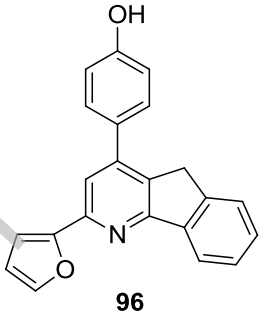
R_1 = phenyl, 2-furyl, 2-pyridyl, 2-thienyl, 3-furyl, 3-pyridyl, 3-thienyl
R_2 = phenyl, 2-furyl, 2-pyridyl, 2-thienyl (Thapa et al., 2011)
R_2 = phenyl, 2-furyl, 2-pyridyl, 2-thienyl, 3-furyl, 3-pyridyl, 3-thienyl (Thapa and Lee, 2012)



XII-XVI

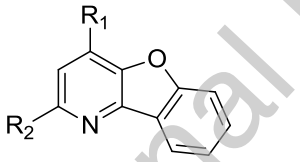


95

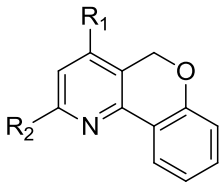


96

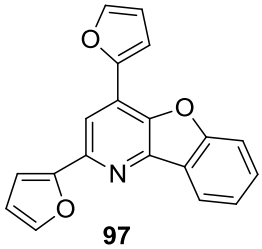
XII. R_1, R_2 = phenyl, 2-furyl, 3-furyl, 2-pyridyl, 3-pyridyl, 2-thienyl, 3-thienyl
XIII. R_1= 2-furyl, 3-furyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-thienyl, 3-thienyl; R_2 = phenyl
XIV. R_1 = 2-furyl, 3-furyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-thienyl, 3-thienyl; R_2 = *o*-, *m*-, *p*-hydroxyphenyl;
XV. R_1= phenyl; R_2 = 2-furyl, 3-furyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-thienyl, 3-thienyl
XVI. R_1 = *o*-, *m*-, *p*-hydroxyphenyl; R_2 = 2-furyl, 3-furyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-thienyl, 3-thienyl



XVII, XVIII, XX

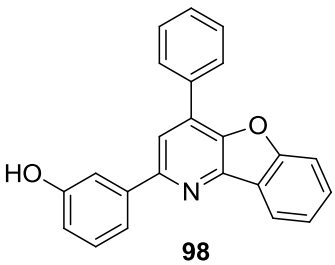


XIX



97

XVII. R_1, R_2 = phenyl, 2-furyl, 3-furyl, 2-pyridyl, 2-thienyl, 3-thienyl
XVIII, XX. R_1 = 2-furyl, 2-thienyl, 3-thienyl; R_2 = phenyl, *o*-, *m*-, *p*-hydroxyphenyl, 2-furyl, 3-furyl, 2-(4-chloro)furyl
XIX. R_1, R_2 = *o*-, *m*-, *p*-chlorophenyl, *o*-, *m*-, *p*-hydroxyphenyl



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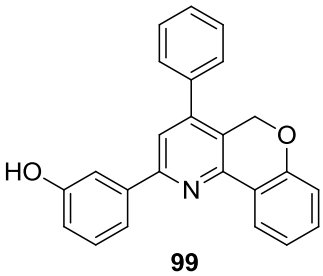

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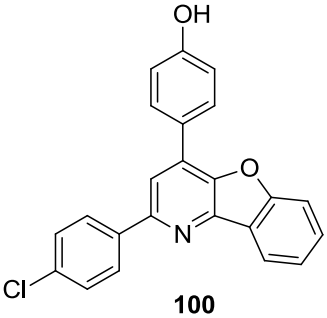
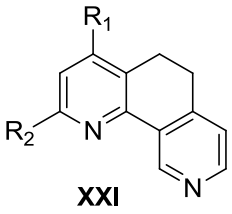


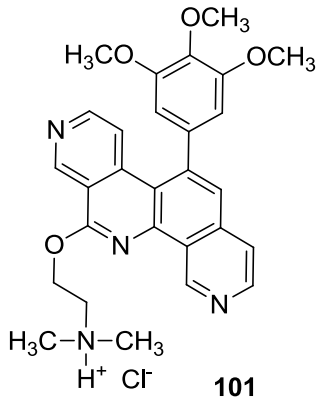
100

Chart 5. Structures of 2,4-diaryl chromenopyridines **XI** and X**IX**, indenopyridines X**II-XVI**, benzofuropyridines **XVII**, **XVIII**, **XX** as dual TopoI/TopoII inhibitors.



XXI

R_1 = 2-pyridyl, 3-pyridyl, 4-pyridyl (Thapa et al., 2012)
R_1 = 2-furyl, 3-furyl, 2-thienyl, 3-thienyl (Thapa and Lee, 2012)
R_2 = 2-pyridyl, 3-pyridyl, 4-pyridyl



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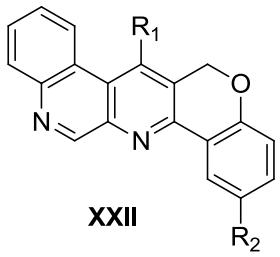
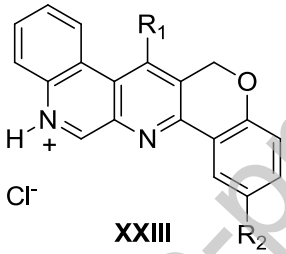
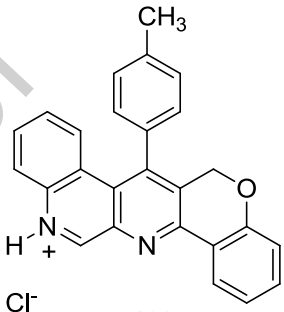


XXII



XXIII



102

R_1 = H, CH_3, *p*-tolyl
R_2 = H, CH_3, Cl, F, Br, $N(CH_2CH_3)_2$

Chart 6. Structures of phenanthroline **XXI** and benzochromenonaphthyridines **XXII-XXIII,** as dual TopoI/TopoII inhibitors.

2.5. Acridines and benzophenazines

2.5.1. Acridine derivatives

2.5.1.1. Acriflavine

Acriflavine **103** (Chart 7), a mixture of two very closely related acridine molecules, was discovered almost 100 years ago and has been used as antimicrobial agent (Wainwright, 2001). It has also been found to be active in sensitive tumor xenograft models in rodents when administered locally or systemically. Its mechanism was ascribed to its ability to intercalate nucleic acids and inhibit TopoII (Kim et al., 1997; Lee et al., 2007; Lee et al., 2009), like almost all acridine derivatives. Moreover, acriflavine is an interesting candidate for cancer drug development thanks to its low cross-resistance and its druggable features according to Lipinski's rule of five (Lipinski et al., 2001) In 2011 acriflavine was identified as a potentially colorectal cancer (CRC) active molecule (Hassan et al, 2011) and it proved to be a dual TopoI/TopoII inhibitor in line with other acridine derivatives, such as DACA (Finlay et al., Baguley et al., 1995).

2.5.1.2. Tacrine

Tacrine, a tetrahydroacridine, is an effective acetylcholinesterase inhibitor useful in neurological disorders such as Alzheimer's disease (Kozurkova et al., 2011; Tumiatti et al., 2010), but it is also able to inhibit topoisomerases and DNA synthesis (Mansouri 2003). In recent years there has been a growing interest in bisintercalators in which the two planar structures connected by an appropriate linker promote DNA binding and increase cancer cell cytotoxicity, in comparison with monointercalators (Wang et al., 2007).

Based on these considerations, in 2015 mono- and bistacrine derivatives **104-107** (Chart 7) were synthesized and biologically tested, showing to be effective DNA-groove binder agents (Janockova, et al., 2015a). Monotacrine derivative **104** and bistacrine derivatives **105-107** were proved to be dual TopoI/TopoII catalytic inhibitors even though they had shown to inhibit TopoI with an order of magnitude higher than TopoII. Moreover, the biological activities of **104-107** were studied using

MTT assay and flow cytometric methods, but all the compounds can only induce apoptosis and mitochondrial membrane depolarization in the HL60 cell line at very high concentrations (Janockova, et al., 2015a).

In an effort to gain more insight into the effects of substitution pattern of the acridine ring, new derivatives **108-110** (Chart 7) were synthesized and they were validated as dual TopoI/TopoII catalytic inhibitors. Moreover, compounds **108**‑**110** effectively inhibited HL-60 cells growth at the relatively low 5 μM concentration (Janockova, et al., 2015b).

2.5.2 Benzophenazine derivatives

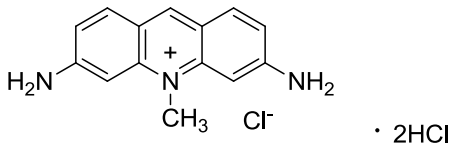
Previous studies on benzophenazines led to the development of a potent antitumor compound, XR 11576, which proved to be a dual TopoI/TopoII poison (Mistry et al., 2002). In 2013 a series of benzo[a]phenazine derivatives **XXIV** (Chart 7) was designed (Zhuo et al., 2013) to obtain insight on the effect of introducing an aminoalkyl side chain on ring B of the scaffold. MTT assays on HeLa, A549, MCF-7, and HL-60 cell lines evidenced a good antiproliferative activity with IC_{50} values ranging from 1.0 to 10 μM for the most compounds of the series. Topo-mediated DNA relaxation assays allowed to correlate the biological activity with dual TopoI/TopoII inhibition; furthermore, mechanism studies carried out on the most interesting compounds, **111** and **112**, indicated that they represent a rare class of dual Topo inhibitors, TopoI poisons and TopoII catalytic inhibitors, by inhibiting the ATP binding domain of hTopoII (Zhuo et al., 2013).

SAR studies revealed that the introduction of the aminoalkyl side chain at the C-5 position of the benzophenazine enhance both Topos inhibition and cytotoxicity. Derivatives with different terminal amino groups did not display discrimination in activities, except for those with the morpholine moiety, which lost in inhibition potency. Moreover, the best performing dual Topo inhibitors showed to be those with unsubstituted D ring ($R_1 = R_2 = H$), while the introduction at R_1 or R_2 of a methoxy or a nitro group shifts the activity from dual Topo inhibitors to potent and selective TopoII

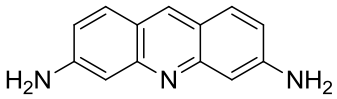
inhibitors. Finally, flow cytometric studies and caspases activation assay showed that this class of compounds induce apoptosis in HL-60 cells (Zhuo et al., 2013).

A new series of benzo[a]phenazine derivatives **XXV** (Chart 7) in which the aminoalkyl side chains were shifted to the nitrogen group on ring C, and a chlorine or a methoxy group were also inserted on rings A and D, was synthesized (Yao, et al., 2015). Almost all compounds turned out to be dual TopoI/TopoII inhibitors at 50 μM concentration, and some of them (e.g. **113** and **114**, Chart 7) exhibited significant dual inhibitory activity also at 25 μM concentration. Moreover, they showed to be TopoI poisons and TopoII catalytic inhibitors in line with the benzophenazines of series **XXI** (Yao, et al., 2015). Derivatives of series **XXV**, evaluated for their cytotoxic activities against four cancer cell lines (HL-60, K-562, HeLa and A549), showed excellent antiproliferative activity which correlate with their inhibition profile; in particular, all the compounds result highly potent against HL-60 cells showing submicromolar IC_{50} values.

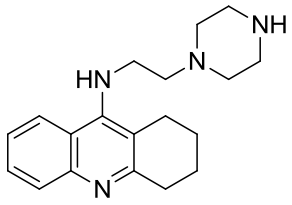
SAR studies indicated that compounds with the dimethylamino ($R = N(CH_3)_2$) moiety showed the most potent dual TopoI/TopoII inhibitory activity, independently from the length of the spacing chain (n = 1, 2, 3). Moreover, the introduction of different groups on ring A or on ring D of the scaffold did produce none gain in the TopoI and TopoII activity, as only compound **114** ($R_4 = OCH_3$) was validated as a good dual TopoI/TopoII inhibitor at 25 μM concentration; in general, the unsubstituted derivatives result more potent respect to their analogues featuring substitution on A (R_3 or $R_4 = OCH_3$) or D (R_1 or R_1 = Cl, OCH_3) ring (Yao, et al., 2015).



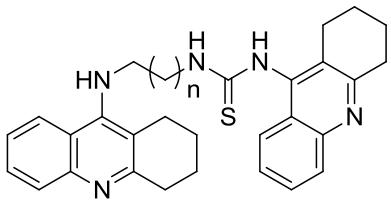
· 2HCl



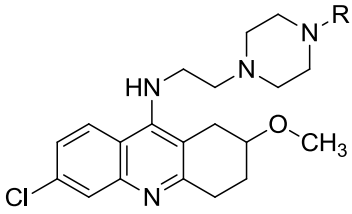
103



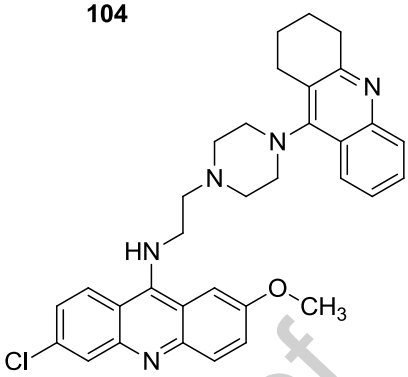
104



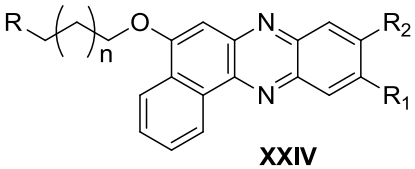
105 n = 1
106 n = 3
107 n = 5



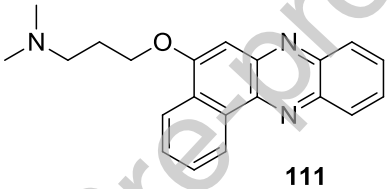
108 R = H
109 R = CH_2Ph



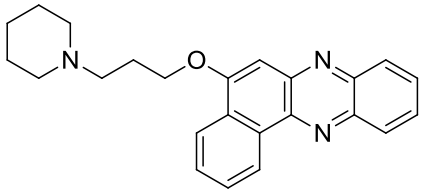
110



XXIV

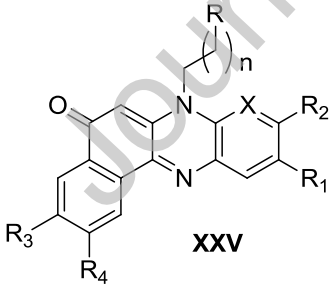


111

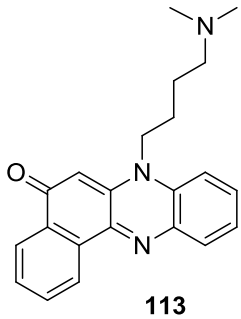


112

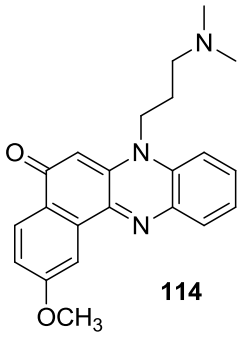
n = 1, 2
R = $N(CH_3)_2$, $N(CH_2CH_3)_2$, $N(CH_2CH_2)_2CH_2$, $N(CH_2CH_2)_2NCH_3$, $N(CH_2CH_2)_2$, $N(CH_2CH_2)_2O$
R_1, R_2 = H, F, Cl, OCH_3, NO_2



XXV



113



114

n = 1, 2, 3
X = N. CH
R = CH_3, $N(CH_3)_2$, $N(CH_2CH_3)_2$, $N(CH_2CH_2)_2CH_2$, $N(CH_2CH_2)_2NCH_3$, $N(CH_2CH_2)_2$, $N(CH_2CH_2)_2O$
R_1 = H, Cl, OCH_3
R_2, R_3, R_4 = H, OCH_3

Chart 7. Structures of acridine (**103-110**) and benzophenazine (**XXIV-XXV**) derivatives as dual TopoI/TopoII inhibitors.

2.6. Miscellaneus

2.6.1. Indenoquinoline derivatives

Starting from the lead indenoquinoline **115** (Tseng et al., 2010) (Chart 8), reported as a potent inhibitor of both TopoI and TopoII (IC_{50} values 3.76 μM and 8.26 μM, respectively), in 2013 C.H. Tseng *et al.* designed and synthesized the series of indenoquinolines **XXVI** (Chart 8) with the aim to develop novel more potent dual TopoI/TopoII inhibitors (Tseng et al., 2013a).

All the newly compounds are characterized by alkylaminoalkyl side chain in 9 and/or in 11 position of the indenoquinoline scaffold.

In particular, all compounds bearing the aminoalkyl side chain at 9 position and a carbonyl at 11 position (X = O, R_1 = H) were inactive, while compounds characterized by a further introduction of an alkylaminoalkyl side chain in the 11 position (X = NOR, R_1 = H), proved to be good inhibitors of both enzymes, confirming the importance of this group on C-11, with two compounds showing better TopoI (IC_{50} values: 2.17 μM and 2.62 μM) and Topo II (IC_{50} values: 3.98 μM and 4.95 μM) inhibitory activities with respect to the lead **115**.

At the end, compounds bearing also a C-6 hydroxyl group (X = NOR, R_1 = OH) gave lower activity as compared to the non-hydroxylated analogues, except compound **116** (Chart 8) resulting a potent dual TopoI/TopoII inhibitor (IC_{50} values: 3.14 μM and 4.80 μM, respectively) more active than the lead compound **115** (Tseng et al., 2013a).

All compounds of series **XXVI** were tested *in vitro* toward MTT experiment on seven different cell lines (HeLa, AGS, A549, PC-3, SKHep, BT483, MRC-5).

The most active compound results compound **117** (Chart 8) showing a high antiproliferative effect, in particular against HeLa and A549 cell lines (IC_{50}: 0.39±0.12 μM and 6.99±3.04 μM, respectively) which correlates with its dual Topos inhibitory activity (IC_{50} values: 2.63 μM and 4.95 μM, for TopoI and II, respectively). Moreover, the pharmacokinetic study of compound **117** revealed its preferable bioavailability, compared to that of **115**. Furthermore, *in vivo* tests have shown that the oral administration of **117** decreases the volume of the tumor in breast cancer.

2.6.2. Isoquinoline derivatives

A series of 3-heteroarylisoquinolinamines (series **XXVII**, Chart 8) and their hydrochlorides have been synthesized assuming that the heteroaromatic rings maintain the planar arrangement necessary to express the inhibitory activities of Topos, (My Van, 2014).

The cytotoxic effects of all compounds of series **XXVII** was evaluated against non-cancerous (MCF-10A) and tumor (T47D, DU145, HCT-15) cells and most of the derivatives were more cytotoxic against HCT-15 cells than camptothecin, etoposide and doxorubicin.

In general, the Topos inhibitory activities were widely dependent upon the position of the methyl group and the type of amine (R) inserted on the isoquinoline scaffold, in addition HCl salts showed inhibition values higher than the corresponding free bases. Most of the 3-heteroarylisoquinolinamines showed a potent TopoI inhibition which correlated with the cytotoxicity, indicating that TopoI is the main target for this class of compounds, (My Van, 2014).

Anyway, piperazine derivatives, in particular compounds **118** and **119**, turned out to be dual TopoI and II inhibitors with % inhibition values of 100 and 59,6 for TopoI and 56 and 40.9 for TopoII at 100 μM, respectively.

A molecular docking study of the most interesting compound **118** showed that the aromatic rings intercalated between DNA bases, the isoquinoline N was associated with Arg364 of TopoI by H-bond, while piperazine moiety was associated with Glu522 of TopoII, through H-bonds.

Finally, flow cytometry indicated that compounds of series **XXVII** arrested cell cycle and caused apoptosis (My Van, 2014).

Based on the monoarylisoquinolines' results (My Van, 2014), some 3,4-diarylisoquinolones and 3,4-diarylisoquinolinamines were synthesized (Khadka, 2015).

MTT assay revealed that some of these compounds were cytotoxic at sub-μM concentrations showing selective toxicity towards cancer cells (T47D, DU145, HCT-15) with no effects on normal cell (MCF10A) (Khadka, 2015).

Anyhow, only for compound **120** (Chart 8) it was possible to correlate the cytotoxic activity (0.1 ± 0.001 μM for T47D and 0.06 ± 0.002 μM for HCT-15) with the dual inhibition of TopoI and II (% inhibition values of 37 for TopoI and 59.9 for TopoII at 100 μM concentration).

2.6.3. Pyrroloquinolinone derivatives

A new series of pyrroloquinolinones bearing different alkylamino side chains (**XXVIII**, Chart 8) was synthesized and evaluated as cytotoxic compounds against three different human tumor cell lines (HeLa, HL-60 and A431) (Dalla Via et al., 2014). All the compounds presented interesting antiproliferative activity, in particular against A431 cells and showed the ability to intercalate DNA, but only the most cytotoxic one (compound **121**, Chart 8) turned out to be a dual TopoI and II inhibitor at 100 μM concentration.

Moreover, molecular modeling simulations were performed to study the interactions between compound **121** and Topos. The results evinced that, in the ternary complex with TopoI, the carbonyl oxygen of compound **121** establishes an H-bond with Thr718-OH while the dialkyldiamine side chain interacted with both DNA and the carboxylate of Glu356. In the case of TopoII, instead, compound **121** establishes only H-bonds with the carboxylate ion of Asp577 and the amide oxygen of Glu477 of the enzyme (Dalla Via et al., 2014).

2.6.4. Ellipticine derivatives

In 2013 novel ellipticinium salts **XXIX** (Chart 8), with improved water solubility, were synthesized and evaluated for their capability to intercalate DNA, to inhibit both TopoI and II and to *in vitro* inhibit the growth of 12 cancer cell lines, in comparison with their non alkylated derivatives **122** and **123** (Deane et al., 2013).

In the DNA unwinding assay all the compounds were able to bind DNA via intercalation preventing TopoI from recognizing the site. In particular, compounds with terminal nitrile or amide display much stronger binding affinities for DNA respect to their carboxylic acid analogues, also in

accordance with the cytotoxicity data. Furthermore, the length of the chain does not seem to influence the activity.

From the results of TopoI cleavage assay, it is not to be excluded that these compounds behave as TopoI inhibitors at high concentrations and TopoI poisons at lower concentrations, in analogy with some reported drugs (Fleury et al., 2000; Wassermann et al., 1990). Some selected compounds were also evaluated for their abilty to inhibit TopoII and most of them showed a partially inhibition, while compounds **124** and **125** completely inhibit TopoII at 100 μM concentration.

The antiproliferative activity of all compounds was tested *in vitro* on 12 different cancer cell lines (HT29, SW480, MCF-7, A2780, H460, A431, DU145, BE2-C, SJ-G2, U87, MIA and SMA). The obtained results evidenced that N-alkylation of ellipticine **122** enhanced the growth inhibition, while N-alkylation of 6-methylellipticine **123** reduced the activity of the new compounds with GI_{50} values in the range of 1.3–28 μM. So the most potent growth inhibitory compound was compound **123** (GI_{50} = 0.47–0.9 μM).

Moreover, the cytotoxic profile observed for all compounds is in accordance with their TopoI inhibitory activity, while it is not consistent with TopoII inhibition, actually compounds **124** and **125**, albeit they are the strongest TopoII inhibitors, showed to be less active than compound **123** (average GI_{50} values of 12.0 and 5.1 μM, respectively) on cell line assays (Deane et al., 2013).

2.6.5. Benzimidazole derivatives

In 2013 was reported a symmetric bibenzimidazole derivative, STK295900, (**126**, Chart 9) as dual TopoI and II inhibitor (Kim et al., 2013).

Indeed, several asymmetric and symmetric bibenzimidazole derivatives, such as Hoechst 33258 and Hoechst 33342, were described as TopoI inhibitors by binding to minor groove of DNA (Chen et al., 1993; Jin et al., 2000) Moreover, symmetric bibenzimidazole derivatives have been reported to bind DNA minor groove and exhibit antitumor activity (Mann et al., 2001) but the mechanism of this activity is not reported.

The results from DNA relaxation assay performed on compound **126** suggested that it is a TopoI poison but it also inhibits TopoII catalytic activity (Kim et al., 2013).

Compound **126**, evaluated for its growth inhibitory effects, is considered an efficient antiproliferative agent against various cancer cell lines, especially MCF7 and HepG2, showing more cytotoxicity on cancer cell lines than normal cell lines, in comparison with camptothecin, etoposide, and Hoechst 33342. Moreover, **126** was able to induce also G2 cell cycle arrest on HeLa cells.

Also dovitinib (**127**, Chart 9), a multi-kinase inhibitor in clinical trial phase III as drug for the treatment of several cancers, is a benzimidazole-quinolinone structurally similar to Hoechst 33258. Due to this similarity, **127** has been evaluated for its capability to bind DNA and to inhibit Topos (Hasinoff et al., 2012). It has emerged that dovitinib intercalates DNA probably fitting its minor groove, as supposed by the docking studies performed (Hasinoff et al., 2012).

In the decatenation assay **127** proved to be a dual TopoI and II poison at low micromolar concentrations.

Since dovitinib binds to the kinase ATP binding site, its ability to bind also ATP binding site of Topos was investigated through enzyme kinetic experiments, in absence of DNA. The results showed that **130** strongly inhibits the DNA-independent ATPase activity of TopoII (Ki of 2.4 μM).

Docking studies, carried out on the X-ray structure of TopoIIα confirmed that **127** inhibits TopoII ATPase activity and competes with ATP for the inhibition of the decatenation activity of TopoIIα.

In this way, the anticancer activity of dovitinib may result not only from its ability to inhibit kinases, but also, in part, from its ability to target TopoI and II.

2.6.6. Naphthoquinones

Naphthoquinone TU100 (**128**, Chart 9) is a novel drug candidate with structural similarity to both anthracyclines and tetrahydroisoquinolines (Kennedy et al., 2011).

Cell viability studies revealed that compound **128** is specific for eukaryotes and induces cell death and that it is able to prevent relaxation of supercoiled plasmid DNA mediated from both Topos, thus resulting a dual TopoI and II inhibitor. Despite its structural similarity with anthracyclines, **128** does not intercalate DNA.

The study of the mechanism of action, which appears unique and involves the capacity of targeting the free enzyme in the absence of DNA, suggested that compound **128** is a slow acting poison that causes irreversible enzymes inactivation.

2.6.7. Glycosylated 2-phenyl -indoles, -benzo[b]thiophenes and -benzo[b]furans

Compounds of series **XXX** (Chart 9) were synthesized as anthracycline mimics and their binding to DNA was verified by fluorescence measurements (Shi et al., 2009).

Compounds **XXX** were also evaluated in a cell viability assay against three solid-tumor (MCF-7, HT 29 and HepG2/C3A) and three non-tumor cell lines. Some compounds were more cytotoxic and selective to MCF-7 line respect to daunomycin and doxorubicin (Shi et al., 2011).

In the gel electrophoresis assays, compounds of series **XXX** behaved as dual TopoI and II inhibitors with compounds bearing a tosylated indole core (X = NSO_2-*p*-$C_6H_4CH_3$) resulting the most potent of the series at 100 μM concentration. Moreover, the best performing compounds were studied to assess their mechanism of inhibition and they were not poisons of both TopoI and II.

Unfortunately, there was no a clear correlation between the dual TopoI and II inhibitory activity and cytotoxicity of the studied compounds of series **XXX**.

2.6.8. Benzo-annulated tryptanthrins

Tryptanthrin **129** is a sublimation product of indigo, also isolated from many plant sources (Honda et al., 1980) reported for many biological activities and in particular it induces apoptosis by downregulation of glutathione S-transferase (GST) expression (Yu et al., 2009).

Evaluated for their inhibitory activity on both TopoI and II, tryptanthrin and its benzo-annulated on quinazolin-4(3H)-one moiety derivatives turned out to be selective TopoI inhibitors, while linear benzoannulation on indolin-3-one ring led to dual TopoI and II inhibitors (Liang et al., 2012). In particular, compound **130** showed to be the most interesting compound (% inhibition values 69.56 for TopoI and 75.93 for TopoII at 100 μM concentration) which also showed good cytotoxicities against the selected human cancer cell lines T47D, DU145, HEK293 and in particular against HCT15.

2.6.9. Naphthalimide derivatives

Naphthalimides are reported as typical DNA intercalators and some of them are also strong TopoII poisons, (Van Quaquebeke et al., 2007; Lv and Xu, 2009).

In 2013, two series of novel oxo-heterocyclic fused naphthalimide derivatives **XXXI** and **XXXII** (Chart 9) (Tan et al., 2013) were synthesized and evaluated for their antiproliferative and inhibitory TopoI and II activities in comparison with their thio-heterocyclic fused analogs (e.g.**131**, **132**, Chart 9) yet reported (Qian et al., 2004; Xu et al., 2005).

The newly-synthesized compounds exhibit potent antiproliferative activity against the three cancer cell lines tested (A549, P388, LO2) although less potent than their sulfur analogues. Moreover, the most interesting and representative compounds **131-134** were investigate for their mechanism of action, resulting DNA intercalators and significant dual TopoI and II inhibitors. Finally, the tested compounds also showed a GC sequence preference which may contribute to enhance tumour selectivity and overcoming drug resistance (Tan et al., 2013).

2.6.10. Podophyllotoxin derivatives

The strong TopoII inhibition by podophyllotoxin derivatives such as etoposide and teniposide is widely reported (Ter Haar et al., 1996; Stähelin et al., 1991; Hande et al. 1998).

In 2013, among a new series of 4β-[4′-(1-(aryl)ureido)benzamide]podophyllotoxin congeners, derivative **135** (Chart 9) turned out to be a dual TopoI and II inhibitor (Kamal et al., 2013).

The docking study evinced for **135** a similar orientation and interactions at DNA TopoI and TopoIIα active sites, compared with the standards, camptothecin and etoposide, respectively.

Evaluated for its cytotoxic activity against selected human cancer cell lines, compound **135** showed a significant antiproliferative activity against the Zr-75-1, MCF7, DWD, and Hop62 cell lines with GI_{50} values ranging from 0.10 to 0.17 μM, and a very potent antiproliferative activity against Colo 205 cell line with a GI_{50} value of 0.01 ± 0.005 μM (Kamal et al., 2013).

Flow cytometric analysis in Colo 205 cells indicated that compound **135** showed strong (78%) G1 cell cycle arrest; moreover ELISA and Western blotting analysis showed also that **135** induces apoptosis by up regulating caspase-3.

2.6.11. Cycloprotoberberine derivatives

Cycloprotoberberine is reported as antiproliferative agent due to its capability to intercalate DNA, and prevent DNA cleavage TopoI mediated (Qin et al., 2007).

In 2013 twenty cycloprotoberberine derivatives were synthesized with various substituents (R) at 9-position and, among them, compounds of series **XXXIII** (Chart 9) showed to be potential antiproliferative agents against HepG2 cells showing above 85% of growth inhibition. They also showed good antiproliferative activities against HCT116 cell lines *in vitro* (Li et al., 2013).

One of the most potent cytotoxic compound of the series, **136**, (Chart 9) was chosen for further investigations. In particular, the growth inhibition assay of **136** was performed on human MCF-7 cell line and doxorubicin–resistant cells (MCF-7/ADrR) using doxorubicin as a reference drug. The obtained results evidenced that compound **136** has promising potency *in vitro* in either naive breast cancer MCF-7 or MCF-7/ADrR cells, suggesting that probably there is no cross drug resistance between cycloprotoberberines and doxorubicin. Mechanism studies showed that **136** significantly inhibited activity of TopoI and TopII with potency similar to that of hydroxycamptothecin and

etoposide, respectively. Moreover, flow cytometric analysis of the DNA profile in the HCT116 cells dimostrated that **136** induces G2/M phase cycle arrest (Li et al., 2013).

2.6.12. Acid oleanolic derivatives

Pentacyclic triterpenes such as oleanolic acid are reported to inhibit TopoI and IIα through a direct - interaction with the enzymes so preventing TopoI- or II-DNA complex formation (Syrovets et al., 2000).

In 2014 ten rationally designed derivatives of oleanolic acid were synthesized based on docking studies and four of them (**137-140**, Chart 10) turned out to be dual TopoI and II inhibitors with activity higher than camptothecin and etoposide, respectively (Ashour et al., 2014). SAR studies highlighted the importance of the C12–C13 double bond of the oleanolic acid skeleton, for the inhibitory activity against both Topos, moreover the insertion of a chain in positions 3 and 17 increases the activity by various degrees.

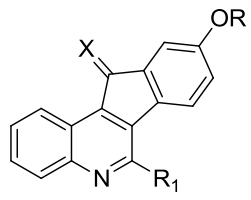
2.6.13. Benzo[f]chromene derivatives

A novel series of halogenated *β*-enaminonitriles **XXXIV** (Chart 10), linked to 9-bromo-1*H*-benzo[*f*]-chromene scaffold, was synthesized and evaluated for the cytotoxic activity against MCF-7, HCT-116, and HepG-2 cancer cell lines (Fouda et al, 2019). Several compounds of this series showed high growth inhibitory activities *versus* all the tumor cell lines tested.

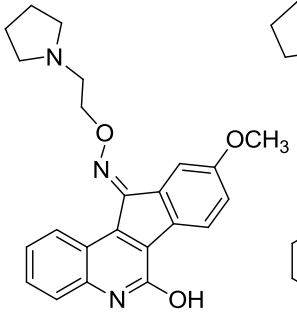
SAR studies revealed that the substitution pattern on the aryl moiety (Ar) is a crucial element of the antitumor activity. In general, the incorporation of an electron-donating group as the methoxy, or with electron-withdrawing ones as bromine, chlorine or fluorine, greatly enhanced the antiproliferative activity (Fouda et al, 2019).

Moreover, annexin V/PI double staining flow cytometric assay performed on some selected compounds revealed that they prompt cell cycle arrest at the G2/M phases.

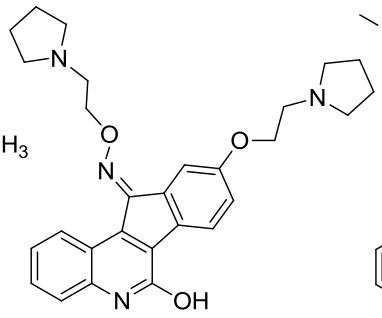
In conclusion, all selected compounds induced DNA TopoI and II mediated relaxation activity, in a dose-dependent manner, in particular compounds with Ar = 3-ClC_6H_4 and Ar = 3,4-$Cl_2C_6H_3$ were described as the most potent dual TopoI and II of the series (Fouda et al, 2019).



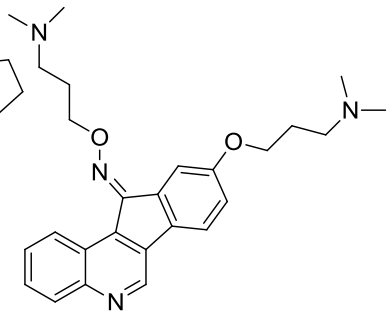
XXVI



115

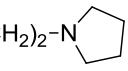


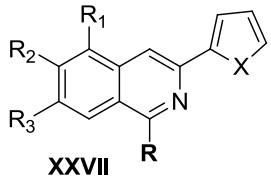
116



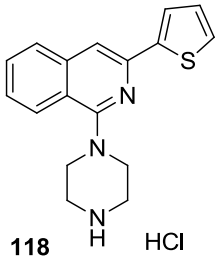
117

X = O, NOR ; R_1 = H, OH

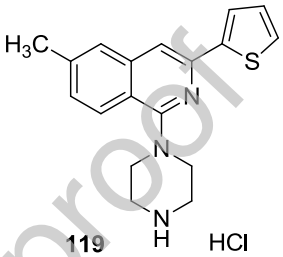
R = -$(CH_2)_2N(Me)_2$; -$(CH_2)_3N(Me)_2$;  ;  ; 



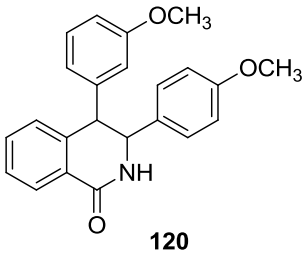
XXVII



118 HCl

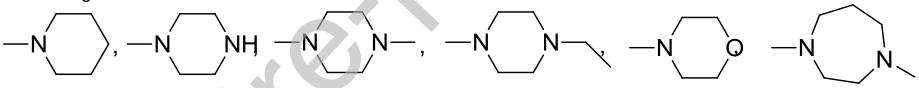


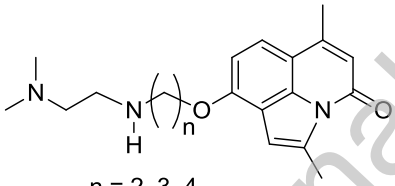
119 HCl



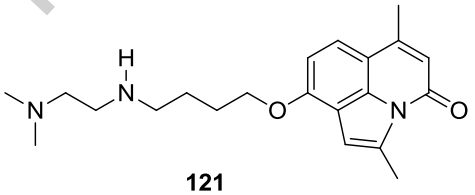
120

X = S, NCH_3; R, R_1, R_2 = H, CH_3

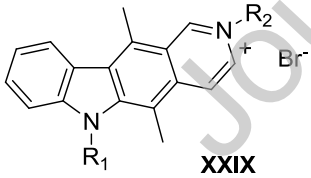
R = N_3, NH_2, $NHCH_2Ph$, 



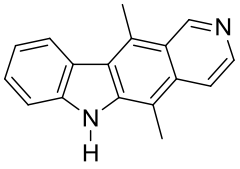
n = 2, 3, 4 **XXVIII**



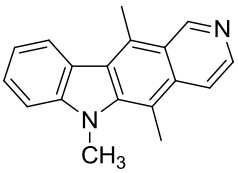
121



XXIX



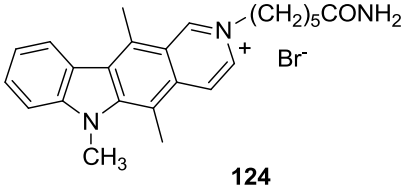
122



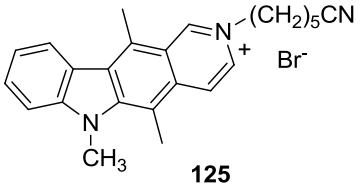
123

R_1 = H, CH_3

R_2 = $(CH_2)_2COOH$, $(CH_2)_4COOH$, $(CH_2)_5COOH$, $(CH_2)_5CONH_2$, $(CH_2)_5CN$, $(CH_2)_5CONHSO_2CH_3$,

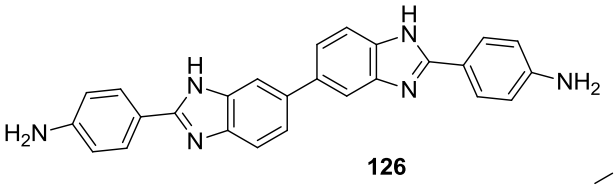


124



125

Chart 8. Structures of indenoquinoline derivatives **(XXVI, 115-117),** isoquinoline derivatives **(XXVII, 118-120**), pyrroloquinolinone derivatives **(XXVIII, 121)**, ellipticine derivatives **(XXIX, 122-125)** as dual TopoI and II inhibitors.



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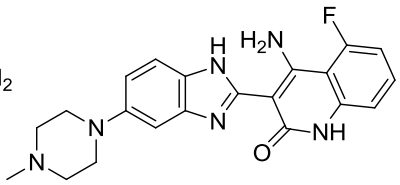

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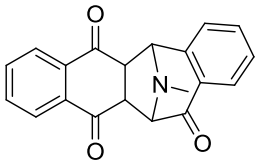

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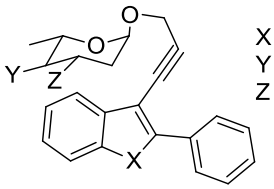
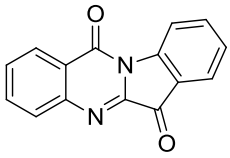


X = O, S, NH, NSO_2p-$C_6H_4CH_3$
Y = OH, NH_2
Z = H, NH_2

XXX



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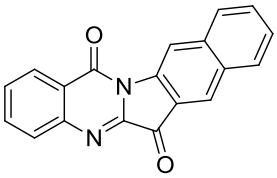

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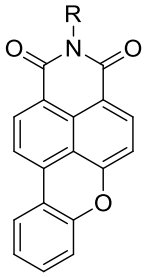
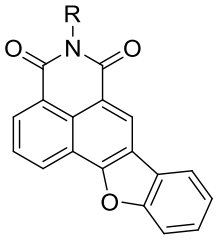
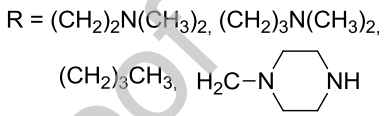


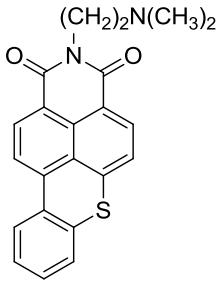
XXXI



XXXII



R = $(CH_2)_2N(CH_3)_2$, $(CH_2)_3N(CH_3)_2$, $(CH_2)_3CH_3$, H_2C-N NH



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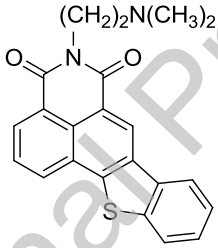

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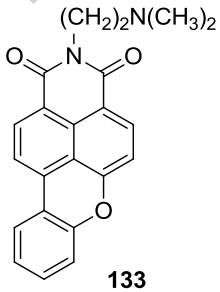

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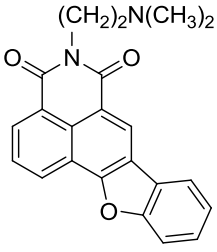

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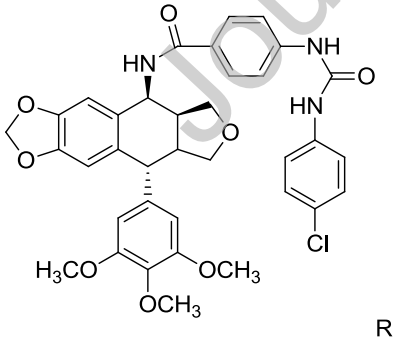

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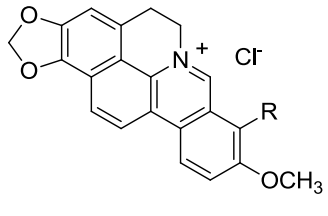
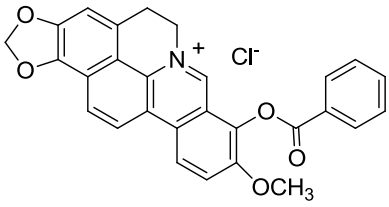


XXXIII

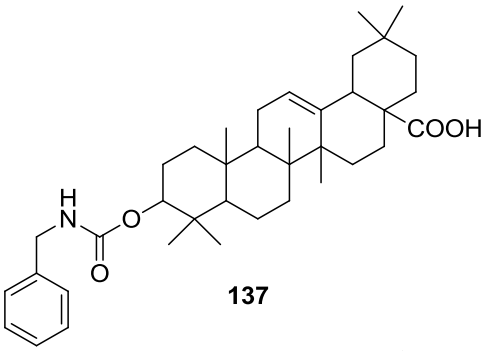


136

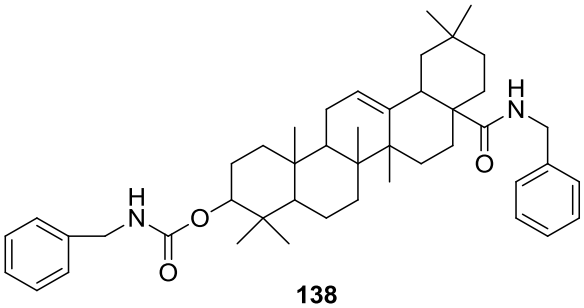
R = $OCOCH_3$, OCOPh, OCOPh-2'-F, OCOPh-2',4'-F_2,
OCOPh-4'-OCH_3,, OCOPh-3',4',5'-$(OCH_3)_3$; piperonyloyloxy

Chart 9. Structures of benzimidazole derivatives **(126-127)**, naphthoquinone **(128)**, glycosylated 2-phenyl -indoles, -benzo[b]thiophenes and -benzo[b]furans **(XXX)**, benzo-annulated tryptanthrins

(**129-130**), naphthalimide derivatives **(XXXI-XXXII**, **131-134),** podophyllotoxin derivative **(135)**, cycloprotoberberine derivatives **(136, XXXIII)** as dual TopoI and II inhibitors.



137



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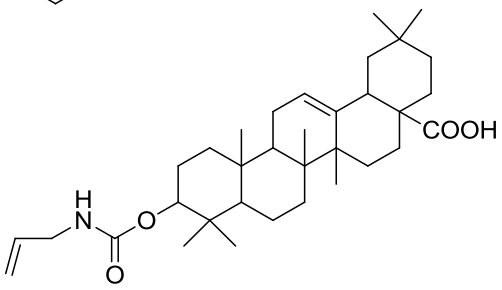

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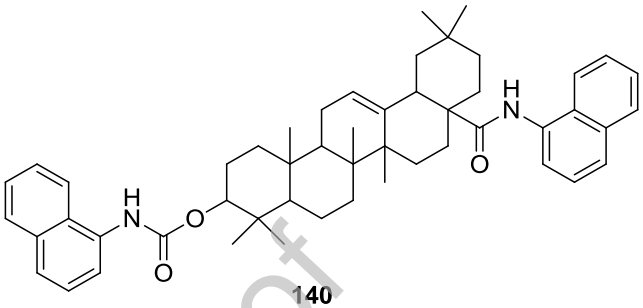

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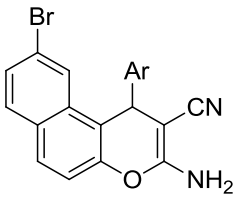


XXXIV

Ar = 4-FC_6H_4, 2-ClC_6H_4, 3-ClC_6H_4, 4-ClC_6H_4
4-BrC_6H_4, 2,4-$F_2C_6H_3$, 2,6-$F_2C_6H_3$,
2,3-$Cl_2C_6H_3$, 2,4-$Cl_2C_6H_3$, 2,5-$Cl_2C_6H_3$,
3,4-$Cl_2C_6H_3$, 3,5-Br_2-2-$OMeC_6H_2$

Chart 10. Structures of acid oleanolic derivatives **(137-140)** and benzo[*f*]chromene derivatives **(XXXIV)** as dual TopoI and II inhibitors.

2. 7. Hybrids and conjugates

A hybrid or a conjugate molecule consists of two pharmacophoric groups condensed or joined by a linker, respectively. These types of compounds are often strategically used to design new drugs exploiting the possible increased potential or improved selectivity profile compared to the corresponding individual drugs (Morphy and Rankovic, 2005). Consequently, several hybrid compounds in which known anticancer compounds have been linked to various molecules able to intercalate DNA, such as 9-anilinoacridine, acridine or quinoline have been reported (Baraldi et al., 2006; Kapuriya et al., 2008; Kakadiya et al., 2010).

2.7.1. Indolizino[6,7-b]indoles

In 2013 a series of novel tetracyclic indolizino[6,7-b]indole derivatives **XXXV** (Chart 11) was designed as hybrids molecule of β-carboline and bis-(hydroxymethyl)pyrroles (Chaniyara et al., 2013). Naturally occurring and synthetic β-carboline derivatives are reported for their antitumor activity through DNA intercalation and ability to inhibit Topo I and TopoII (Deveau et al., 2001); bis(hydroxymethyl)pyrroles were observed to induce DNA cross-linking, displaying significant antitumor activity (Anderson and Halat, 1979).

The preliminary antiproliferative activity of all compounds of series **XXXV** was evaluated using the CCRF-CEM cell line and the best performing compounds were selected for a further *in vitro* evaluation on some human solid tumors: PC3, OECM1, MX-1, HCT-116, HT-29, CL141T, A549, and H460. The results obtained revealed that the compounds have a significant antitumor activity with no cross resistance and, in general, the bis(alkylcarbamate) derivatives are more cytotoxic than the corresponding bis-(hydroxymethyl) derivatives against all the tumor cells tested (Chaniyara et al., 2013). Based on the *in vitro* activity, compounds **141-143** were selected to evaluate their therapeutic efficacy against human solid tumors in xenograft models: compound **141** was more potent than irinotecan against the human colon and lung tumor models, being also less toxic towards the host.

Studies carried out to identify the mechanism of action of these compounds revealed that they induce DNA cross-linking and exhibit dual TopoI/TopoII inhibitory activity. Moreover, studies on the levels of the apoptotic proteins, caspase-3, caspase-7, and PARP suggested that compounds of series **XXXV** are able to induce apoptosis.

Compound **141** turned out to be the most interesting of series **XXXV** and it may potentially be selected as a candidate for preclinical studies (Chaniyara et al., 2013).

2.7.2-Naphthalimide–cyclam conjugates

Cyclam (1,4,8,11-tetraazamacrocycle) is a macrocyclic derivative, in particular a tetraazamacrocycle, with medical applications (Liang et al., 2006; Liang and Sadler, 2004). Moreover, lipophilic substitutions have also proved to increase the antitumor activity (Sibert et al., 2002). Amonafide, a naphthalimide in phase III clinical trial against secondary acute myeloid leukemia (Stone et al., 2015), can intercalate DNA thanks to its basic side chain and it also strongly inhibits TopoII.

Based on these considerations, in 2015, two series of naphthalimide–cyclam conjugates **XXXVI** and **XXXVII** (Chart 11) in which cyclam was introduced at the 2- or 6-position of the naphthalimide replacing the basic side chain, were synthesized (Tan et al., 2015).

Compounds of series **XXXVI** and **XXXVII** were evaluated for their antiproliferative activity against three human cancer cell lines: A549, HeLa and HCT116. The data obtained highlighted a good antiproliferative activity for both series of derivatives with a lipophilic chain (R = octyl, dodecyl), with compounds of series **XXXVII** resulting more potent than those of series **XXXVI** and also respect to amonafide and cyclam.

The best performing compounds (R = octyl, dodecyl) of series **XXXVI** and **XXXVII** proved to possess strong dual TopoI/TopoII inhibition activity. In particular, no difference on TopoII inhibition was observed between compounds of series **XXXVI** and **XXXVII**, whereas derivatives of series **XXXVI** showed lower TopoI inhibitory activity than those of series **XXXVII**, in

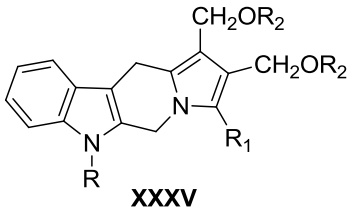
accordance with their cytotoxic activities. The most representative compound of series **XXXVII** (R = octyl) exhibited also moderate DNA intercalation activity. Moreover, molecular modeling studies highlighted its possible interaction with the target by forming TopoI or TopoII/DNA/drug ternary complexes. Finally, derivatives of series **XXXVI** and **XXXVII** with lipophilic alkyl chains (R = octyl, dodecyl) showed to efficiently induce apoptosis (Tan et al., 2015).

2.6.3. N-4-piperazinyl-ciprofloxacin-chalcone hybrids

Fluoroquinolones have been recently reported as antitumor agents (Azema et al., 2009); among them, ciprofloxacin inhibits mitochondrial TopoII and it could be used for the experimental adjunctive therapy of lung cancer (Kloskowski et al., 2011). In addition, some chalcone derivatives resulted of great interest thanks to their cytotoxic properties (Won et al., 2005; Achanta et al., 2006) and, in particular, a 2-phenylquinoline/chalcone hybrid was recently reported as highly potent antiproliferative agent against non-small cell lung cancers and breast cancers (Tseng et al., 2013b). Based on these considerations and on studies reporting that the introduction of a N-4-piperazinyl moiety on the quinolone scaffold may promote the antiproliferative and TopoI/TopoII activity of the compounds (Yogeeswari et al., 2005; Alovero et al., 2000), in 2013 a series of C-7-piperazinyl-ciprofloxacin-chalcone hybrids **XXXVIII** (Chart 11) was synthesized (Abdel-Aziz et al., 2013). Most of the new hybrids showed good dual TopoI/TopoII inhibitory activity at 100 μM, with **144** (Ar = 4-OCH_3-C_6H_4**)** and **145** (Ar = 3,4-OCH_2O-C_6H_3) resulting the most potent ones. Indeed, **144** and **145** showed % inhibition values of 67.6 % and 91.3 % for TopoI and 84.3 % 85.7 % for TopoII, respectively, at 100 μM concentration, comparable to that of references camptothecin and etoposide. Moreover, by means of DNA unwinding assay, their inhibitory activities were attributed to a direct interaction with Topos without binding to DNA.

Some compound of series **XXXVIII** including **144** and **145**, were selected by the National Cancer Institute (NCI) for *in vitro* anticancer screening and five-dose full NCI60 cell panel assay revealed

that there is no direct correlation between their antiproliferative activity and inhibitory effect against TopoI and TopoII. Anyway, the obtained results indicated that introduction of the chalcone moiety into the N-4-piperazinyl group of ciprofloxacin increases dramatically the anticancer activity of the compounds (Abdel-Aziz et al., 2013).

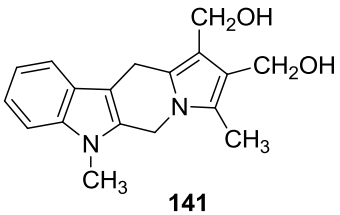


XXXV

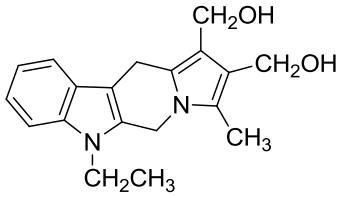
R = CH_3, CH_2CH_3, CH_2Ph

R_1 = CH_3, CH_2CH_3, C_6H_5, 4-F-C_6H_4, 4-Cl-C_6H_4, 3,4-di-F-C_6H_3, 3-Cl,4-F-C_6H_3, 4-OCH_3, 3,4-$(OCH_3)_2$, 3,4,5-$(OCH_3)_3$

R_2 = H, $CONHCH_2CH_3$, $CONHCH(CH_3)_2$



141



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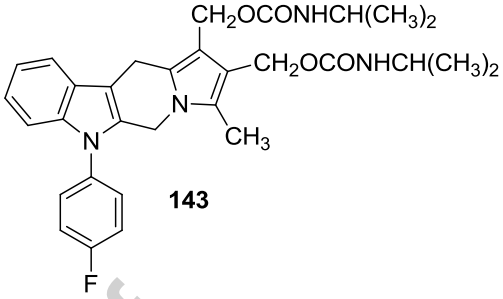

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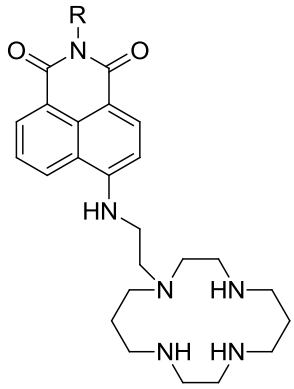
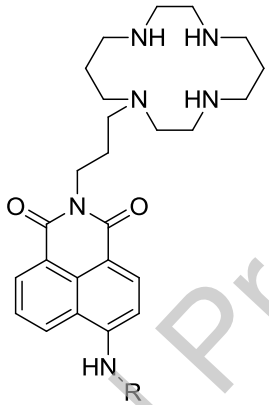
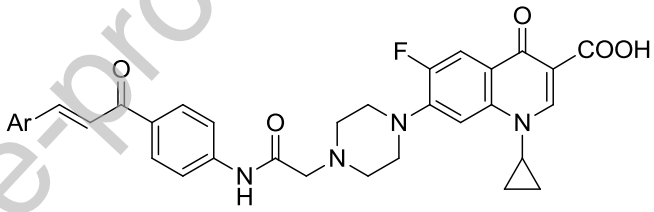


XXXVI



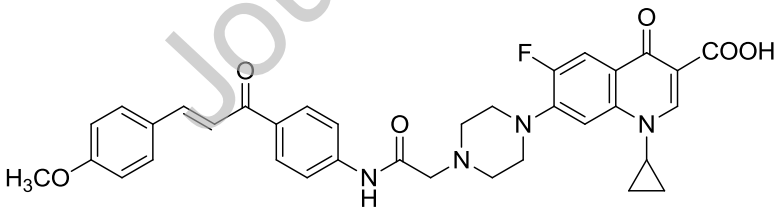
XXXVII

R = CH_3, C_4H_9, C_8H_{17}, $C_{12}H_{25}$

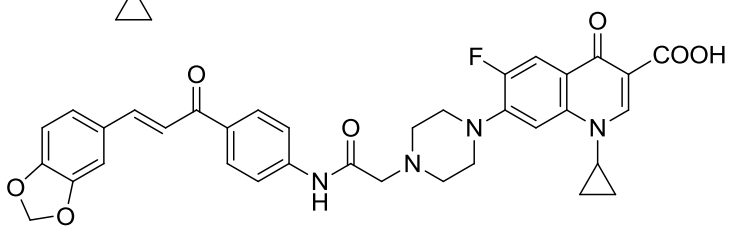


XXXVIII

Ar = -C_6H_5, 2-Cl-C_6H_4, 3-Cl-C_6H_4, 4-Cl-C_6H_4, 4-OCH_3-C_6H_4, 3,4-$(OCH_3)_2$-C_6H_3, 3,4,5-$(OCH_3)_3$-C_6H_2, h: 3-NO_2-C_6H_4, 2,4-$(CH_3)_2$-C_6H_3, 3,4-OCH_2O-C_6H_3.



144



145

Chart 11. Structures of hybrids and conjugates (**XXXV-XXXVIII**) as dual TopoI and TopoII inhibitors.

3. Multitarget DNA Topoisomerases and Tyrosyl−DNA phosphodiesterases inhibitors

The drug resistance that occur for TopoI or TopoII inhibitors is mainly due to DNA repair systems, including tyrosyl-DNA phosphodiesterases I and II (TDP1 and TDP2) (Pommier et al., 2006; Maede et al., 2014; Pommier et al., 2014). In this context, the new strategy regarding the development of multiple Topo/TDP targeting agents becomes relevant to obtain more efficient anticancer drugs (Nguyen et al., 2012).

3.1. Dual Topoisomerase I (TopoI) and Tyrosyl−DNA phosphodiesterase I (TDP1) inhibitors.

As TDP1 counteracts the action of TopoI inhibitors, it is possible that TDP1 inhibitors may potentiate the cytotoxic effects of TopoI inhibitors in antitumor combination therapy (Dexheimer et al., 2008).

In the past decade, numerous studies have been carried out to find dual TopoI/TDP1 inhibitors as agent with potentially enhanced anticancer activity and selectivity.

3.1.1. Indenoisoquinolines

Indenoisoquinolines, as indotecan or indimetican, are reported in literature as potent TopoI inhibitors (Pommier and Marchand, 2012). With the aim to develop the first dual TopoI/TDP1 inhibitors, the screening of a library of indenoisoquinolines was performed (Nguyen et al., 2012), and three molecules (**XXXIX**, n = 10-12, Chart 12) synthetized by Morell *et al.* (Morrell et al., 2007b) were selected for their ability to inhibit TDP1. Surprisingly, they did not present activity against TopoI, differently from their analogues with a shorter side chain (n = 2-4) that were good TopoI inhibitors (Davies et al., 2004). This aspect suggests that TDP1 inhibitors might be found among TopoI inhibitors featuring short chain linkers.

Nguyen *et al*. confirmed that the indenoisoquinolines within the primary amine (**XXXIX**) are able to inhibit TDP1 (IC_{50} range, 13-55 μM) and compounds with a longer side chain (n = 10-12) showed

higher TDP1 inhibition with respect to their homologous with shorter linker; for the TopoI inhibition, the trend is exactly the opposite. Moreover, the presence of a positive charge was important for TDP1 inhibition. The most active compounds (IC_{50} = 22-29 μM) were characterized by 3-aminopropyl and 4-aminobutyl side chains and they represent the first dual TopoI-TDP1 inhibitors ever reported (Nguyen et al., 2012).

Based on preliminary studies (Nagarajan et al., 2006) demonstrating that bis(indenoisoquinolines) could be considered as potential TDP1 inhibitors, Nguyen *et al.* synthetized also a class of bis(indenoisoquinolines) with the aim to obtain molecules with significant dual TopoI/TDP1 activity. Among them, compound **146** (Chart 12) results the most potent inhibitor of both TopoI and TDP1 (IC_{50} = 1.5 μM and 1.9 μM against rec. and WCE TDP1, respectively) (Nguyen et al., 2012).

Subsequent studies highlighted the possibility that the introduction of an ester or a carboxylic acid on the molecules could increase the TDP1 inhibition. In fact, TDP1 active site presents two histidine and two lysine residues, so the carbonyl moiety could establish a hydrogen bond with these residues (Dexheimer, 2008). In this view, in 2013 Conda-Sheridan *et al*., synthetized new classes of compounds possessing an ester (**XL**) or a carboxylic acid (**XLI**) at 3-position, with methyl or amminoalkyl substituents (R_2) on the nitrogen of the indenoisoquinoline scaffold (Chart 12) (Conda-Sheridan et al., 2013). Only the esters (series **XLI)** with the aminopropyl side chain were active, and the activity of the molecules decreased when more methylene units were added to the ester side chain.

For this reason, ten compounds characterized by an aminopropyl chain, previously reported as TopoI inhibitor (Nguyen et al., 2012; Morrell et al., 2006; Morrell et al., 2004), were selected and the primary amine was removed to evaluate how this variation could affect the inhibitory profile of the molecules. The primary amine was replaced with groups of various nature as azide, imidazole, morpholine or bromide; all the obtained compounds were inactive or weakly active on both TopoI and TDP1. This study confirmed the importance of the primary amino side chain on the indenoisoquinoline scaffold to exhibit TopoI/TDP1 inhibitory activity.

Finally, in this study emerged that the presence of a methoxy group at the 9-position of the indenoisoquinoline system led to compounds with good TDP1 inhibition, while changing the position from C9 to C8, or C7 decreased the inhibitory activity of the compounds (Conda-Sheridan et al., 2013).

Pursuing the study on the indenoisquinolines scaffold, in 2014 Lv *et al*. (Lv et al., 2014), evaluated how the introduction of substituents at 2-position of the system could affect the dual TopoI/TDP1 inhibitory activity of the molecules by a structure-based drug design approach. Molecular docking studies were performed on a selected lead compound, the 2-idrossiindenoisoquinoline **147** (Chart 12), reported in literature as a TopoI inhibitor, (Cinelli et al., 2012) and on its newly synthesized derivatives **147-151** (Chart 12) and the results suggested that the aminoalkyl substituents attached to O-2, next to the cleaved DNA strand, could target the carboxylate of Asp533 of TopoI.

Moreover, the indenoisoquinoline core could be accommodated within the catalytic region of TDP1, while, as already reported, the aminopropyl side chain could increase the affinity toward TDP1, binding a hydrophobic region of the enzyme (Davies et al., 2004).

Enzyme inhibition results of the new compounds **147-151** with both Top1 and TDP1 evinced that the most active compounds get a secondary or a tertiary amine, whereas molecules within a primary amine result inactive (Lv et al., 2014).

Based on the studies carried out by Morrel and Cinelli (Morrell et al., 2006; Cinelli et al., 2012), Nguyen *et al.* developed three series of 3-nitroindenoisoquinolines with a hydroxyl (and a methoxy) group on the D-ring**, XLII, XLIII, XLIV** (Chart 12) (Nguyen et al., 2015).

The presence of a hydroxyl group on the molecules gives the opportunity to synthesize prodrugs and/or to obtain systems with characteristics more suitable for parental formulations. The introduction of a 3-nitro group enhances the potency of these compounds, because it establishes a hydrogen bond with a residue of Asn722 located in the DNA strand (Morrell et al., 2007a).

These compounds were tested for their ability to inhibit both TopoI and TDP1 and for their antiproliferative effect, using camptothecin as standard. TopoI inhibition was recorded as the ability

of a drug to poison TopoI and induce enzyme-linked DNA breaks in the TopoI-mediated DNA cleavage assay. TDP1 inhibition was evaluated as the capacity of a molecule to inhibit the enzyme-catalyzed hydrolysis of the phosphodiester linkage among tyrosine and the 3′-end of a DNA substrate, to avoid the generation of an oligonucleotide with a free 3′-phosphate. In general, all the compounds were able, at least, to inhibit TopoI, and they appeared to be inactive or weakly active toward TDP1. Specifically, only compounds with the primary amine side chain result TDP1 inhibitors, whereas their analogues with the morpholine or the imidazole groups are, apart from a few exceptions, inactive toward TDP1. The most interesting results were obtained for compounds **152-158** (Chart 12) which were able to inhibit both TopoI and TDP1.

Compounds of series **XLIII** (9-hydroxy, 8-methoxy) showed only moderate (IC_{50} values between 12 and 37 μM) activity against TopoI.

The most active compounds (**155-158)** belonging to series **XLII** feature a hydroxy group at 8- or 9-positions and this renders the substituents distant enough to establish a hydrogen bond with the phosphodiester DNA backbone. Compound **156** (8-hydroxy, R = NH_2) is the most active of all three series and is a very potent dual TopoI/TDP1 inhibitor (95-100% TopoI inhibition activity at 0.1-10 μM concentrations and IC_{50} value of 1−12 μM toward TDP1), with an activity similar to the bis(indenoisoquinoline) **146** previously described (Nguyen et al., 2012).

To enhance TopoI inhibitory activity, in 2016, Beck *et al*. substituted the 3-nitro group on the A ring with a fluorine or a chlorine atom and get the fusion of dioxolane ring to the 8- and 9-positions of the indenoisoquinoline scaffold (Beck et al., 2016). The design is in accordance with previous SAR studies that reported a modest increase in TopoI activity exploiting these substitutions (Nagarajan et al., 2004). All the obtained compounds turned out to be good to high TopoI inhibitors, and two derivatives, the fluorinated **159** and the chlorinated **160** (Chart 12) were able to inhibit also TDP1 and TDP2, enhancing their therapeutic utility. Both **159** and **160** possess a hydroxyethylaminopropyl side chain, showing that this moiety could improve the TDP1 and TDP2 inhibitory activity, as well as TopoI inhibitory activity. Compound **163** shows 50-75% for TopoI

poisoning activity (0.1-10 μM concentrations), IC_{50} 8.7 μM against TDP1, and IC_{50} 10.2 μM against TDP2; the most active compound **160** shows 75-95% for TopoI poisoning activity at 0.1-10 μM concentrations, IC_{50} 6.3 μM against TDP1, and IC_{50} 9.1 μM against TDP2 (Beck et al., 2016).

3.1.2. Oxynitidine derivatives

As natural products represent an essential resource for medicinal chemistry and drug development (Rodrigues et al., 2016; Xiao et al., 2016), in 2018, Zhang X.R. *et al*., screened an in house library and identified several compounds potentially able to inhibit TopoI (Zhang et al., 2018). Among them products with an oxynitidine scaffold were an interesting chemotype for the development of novel dual TopoI/TDP1 inhibitors, in accordance with previous reported results (Beck et al., 2016). Hence, two series (**XLV**, **XLVI**) (Chart 12) of compounds were synthetized and biologically evaluated for their cytotoxicity toward several cancer cell lines (Zhang et al., 2018).

Among the obtained compounds, the most active molecule belongs to series **XLV** and it is substituted with a dimethylaminoethyl group (n = 2; R = $N(CH_3)_2$) linked to the nitrogen atom of the system; this aspect provides that bigger groups at the 5-position reduced the ability to inhibit TopoI. Molecular modelling studies were performed to evaluate the interactions of the compound with the TopoI-DNA complex, and evidenced that the lactam oxygen establishes a hydrogen bond with an arginine residue, underlining the relevance of a hydrogen bond acceptor. Moreover, this compound turned out to be also a weakly TDP1 inhibitor (inhibition activity of 12% at 100 μM).

As regard TDP1 inhibition, the most active compound belongs to series **XLVI** and it is substituted with a pyrrolidinyl ethyl side chain (TDP1 inhibition activity of 92% at 100 μM), but it was not active toward TopoI. In silico docking studies evidenced that the nitrogen of the pyrrolidyl moiety establishes hydrogen bonds with two TDP1 catalytic residues (His493 and Asn283), highlighting the important role of the side chain to inhibit TDP1.

MTT experiment was assessed to evaluate the cytotoxicity of compounds toward different human cancer cell lines (HCT116, CCRF-CEM, DU-145, A549, Huh7) and the obtained results

demonstrated that the most potent TopoI inhibitor presented also the strongest cytotoxicity toward these cell lines, underlining that shorter side chain at 5-position led to higher cytotoxicity (Zhang et al., 2018).

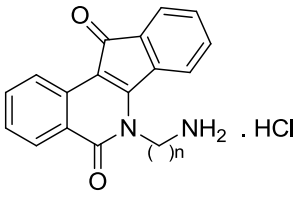
3.2. Multi Topoisomerase I (TopoI) and Tyrosyl−DNA phosphodiesterase I and II (TDP1 and TDP2) inhibitors

More recently, it was recognized that TDP2 also promotes repair of TopoI-mediated DNA damage in the absence of TDP1 and that cells lacking both TDP1 and TDP2 are more sensitive to TopoI inhibitors than TDP1-deficient cells (Zeng et al., 2012; Maede et al., 2014). The functional relationships among these enzymes make both TD1 and TDP2 attractive targets for cancer treatment in combination with TopoI inhibitors.

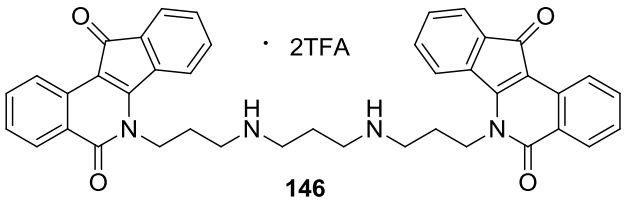
In this view, 2017, Wang *et al.*, taking **161** as lead compound, developed a series of 7-azaindenoisoquinolines **161-167** (Chart 12) as multiple TopoI/TDP1/TDP2 inhibitors (Wang et al., 2017). Briefly, the methoxy and nitro group at the 9- and 3-positions of the scaffold, respectively, were maintained, as suggested by the literature (Kiselev, et al., 2014) describing compound **161 (**Chart 12) as potent TopoI inhibitor. Analogously, the nitrogen atom at 7-position remained unmodified because of a previous study clearly demonstrated its role to confer high TopoI inhibition (Kiselev et al., 2012; Kiselev et al., 2011). Then, the lead compound compound **161** was subjected to molecular modeling studies to explore its binding mode in the active sites of TopoI, TDP1 and TDP2, and, based on the obtained results, (Wang et al., 2017) different hydrophilic side chains were introduced in N-6 to investigate their effect on the biological activity.

Concerning TopoI inhibition, results clearly demonstrated that compounds featuring a secondary amine at the 6-position were more active with respect to those with a tertiary one**.** When tested for their inhibitory activity toward TDP1, compounds **162-164** and **166-167** showed to be the most active, with compound **167** being also the most potent TDP2 inhibitor of the series (Wang et al., 2017).

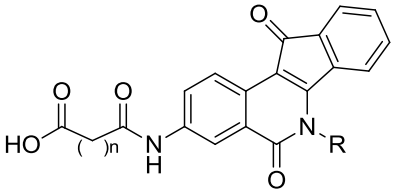
Overall, compound **164** showed the most interesting inhibition profile toward the three enzymes. Molecular modeling studies for **164** in the active site of TopoI and TDP1 and for compound **167** in the TDP2 one were also performed, highlighting that modification of the side chain of the 7-azaindenoisoquinolines can modulate the inhibitory potency toward TopoI, TDP1, and TDP2 (Wang et al., 2017). Compounds **162-166** were also valuated for their cytotoxicity against 60 human cancer cell lines (NCI-60), (Shoemaker, 2006) showing to be potent cytotoxic anticancer agents with GI_{50} in the low to submicromolar range. In addition, these compounds induced greater DNA damage in cancer cells with respect to normal ones.



XXXIX n = 2-12



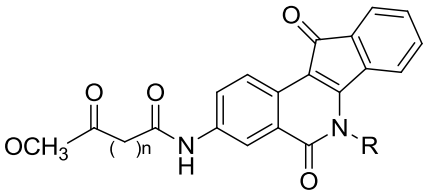
146



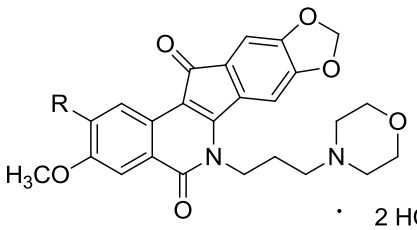
XL

n= 0-**4**

R= CH_3; $(CH_2)_3NHBOC$; $(CH_2)_3NH_2$

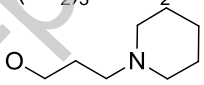


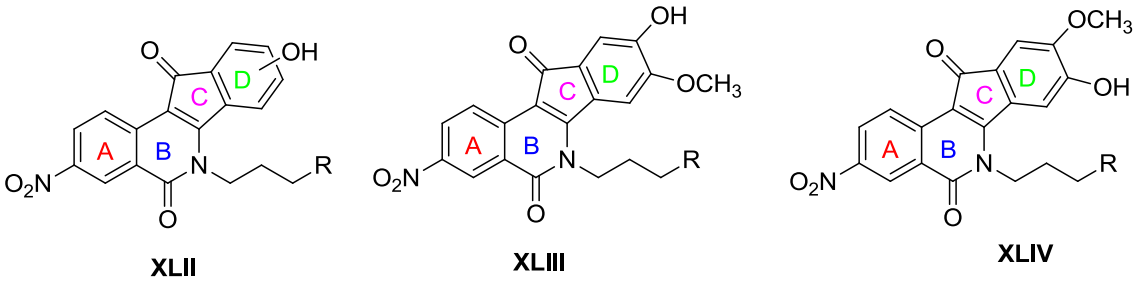
XLI

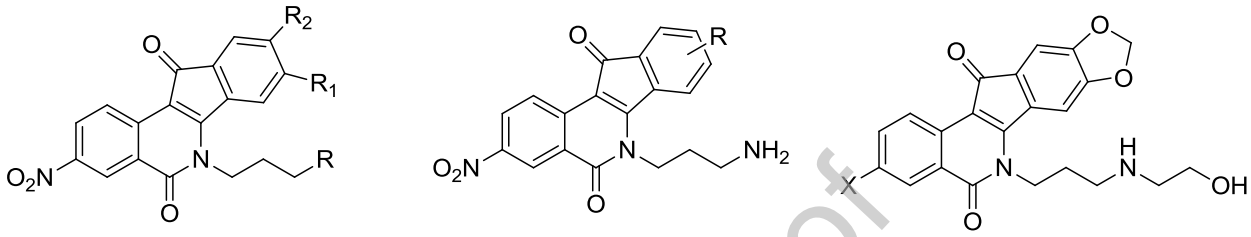


147-151

147 R = OH
148 R = $O(CH_2)_3NH_2$
149 R = $O(CH_2)_3N(CH_3)_2$
150 R = $O(CH_2)_3NHCH_2CH_3$
151 R =





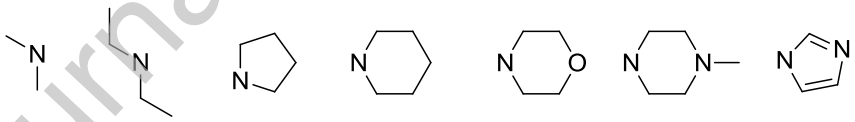


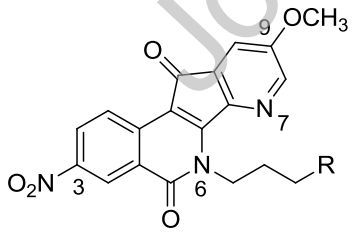
152 R = NH_2, R_1 = OH, R_2 = OCH_3
153 R = imidazole, R_1 = OH, R_2 = OCH_3
154 R = NH_2, R_1 = OCH_3, R_2 = OH

155 R = 7-OH
156 R = 8-OH
157 R = 9-OH
158 R = 10-OH

159 X = F
160 X = Cl

n = 2, 3; R =





161-167

161 R = NH_2
162 R =
163 R = $NHCH_3$
164 R =
165 R = $NHCH(CH_3)_2$
166 R = $NHCH_2CH_3$
167 R =

Chart 12. Structures of multitarget DNA Topoisomerases and Tyrosyl−DNA phosphodiesterases inhibitors **XXXIX-XLVI**.

4. Conclusions

DNA topoisomerases (Topos) are essential enzymes that regulate the topological states of DNA and are involved in several cellular processes, such as replication, recombination, and transcription.

The discovery of new drugs capable to inhibit topoisomerase enzymes is in evidence in anticancer therapy. Actually, many TopoII but also TopoI inhibitors are marketed for their usage in the clinical oncology practice. However, despite their clinical undoubted efficacy the use of these inhibitors is limited as many tumours develop specific resistance to these agents; indeed it is widely reported that TopoI levels become down-regulated after treatment with its specific inhibitors, whereas TopoII levels increase and viceversa, leading to the failure of the therapies. In this view, targeting both TopoI and TopoII simultaneously should lower the potential of developing resistance against such inhibitors but also of side effects thanks to the use of lower doses, given the synergistic effect of the dual activity. More recently, tyrosyl-DNA phosphodiesterases I and II (TDP1 and TDP2) are disclosed as the main DNA repair system responsible for the drug resistance in anticancer therapy involving TopoI or TopoII inhibitors. Consequently, a new strategy has emerged regarding the development of multiple Topo/TDP inhibitors, with the goal to develop compounds able to produce a synergistic anticancer effect.

This paper reviews the current status and the recent studies concerning dual TopoI and II inhibitors, but also inhibitors able to simultaneously inhibit Topo and TDP enzymes.

In particular, the mode of action of the compounds is described focusing the attention on their reported capability to exert cytotoxicity against tumor cell lines *in vitro* and *in vivo* (if carried out) and to affect the activity of both TopoI and II or Topos and TDP. Moreover, the enzyme inhibition profiles, when reported, are indicated and, when possible, SAR results are discussed.

As a summary, the properties of the most interesting compounds reviewed have been schematized in table 1.

It results that none of the derivatives exhibited a better activity than that of the first line treatments in clinic. However, the researches in the field of dual topoisomerases inhibitors, also in the past decade, are still limited. In particular, most of the *in vivo* antitumor studies are limited in the acridine derivative **103** (Hassan et al., 2011); the evodiamine analogues **10-12** (Dong et al., 2012) and synthetic compounds as **98** (Known et al., 2015), **101** (Aichinger et al., 2020), **117** (Tseng et al., 2013a) and **141** (Chaniyara et al., 2013). In general, few *in vivo* tests of dual topo inhibitors have been reported. Further *in vivo* studies should be done for those compounds with good cytotoxities *in vitro*, such as multiple TopoI/TDP1/TDP2 inhibitors azaindenoisoquinolines **161-166** (Wang et al., 2017), (Table 1) to evaluate their real therapeutic potential.

Of note, although some of the compounds exhibited significant cytotoxity towards tumor cells, the selectivity between tumor cells and normal cells have not been investigated in most cases and this could directly impact on their future prospect of clinical application.

In the last decade, in the quest for new dual topoisomerases inhibitors, many strategies have been used, such as scaffold hopping, molecular hybridization, bio isosteric replacement, conformational restriction, as well as guiding by the available SAR results. During the design process, the binding patterns of representative compounds may offer inspiration for discovering more potent topo inhibitors; these findings highlighted the importance to perform, for example, in silico docking to gain experimental evidence on the interactions that the herein described compounds establish by binding to TopoI and TopoII.

In this context, the aim of the present review is to provide new perspectives on existing data regarding multiple modulators of topoisomerases and/or tyrosyl-DNA phosphodiesterases, in order to promote future researches aimed to the development of increasingly potent, efficient and safe anticancer agents for the clinical usage.

Table 1. Properties of the most interesting compounds reviewed.

Cmp	Class	Enzymatic activity	Citotoxicity *in vitro*	Citotoxicity *in vivo*	Other	Ref.
10-12	Evodiamine (natural compound) derivatives	TopoI poisons, TopoII catalytic inhibitors Concentration value = 50 μM	GI_{50} = 0.003 μM for A549,MDAMB-435, HCT116	Inhibition of the tumor growth at the dose of 2 mg/kg on HCT116 xenograft model	No DNA intercalation	Dong et al., 2012
17	Cinnamonum constituent (natural compound)	TopoI and TopoII inhibitor Concentration value = 20 μM	IC_{50} = 12.11 μM for NCIH520	Inhibition of the tumor growth at the dose of 10 or 20 mg/kg/day on NCIH520 xenograft model	Apoptosis activating caspases 3 and 9	Perng et al., 2016 Liu et al., 2017
Δ-21, **Λ-21**	Ruthenium(II) complexes	TopoI and TopoII poisons IC_{50} = 3-15 μM	IC_{50} = 40.1 μM for Hep-G2	-	DNA intercalation; apoptosis in Hep-G2 cells	Zhang et al., 2013
98	Benzofuropirydine	TopoI and TopoII catalytic inhibitor Concentration value = 100 μM	IC_{50} = 0.15 μM and 0.83 μM for HCT15 and T47, respectively	Significant inhibition of tumor growth in HCT15 xenograft model	No DNA intercalation; Apoptosis and G1 arrest in T47D cells	Kwon et al., 2015
101	Phenanthroline	TopoI and TopoII poison Concentration value ≥ 1 μM	broad-spectrum effect at nM concentrations on NCI-60 DTP	Reversible toxic effects at 5 mg/Kg body weight on athymic nude mice	Potent DNA-damaging properties	Meier et al., 2017 Aichinger et al., 2020
103	Acridine	TopoI and TopoII inhibitor Concentration value = 5 μM	IC_{50} = 1.38 μM for CRC	HIF-1α inhibition associated to inhibition of tumor growth *in vivo*	DNA intercalation; druggable features (Lipinski's rule)	Hassan et al, 2011
114	Benzophenazine	TopoI poisons, TopoII catalytic inhibitor Concentration value = 25 μM	IC_{50} = 0.22 μM for HL-60	-	DNA intercalation	Yao, et al., 2015
117	Indenoquinoline	TopoI and TopoII inhibitor IC_{50} values: 2.63 μM and 4.95 μM, respectively	IC_{50}: 0.39 μM for HeLa	Oral administration of 10 mg/kg dramatically reduced tumor growth in BT483 xenograft	No DNA intercalation	Tseng et al., 2013a
141	Indolizinoindole	TopoI and TopoII inhibitor Concentration value = 25 μM	broad spectrum of antitumor activity against many solid	At 25 mg/kg suppression of tumor growth in HT-29	DNA double-strand cross-linking;	Chaniyara et al., 2013

			tumor and no cross resistance	xenograft models	Apoptosis modulating caspase-3, caspase-7, and PARP	
161-167	Azaindenoisoquinolines	Multiple TopoI/ TDP1/TDP2 inhibitors IC_{50} values: <1 µM, 1-12 µM, 12-37 µM, respectively	GI_{50} in the low to subµM range against 60 human cancer cell lines (NCI-60)	-	greater DNA damage in cancer vs normal cells	Wang et al., 2017

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Graphical abstract