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Kinase targets in CNS drug discovery

Originally thought to be nondruggable, kinases represent attractive drug targets for pharmaceutical companies and academia. To date, there are over 40 kinase inhibitors approved by the US FDA, with 32 of these being small molecules, in addition to the three mammalian target of rapamycin inhibitor macrolides (sirolimus, temsirolimus and everolimus). Despite the rapid development of kinase inhibitors for cancer, presently none of these agents are approved for CNS indications. This mini perspective highlights selected kinase targets for CNS disorders, of which brain-permeable small-molecule inhibitors are reported, with demonstrated preclinical proof-of-concept efficacy. This is followed by a brief discussion on the key challenges of blood–brain barrier penetration and selectivity profiles in developing kinase inhibitors for CNS disorders.

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Keywords: blood–brain barrier • brain cancer • dual-specificity kinase inhibitors • GSK-3 • kinase • LRRK2 • neurodegenerative diseases

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Kinases as drug targets

Constituting a major part of phosphotransferases (EC 2.7 enzymes) in the human genome, kinases mediate the phosphorylation of various biological substrates including lipids, sugars and proteins, thereby regulating various important cellular functions, such as cell growth, proliferation, cell survival and metabolism. The protein kinases account for a large proportion of the phosphotransferases and can be divided into three main classes: Ser/Thr kinases, Tyr kinases and mixed protein kinases. Ser/Thr kinases phosphorylate the hydroxyl group of either serine or threonine residues, whereas Tyr kinases phosphorylate only tyrosine residues. The mixed kinases can phosphorylate the hydroxyl group of Ser, Thr and Tyr residues and thus termed dual-specificity kinases. In addition, lipid phosphorylation, mainly for the phosphoinositides (PI) family, is tightly controlled by PI kinases. To date, the human kinome comprises 518 protein kinases and 20 lipid kinases, contributing to approximately 1.7% of human genes [1].

As virtually all signal transduction is tightly regulated by the reversible phosphorylation processes involving kinases and phosphatases, malfunctioning of kinase activity has been linked to a wide range of diseases, including cancer, CNS disorders, vascular and chronic inflammatory conditions. In the past two decades, protein kinases have emerged as prominent drug targets which are being perused by pharma and academia. Previously thought to be nondruggable, presently more than 40 kinase inhibitors, including small molecules and antibodies, have been approved by the US FDA [2]. It must be noted that small molecules account for the major proportion of these agents [3,4], with 32 of them listed in Table 1 (last updated in August 2016). Idelalisib is the only lipid kinase inhibitor, with all the other 31 inhibi-

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tors being protein kinase inhibitors. The major driving force for the development of kinase inhibitors has been the demand for cancer therapeutics, with the approval of imatinib (Gleevec®) in 2001 for chronic myeloid leukemia as a defining moment, paving the way for kinase inhibitor drug discovery. Strikingly, out of the 32 agents, only two are used for noncancer indications: tofacitinib for rheumatoid arthritis and nintedanib for idiopathic pulmonary fibrosis and none for CNS indications. Moreover, the long-term use of some kinase inhibitors for peripheral oncology has been associated with reports of metastases to the CNS, particularly for those inhibitors that are not CNS-penetrant. Brain metastases in lung and breast cancer have particularly stood out, further emphasizing the urgent, unmet chemotherapy needs related to the CNS malignancy [5]. A recent perspective article summarizes the CNS penetration extent of the approved kinase inhibitors and their potential use for treatment of brain cancer, assessing the free brain-to-plasma ratios and whether or not they interfere with P-glycoprotein or breast cancer resistance protein transporters [6].

The focus of this mini perspective is therefore to highlight kinase targets for CNS indications with known brain-penetrant small-molecule inhibitors during the last decade. Previous review article by Chico *et al.* [8] discusses the main challenges in developing kinase inhibitors for CNS conditions as well as providing some examples of CNS protein kinase targets. Another more recent review article [2] provides a comprehensive analysis of kinase targets currently in clinical use and in clinical trials. Herein a brief update of selected kinase targets for CNS indications is presented with emphasis on the kinase targets with reported small molecules that have been shown to be brain-permeable, and/or demonstrating efficacy in animal models. GSK-3 and LRRK2 are the two outstanding kinase targets for CNS indications that have received much attention by both Pharma and academia. Other emerging kinase targets, such as DYRK1A and cyclin-dependent kinase 5 (CDK5) will also be discussed. This is followed with concluding remarks on the issues of blood–brain barrier penetration and true selectivity profiles during the development of small-molecule kinase inhibitors.

Established kinase targets for CNS indications

GSK-3

GSK-3 is a ubiquitously expressed Ser/Thr kinase with versatile functions in many key aspects of physiological events, such as metabolic homeostasis, signal-to-gene transcription, cell growth/death and neurogenesis. Two isoforms: GSK-3α (51 kDa) and GSK-3β (47 kDa) are present in mammals, with the β-isoform being highly expressed in the nervous system. Initially reported to be the kinase that phosphorylates and inactivates glycogen synthase, more than 50 protein substrates for GSK-3 have been identified. Regulation of GSK-3 is achieved via the phosphorylation of amino acid residues Tyr279/Tyr216 (GSK-3α/β) and Ser21/Ser9 (GSK-3α/β), leading to its subsequent activation or inhibition, respectively.

Table 1. Current US FDA-approved small-molecule kinase inhibitors (as of August 2016).

Year	FDA-approved small-molecule kinase inhibitor					
2001	Imatinib	–	–	–	–	–
2002	–	–	–	–	–	–
2003	Gefitinib	–	–	–	–	–
2004	Erlotinib	–	–	–	–	–
2005	Sorafenib	–	–	–	–	–
2006	Dasatinib	Sunitinib	–	–	–	–
2007	Nilotinib	Lapatinib	–	–	–	–
2008	–	–	–	–	–	–
2009	Pazopanib	–	–	–	–	–
2010	–	–	–	–	–	–
2011	Ruxolitinib	Vemurafenib	Vandetanib	Crizotinib	–	–
2012	Bosutinib	Regorafenib	Cabozantinib	Axitinib	Tofacitinib	Ponatinib
2013	Dabrafenib	Trametinib	Afatinib	Ibrutinib	–	–
2014	Idelalisib	Nintedanib	Ceritinib	–	–	–
2015	Lenvatinib	Palbociclib	Osimertinib	Cobimetinib	Sonidegib	Alectinib

Adapted from [3,7]

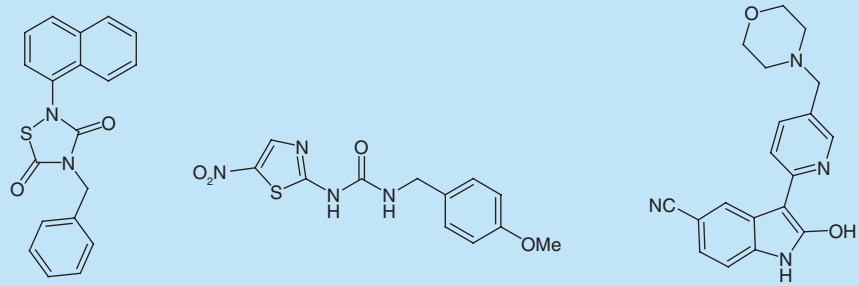
Lithium, the mainstay treatment for bipolar disorder, directly inhibits GSK-3, and many mood-stabilizing behavioral aspects of lithium action are mirrored by GSK-3 inhibition [9]. Overactivation of GSK-3 has been implicated in numerous other CNS diseases, including Alzheimer's disease (AD) [10], mood disorders [11], brain cancer [12] and autism spectrum disorders [13], consistent with its multifunctional activities on many *bona fide* protein substrates. In this regard, it is not surprising that a myriad of chemical scaffolds have been reported as small-molecule GSK-3 inhibitors (Figure 1). The most clinically advanced GSK-3 inhibitor for CNS indication is tideglusib (NP-12) for use in AD. However, in its Phase II study it did not meet end point criteria [14]. AR-A014418 and AZD-1080 are brain-permeable GSK-3 inhibitors showing efficacies in animal models of AD [15,16]. For mood disorders, the maleimide ING-135 (aka BIP-135) has been demonstrated to exert antimanic effects *in vivo* [17]. A more recent review summarizes a body of evidence that links inhibition of GSK-3 to the amelioration of cognitive impairments in various animal models of CNS disorders, such as AD, Fragile X syndrome, Down syndrome, traumatic brain injury and others [18]. In addition, there is a growing number of reports supporting the use of GSK-3 inhibitors for the treatment of brain cancer, with brain-permeable 9-ING-41 at the forefront, having received accelerated FDA orphan drug designation for treatment of glioblastoma in early 2016 [19]. Some safety concerns were raised in that long-term GSK-3 inhibition could lead to oncogenic effects. However, this can be argued by the observation that long-term use of lithium in patients with bipolar disorder since 1970s has not been associated with oncogenicity. Collectively, GSK-3 represents the most popular kinase target for CNS indications, although clinically approved inhibitors are yet to reach the market.

LRRK2

Mutations in LRRK2 represent the most common genetic cause of Parkinson's disease (PD). Since its discovery in 2004, more than 50 genetic variants have been reported, with G2019S being the most common form [20]. The phenotype of patients with LRRK2 mutations is essentially identical to the idiopathic PD, suggesting the therapeutic potential of targeting this kinase. Majority of the LRRK2 mutations within its catalytic domain lead to the hyperactivation of the kinase function. These findings have culminated in drug discovery efforts to develop brain-penetrant LRRK2 inhibitors (Figure 2) and to assess their potential as PD therapeutics [21,22]. Genentech's aminopyrimidines exemplified by GNE-7915 and GNE-0877 were first disclosed as selective LRRK2 inhibitors with unbound brain/unbound plasma ratio of approximately 0.5 [23]. More recently, Pfizer's PF-06447475 [24], Elan's LRRK2 quinoline 14 [25], Novartis' LRRK2 indolinone 11 [26] and HG-10-102-01 [27] have also been reported as brain-penetrant small-molecule LRRK2 inhibitors with target engagement in the brain. Some key questions for targeting LRRK2, however, revolve around the lack of information on the endogenous substrates of LRRK2 and the exact mechanisms of their regulation [28]. This dearth of information has so far necessitated the development of surrogate biological conditions for testing of LRRK2 inhibitors. Peripheral organ toxicity, particularly in lung and kidney [29], has also been raised as a major safety concern associated with LRRK2 inhibition. Future work on the LRRK2 inhibitors would therefore be needed to address the observed safety issues together with establishing proof-of-efficacy in preclinical models of PD.

p38 MAPKs

MAPKs are a superfamily of Ser/Thr kinases that comprises three main signaling pathways: the p38 kinases (p38α, p38β, p38γ and p38δ), the c-Jun N-terminal kinases (JNKs [JNK1, JNK2, JNK3]) and extracellular signal-regulated kinases (ERKs [ERK1 and ERK2]). To complete the MAPK cascades [30], upstream of the MAPKs are the MAPK kinases (MAP2Ks) and MAPK kinase kinases (MAP3Ks, e.g., mixed lineage kinases (MLKs)), whereas downstream of the MAPKs are MAPK-activated protein kinases (MAPKAPKs), such as MAPK-activated protein kinase 2 (MK2), ribosomal S6 kinases (RSKs) and MAPK-interacting protein kinases (MNKs). In particular, activation of p38 MAPK has been well established in AD patients and AD animal models [31]. The p38α MAPK represents a kinase target that has been targeted with a brain-permeable, orally active small molecule MW01-2-069A-SRM (Figure 3) and studied in an animal model of AD [32]. Administration of MW01-2-069A-SRM suppresses the upregulation of proinflammatory cytokines in mice as well as attenuating synaptic protein loss and behavioral deficits. Subsequently, MW01-18-150SRM has also been reported as a p38α MAPK inhibitor that suppresses associative and spatial memory deficits in transgenic animal models of AD [33]. VX-745 is currently in Phase II clinical trial for AD patients with mild cognitive impairment [34]. So far, however, no p38α inhibitors are clinically used as a result of either lack of efficacy or undesirable side effects stemming for inhibition of other anti-inflammatory molecules [28]. As such, MK2, which is a protein kinase downstream of the p38α MAPK, has recently emerged as a promising kinase target that is also a key regulator of cell-



Tideglusib

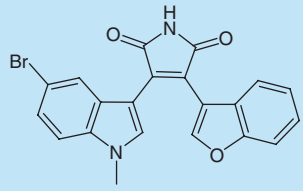
logP 3.69
logD 3.69
TPSA 65.92
MW 334.39
HBD 0
pKa -1.5

AR-A014418

logP 2.02
logD 1.74
TPSA 140.32
MW 308.31
HBD 2
pKa -0.4

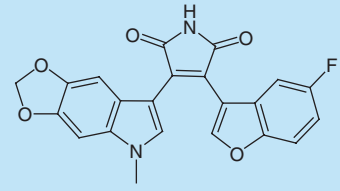
AZD-1080

logP 1.91
logD 1.84
TPSA 85.17
MW 334.37
HBD 2
pKa 5.5



ING-135 (BIP-135)

logP 4.29
logD 4.01
TPSA 64.24
MW 421.24
HBD 1
pKa -6.8



9-ING-41

logP 3.07
logD 2.68
TPSA 82.7
MW 404.35
HBD 1
pKa -7.1

All six of the calculated physiochemical parameters were obtained from ACD/Labs Percepta 2016 Build 2911 (13 Jul 2016).

Figure 1. Brain-penetrant GSK-3 inhibitors.
HBD: Hydrogen bond donor; TPSA: Topological polar surface area.

cycle checkpoints [35]. In addition, Chk1 and Chk2 as well as Wee1 kinases have also received attention due to their inhibitory effects on DNA-damaging response caused by chemotherapeutic agents.

Emerging kinase targets for CNS indications

DYRK1A

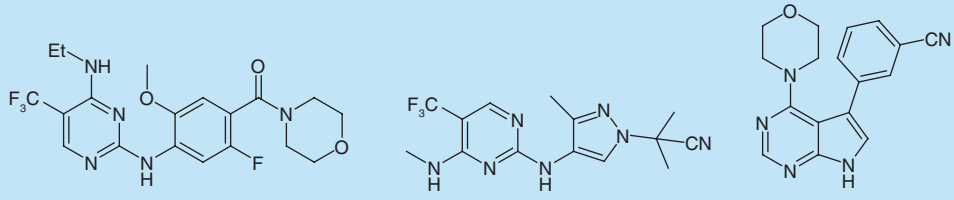
DYRK1A is member of the CMGC family that comprises CDKs, MAPKs, GSKs and CDK-like kinases. One of the most studied substrates of DYRK1A is the T protein, and DYRK1A has been shown to be an important priming kinase before further T hyperphosphorylation occurs by GSK-3β [36,37]. Overexpression of DYRK1A has been associated with cognitive deficits and a number of reported small-molecule DYRK1A inhibitors have been studied in AD models [38]. Harmine and its analogs (Figure 4) represent one of the earliest reported DYRK1A inhibitors, but the studies are mostly limited to *in vitro* activities. Epigallocatechin gallate is a natural constituent of green tea that has been reported as a modest DYRK1A inhibitor (IC_{50} = 330 nM) [39] and underwent Phase II clinical trials in patients with early AD [40] and multiple sclerosis (MS). Other small molecules, such as leucettine L41 and others have been previously reviewed [38]. More recently, quinazolines EHT5372 and EHT1610 have been reported as highly potent and selective DYRK1A/B inhibitors [41].

CDK5 & CDK4/6

CDK5 is a Ser/Thr kinase that belongs to the family of CDKs, of which 13 members have been identified. Known to be required for mammalian brain development [42], CDK5 was shown to be involved in the T phosphorylation alongside GSK-3. Overexpression of CDK5 and p25 (a truncated version of p35) complex hyperphosphorylates T and subsequently promotes neurodegeneration in AD [43]. Roscovitine (seliciclib) is a typical CDK5 inhibitor, although it also inhibits other CDKs to a weaker extent, particularly CDK2 (Figure 5). Both isomers of roscovitine are brain penetrant and the (*S*)-isomer was demonstrated to be neuroprotective in animal models of stroke [44]. Other molecules that have been shown to inhibit CDK5 include bellidin [45], alsterpaullone [46] and indirubin 3′-oxime [47], with the latter two compounds also known as GSK-3 inhibitors. Abemaciclib, an inhibitor of CDK4/6, has been recently shown to increase survival time in U87 glioblastoma model [48]. It is reported to be brain permeable with free brain/free plasma ratio of approximately 0.2 in mice, despite being a substrate for P-glycoprotein and breast cancer resistance protein.

ROCK

Rho-associated coiled-coil containing kinase (ROCK) is a ubiquitous Ser/Thr kinase that is activated by Rho GTPases and belongs to the protein kinase-A, -G and -C (AGC) family of kinases. Since its discovery



GNE-7915

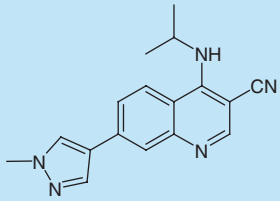
logP 2.89
logD 2.89
TPSA 88.61
MW 443.4
HBD 2
pKa 3.8

GNE-0877

logP 2.41
logD 2.41
TPSA 91.45
MW 339.32
HBD 2
pKa 4

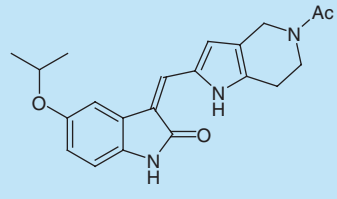
PF-06447475

logP 2.15
logD 2.14
TPSA 77.83
MW 305.33
HBD 1
pKa 5.9



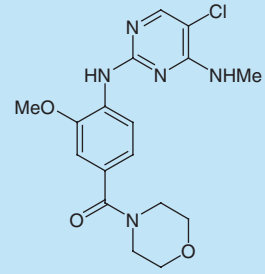
Elan's quinoline 14

logP 2.66
logD 2.66
TPSA 66.53
MW 291.35
HBD 1
pKa 4.7



Novartis' indolinone 11

logP 2.33
logD 2.33
TPSA 74.43
MW 365.43
HBD 2
pKa 2.6



HG-10-102-01

logP 1.87
logD 1.87
TPSA 88.61
MW 377.83
HBD 2
pKa 4.8

All six of the calculated physiochemical parameters were obtained from ACD/Labs Percepta 2016 Build 2911 (13 Jul 2016).

Figure 2. Brain-penetrant LRRK2 inhibitors.
HBD: Hydrogen bond donor; TPSA: Topological polar surface area.

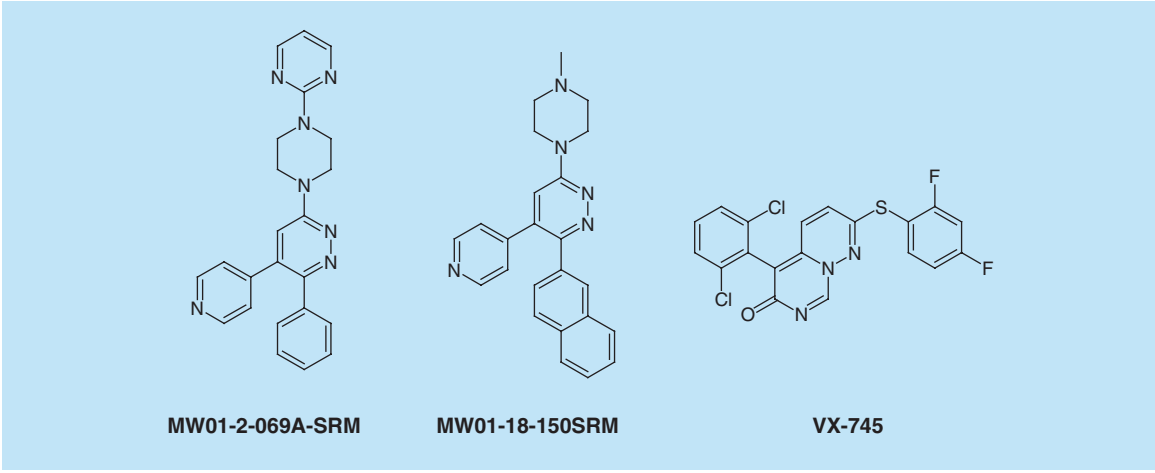


Figure 3. Brain-penetrant inhibitors of p38 MAPKs.

in 1996, inhibition of ROCK has been demonstrated to be beneficial in a range of CNS diseases, including AD, MS, stroke and neuropathic pain [49]. Two isoforms are present, namely ROCK I and ROCK II, with fasudil and Y27632 (Figure 6) as the two most well-studied compound tools, but with low blood–brain barrier penetration. Thus far, there has been limited number of reports describing brain penetration of ROCK inhibitors. One more recent example is the use of KD025 as a ROCK II inhibitor in animal models of focal cerebral ischemia [50].

Tyrosine kinases & other kinases for MS

MS is a chronic autoimmune disorder of the brain and spinal cord manifested in demyelination, gliosis and neuroaxonal degeneration. Fingolimod is the first

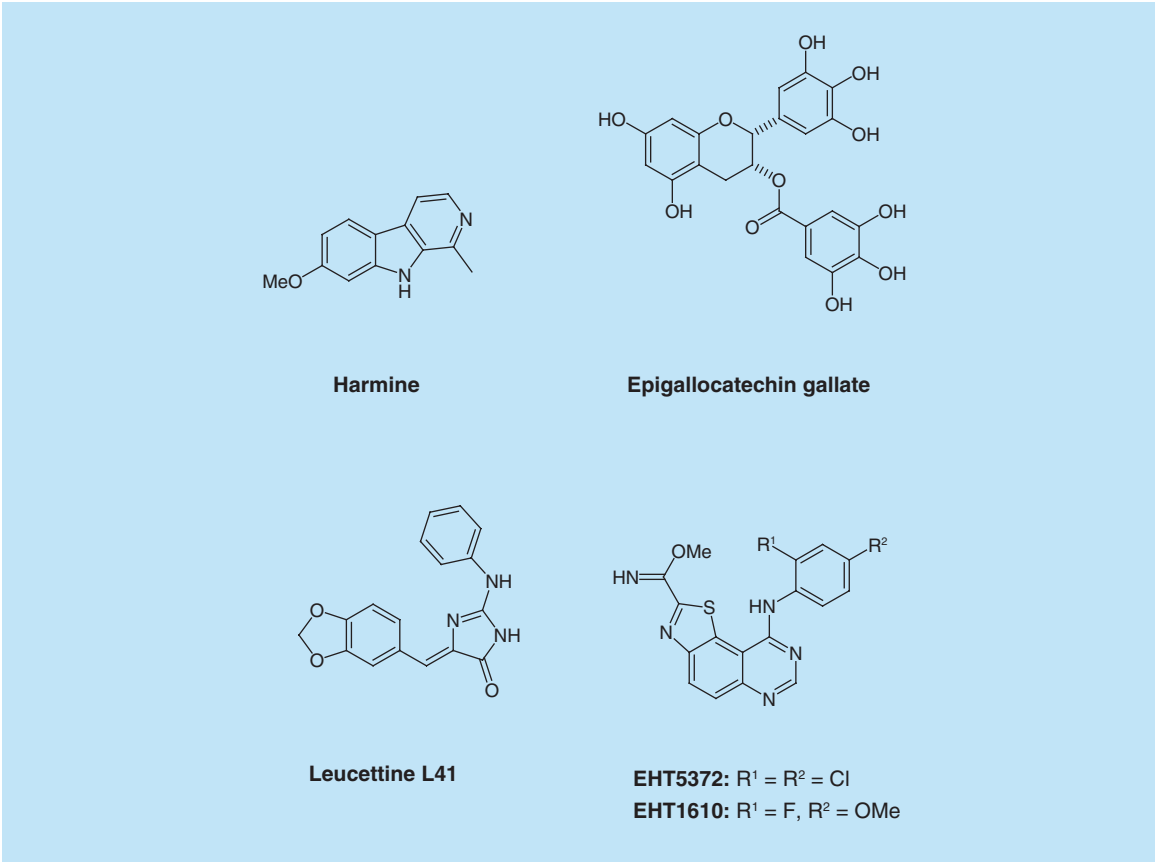


Figure 4. Examples of small-molecule DYRK1A inhibitors.

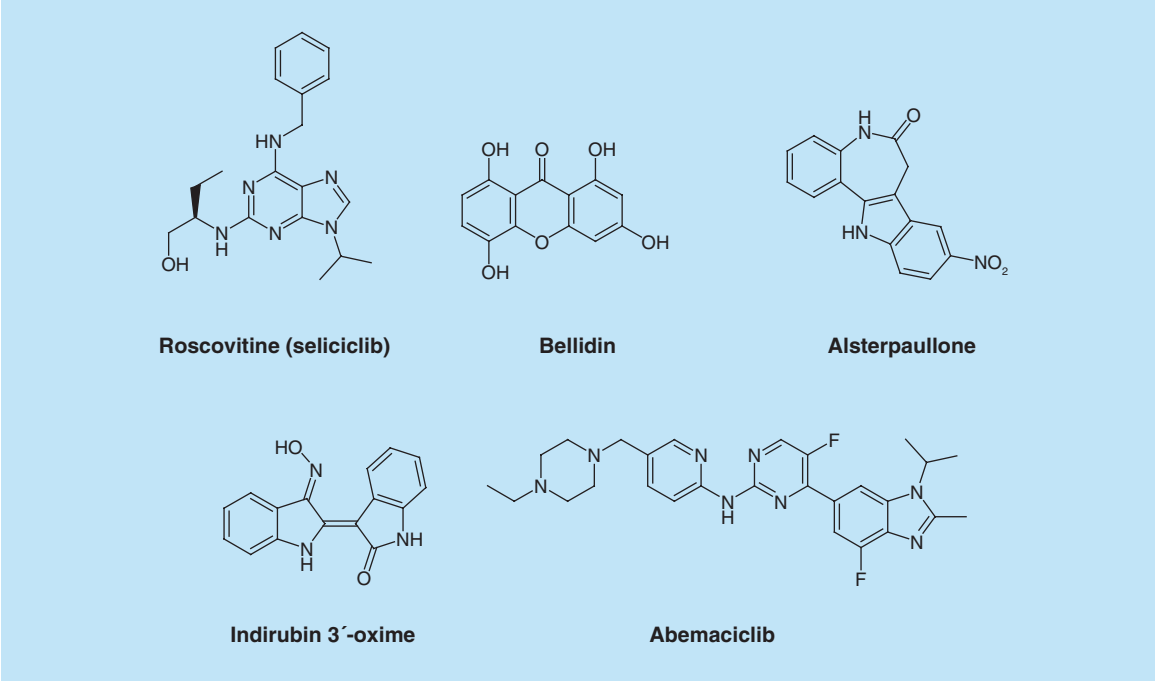


Figure 5. Brain-penetrant inhibitors of CDK5 and CDK4/6.

approved oral treatment in MS, possessing a unique mechanism of action involving kinase-mediated activities. It is a prodrug that becomes phosphorylated by sphingosine kinase 2 once entered the brain to form fingolimod phosphate. The binding of fingolimod phosphate to sphingosine 1 phosphate receptor (S1P1) and other subtypes (S1P2/3/5) results in a long-lasting internalization of S1P1 receptors, thereby effectively leading to downmodulating S1P1-mediated inflammatory responses [51]. Masitinib, a selective tyrosine kinase inhibitor, is currently undergoing a Phase IIb/III clinical study in patients with primary progressive or secondary progressive MS (NCT01433497) [52]. In an experimental autoimmune encephalomyelitis model of MS, treatment with GSK-3 inhibitor lithium repressed disease severity [53] and a Phase I/II clinical trial of lithium in MS patients has just been completed (NCT01259388). More recently, inhibition of RIPK2 has also been reported to reduce progression in experimental autoimmune encephalomyelitis mouse model [54].

Conclusion & future perspective

Kinases have established themselves as *bona fide* therapeutic targets for drug development, as evidenced in the increasing number of FDA-approved agents and the paradigm shift in solid cancer therapy toward small molecule or antibody-based kinase inhibitors. While the development of kinase inhibitors is still mainly concentrated in field of oncology, a number of kinase-targeting candidate therapeutics discussed above have entered various stages of clinical evaluation for CNS indications. It is noteworthy to mention the increasing trend of academic institutions as the drivers of CNS-targeting kinase inhibitor drug discovery efforts. A holistic approach toward assessment of drug-likeness properties affecting overall brain penetrance, for example Pfizer's CNS multiparameter optimization[55], which takes into account ClogP, ClogD, MW, total polar surface area, number of hydrogen bond donors and pKa of ionizable groups, has been useful in prioritizing lead candidates. In this context, a majority of the currently approved kinase inhibitors listed in Table 1 have MWs of over 500 g/mol, accompanied with high number of hydrogen bond donors and total polar surface area, thus limiting their CNS penetration. In addition to the free brain/free plasma ratio determination, demonstrating target engagement for the intended kinase inhibitors is of paramount importance. Toward this end, newer methodologies to determine drug penetration and target engagement in brain tissue have started to emerge. The use of MALDI mass spectrometry imaging to monitor drug transit through the blood–brain barrier without the need of radiolabeling was recently reported [56]. Mass spectroscopy based assays have also been demonstrated to be useful in identifying direct target engagement biomarkers for 3-PI-dependent kinase-1 inhibitors [57].

Although the majority of drug developers in the last two decades have focused on selective molecules targeting one specific biological target of interest (single-targeting), there has been a slow yet emi-

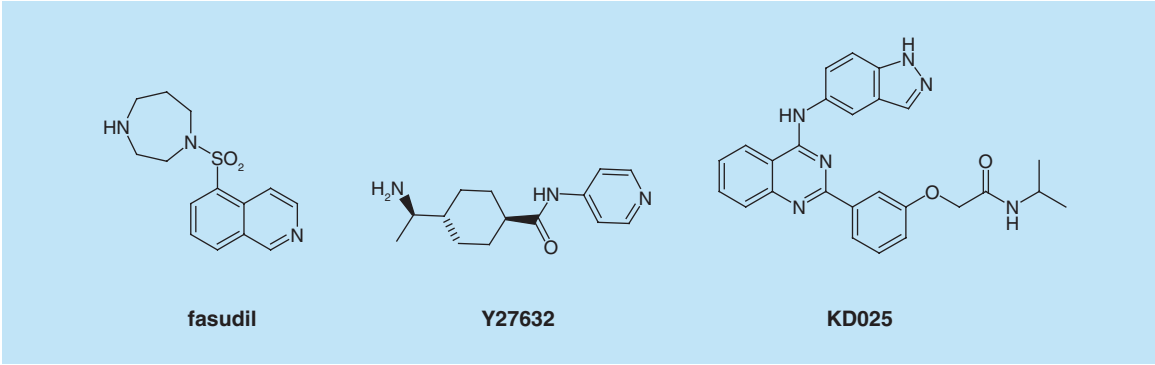


Figure 6. Selected ROCK inhibitors.

nent transition toward the development of molecules imparting their therapeutic action via a set of molecular targets instead (multitargeting). This trend is also currently observed in kinase drug discovery for cancer and CNS indications. The promiscuity has been long thought to be associated with unwanted adverse side effects and toxicity profiles. One such example is the observed toxicity profile of lithium as a result of its multiple mechanisms of action [9] not only on GSK-3 but also on inositol monophosphatase as well as other kinases, such as casein kinase 2, p38-regulated/activated protein kinase and MAPKAPK2 among others [58]. Furthermore, clinical investigations on the use of tamoxifen for bipolar mania revealed adverse side effects as a consequence inhibiting several isoforms of protein kinase C [59–62]. On the other hand, lessons learned from the failed clinical trials for CNS indications and increasing understanding of the complex etiology of the CNS diseases may imply that cleaner, more selective drugs are less likely to work in the clinic compared with drugs that target a set of molecular pathways implicated in the disease pathophysiology. Even among those regarded as 'selective' kinase inhibitor, caution must be exercised as not all selective kinase inhibitors for a particular kinase target are created equal. Different ATP concentrations used during the functional assay and the selection of representative kinases being tested for its selectivity profile make comparison difficult in determining the true extent of the compound's selectivity. A comprehensive kinome screening profile as well as examination at common neuroreceptors, enzymes and transporters, would be a more accurate measure of true selectivity, but at the expense of the associated high costs. Owing to the complex array of neuroreceptors and subsequent downstream signaling pathways, it is likely that kinases will constitute a major drug target for CNS indications in the next 5–10 years.

An increasing number of kinase targets are being discovered as key modulators of drugs' therapeutic effects, for example, the importance of GSK-3 and eukaryotic elongation factor 2 kinase in the antidepressant effects of ketamine. For AD, recently reported inhibitions of 3-PI-dependent kinase-1 [63] and eukaryotic initiation factor 2α [64] were demonstrated to alleviate memory impairments in animal models of AD. There has also been an upward trend toward improving the brain penetration extent of the currently FDA-approved small-molecule kinase inhibitors. For example, among the nine approved VEGFR inhibitors, cabozantinib has been reported not to be a Pgp substrate, and was subsequently evaluated in a Phase II study of glioblastoma [65,66]. EGFR is yet another popular kinase target with six agents currently FDA-approved and quinazoline AZD3579 has recently been developed as an orally active, brain-penetrant EGFR inhibitor that induces regression of brain metastases in a mouse model [67]. Overall, kinases represent *bona fide* targets for CNS drug discovery and the future clinical use of brain-permeable kinase inhibitors will continue to have a significant impact in the treatment of human conditions.

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Executive summary

Kinases as drug targets

- Kinases mediate the phosphorylation of lipids, sugars and proteins. To date, 518 protein kinases and 20 lipid kinases are known.
- Thirty two small-molecule kinase inhibitors are currently approved by the US FDA. Only two are used for noncancer indications, and none for CNS indications.

Established kinase targets for CNS indications

- GSK-3 is a ubiquitously expressed serine/threonine kinase with versatile functions. Two isoforms (α and β) are present in mammals, with the β-isoform being predominantly expressed in the nervous system.
- Hyperactivation of GSK-3 has been implicated in numerous CNS disorders, including Alzheimer's disease (AD), mood disorders, brain cancer and autism spectrum disorders.
- Inhibition of GSK-3 has been linked to the amelioration of cognitive impairments in various animal models of CNS disorders, such as AD, Fragile X syndrome, Down syndrome, traumatic brain injury and others.
- A GSK-3 inhibitor, 9-ING-41 received an accelerated FDA orphan drug designation for treatment of glioblastoma.
- LRRK2 is an established kinase target as a potential therapeutic for Parkinson's disease.
- More than 50 genetic variants of LRRK2 have been reported, and the phenotype of patients with LRRK2 mutations closely resembles that of idiopathic Parkinson's disease.
- Brain-penetrant small-molecule LRRK2 inhibitors include: GNE-7915, GNE-0877, PF-06447475 and others.
- p38α MAPK represents a kinase target for AD, with reported brain-penetrant molecules, such as MW01-2-069A-SRM and VX-745.

Emerging kinase targets for CNS indications

- DYRK1A is a member of CMGC kinase family that includes CDKs, MAPKs, GSKs and CDK-like kinases.
- DYRK1A is an emerging kinase target for neurodegenerative diseases and cancer.
- Roscovitine, a brain-penetrant CDK5 inhibitor, has been shown to be neuroprotective in animal models of stroke.
- Abemaciclib, a brain-penetrant inhibitor of CDK4/6, has been shown to increase survival time in U87 glioblastoma model.
- ROCK is a serine/threonine kinase that is activated by Rho GTPases and belongs to the AGC family of kinases. Two isoforms are known: ROCK I and ROCK II.
- Despite its potential therapeutic target in a range of CNS disorders, including AD, multiple sclerosis (MS), stroke and neuropathic pain, there has been a dearth of brain-penetrant ROCK inhibitors. KD025 represents a recent example of ROCK II inhibitor that has been shown to be effective in animal models of focal cerebral ischemia.
- Fingolimod, currently approved oral drug for MS, is a prodrug that undergoes phosphorylation by sphingosine kinase 2 in the brain to form fingolimod phosphate.
- Tyrosine kinase inhibitors, such as masitinib and CC-292, show promising results in preclinical studies of inflammatory disorders.
- Other kinases, such as Bruton's tyrosine kinase, GSK-3 and RIPK2 have also been reported to be beneficial in animal models of MS.

Conclusion & future perspective

- Kinases are *bona fide* therapeutic targets in the current oncology pipeline, and will continue their strong trend as drug targets for CNS indications.
- The extent of blood–brain barrier penetration is a key aspect in development of small molecules for CNS disorders.
- Caution must be exercised when describing selectivity profiles of reported kinases inhibitors. There has been a paradigm shift from single-targeting to multitargeting for the development of kinase inhibitors.
- More kinase targets are being discovered as key modulators in CNS therapy. Brain cancer is likely to be the clinical application for the future kinase inhibitor for CNS indication.

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Editorial

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Future Medicinal Chemistry

Beyond the wall: can D-amino acids and small molecule inhibitors eliminate infections?

"Recently, the use of small molecules produced by bacteria became a prominent strategy of biofilm dispersal in medicinal microbiology."

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Bacterial multicellular communities called biofilms thrive in a variety of conditions, as they provide significant benefits to the resident bacteria. In clinical settings, biofilms are often associated with persistent infections, and their formation can have deadly outcomes. In a biofilm, the bacteria are effectively sheltered from environmental insults. For example, biofilm cells can be up to 1000 times more resistant to antibiotics than planktonic (free-living) cells [1,2]. The mechanisms supporting this resistance are poorly understood.

Biofilms are tightly held together by a self-produced organic extracellular matrix, as well as by biogenic minerals [1,3]. The extracellular matrix is composed of aggregated proteins, exopolysaccharides and nucleic acids often tightly associated with the bacterial cell envelope [1]. However, once the nutrients available to the community are exhausted, the biofilm state is no longer beneficial for the bacteria – and it is actively dispersed. In the last decade, it was discovered that bacteria often generate small molecules to aid dispersal. Those molecules transcend the diffusion limiting environment and disrupt the complex extracellular matrix encapsulating biofilm cells [4]. Recently, the use of small molecules produced by bacteria became a prominent strategy of biofilm dispersal in medicinal microbiology [4].

Here, we discuss small molecules that interfere with biofilm formation. We will focus on the complex mode of action and applications of D-amino acids (DAAs) – biofilm inhibitors and dispersal agents which are widely produced by bacteria. We will also discuss how the insights from the study of DAAs can be applied to the study of additional small molecule biofilm inhibitors.

Nearly, all bacteria synthesize a cell wall located outside of the cell membrane. This strong yet elastic network counteracts osmotic pressure, maintains cell shape and serves as a protective barrier against physical, chemical and biological assaults [5,6]. In both Gram-positive and Gram-negative bacteria, the cell wall is composed of a peptidoglycan (PG) polymer – glycan chains (alternating *N*-acetylglucosamine) and *N*-acetylmuramic acid) cross-linked by short peptides. Those are called stem peptides, and normally include the canonical (DAAs), D-Alanine and D-Glutamate (or the amidated form, D-Glutamine). Unlike L-amino acids, DAAs are not used for ribosomal synthesis of proteins. Instead, their main role is as bacterial cell–wall constituents [5,6], though some exceptions have been observed [7]. Incorporation into PG is independent of DAA production; strains that fail to produce DAAs can nonetheless incorporate them into PG

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at the terminal position of the stem peptide. Diverse Gram-negative and Gram-positive bacteria also produce nonconventional DAAs – other than D-Alanine and D-Glutamate. Once incorporated into the stem peptides of the PG instead of D-Alanine, those DAAs reduce the transpeptidation of PG [6,8].

> "The specific role proposed for D-Tyr-tRNA deacylase (DTD) in translational quality control of biofilm development makes this enzyme a compelling therapeutic target for biofilm infections"

In 2010, my colleagues and I have reported that two Gram-positive bacteria – the beneficial *Bacillus subtilis* and the pathogenic *Staphylococcus aureus* – may utilize self-produced non-canonical DAAs to inhibit and disperse established biofilms [9]. It was observed that biofilm interference by DAAs is partially or fully rescued by addition of D-Alanine [9,10]. In agreement with the hypothesis that D-Alanine blocks the incorporation of DAAs into the bacterial envelope, incorporation of ^{14}C-D-Leucine into the cell wall is largely inhibited by applying access to D-Alanine [10]. The specific mechanisms that link cell–wall interference and biofilm formation remain to be determined. Intriguingly, macro-analysis of *B. subtilis* biofilms indicated that DAAs induced a malfunction of the anchoring of the extracellular matrix to the cell wall [10,11]. DAAs can also directly interfere with protein translation, leading to production of defective proteins. In some cases, aminoacyl tRNA synthase, an enzyme responsible for charging of tRNA with L-amino acids may instead load tRNA with DAAs, resulting into the formation of a highly toxic DAA–tRNA complex. It was first reported in *Escherichia coli* and *B. subtilis* that tRNA molecules are charged with D-tyrosine in the presence of tyrosyl tRNA synthetase [12], followed by similar reports on D-Valine, D-Aspartate and D-Tryptophan. Noncanonical DAAs were suggested to have a global role in inhibition of protein translation [13], although, this hypothesis was never tested directly, and a later study found no change in the overall protein levels in treated biofilm cells [10].

The proofreading activity of D-Tyr-tRNA deacylase (DTD) is responsible for hydrolyzing DAA–tRNA complex. It cleaves the bond formed between DAA and tRNA, freeing the tRNA to reconnect to the correct L-amino acid. In *B. subtilis,* the presence of a defective *dtd* allele sensitized the cells to the application of DAAs [10,13], which would be consistent with a general toxic effect of tRNA-DAA on translation. However, the biofilm inhibition clearly occurred at sub-toxic concentrations even in the presence of a defective *dtd* allele [10]. In addition, and in contrast to the expectations that the addition of D-Alanine will further impair translation and thus biofilm growth, its application actually rescued biofilm formation [9,10]. Furthermore, DAAs induced a translational response identical to a response induced by a canonical cell–wall stress [10]. Overall, those findings suggest an additional, unknown link between protein synthesis, cell–wall stress and the assembly of macrostructures. The specific role proposed for DTD in translational quality control of biofilm development makes this enzyme a compelling therapeutic target for biofilm infections [7].

DAAs interfere with biofilm formation & promote antibiotic sensitivity of biofilm

Initially, DAAs and their effects on biofilm were studied in *B. subtilis*, a Gram-positive root associated bacterium [1]. Since then, DAAs became an example of successful translational medicine approach – demonstrating how basic principles uncovered in a model organism can be translated into therapeutic strategies relevant to pathogens. The first pathogen model to be evaluated for DAAs sensitivity was the Gram-positive pathogen *S. aureus* [14,15]. Biofilms formed by *S. aureus* play a critical role in many device-related infections, infective endocarditis, urinary tract infections and acute septic arthritis. *In vitro*, *S. aureus* biofilms were found to be inhibited and dispersed by various DAAs. Later, local delivery of submolar concentrations of DAAs from biodegradable polyurethanes (PUR) scaffolds inhibited biofilm formation by clinical isolates of *S. aureus* both *in vitro* and *in vivo*. An equimolar mixture of D-Met:D-Pro:D-Trp shifted the dose–response curve toward lower doses compared with the individual DAAs and exhibited minimal cytotoxicity at concentrations that are effective at dispersing biofilms. Furthermore, the addition of DAAs enhanced the activity of several antibiotic classes against biofilms of genetically distinct isolates of methicillin-resistant *S. aureus*. The greatest synergy was observed in the combination of DAAs and rifampin. Interestingly, the authors report similar trends for the Gram-negative pathogen multidrug-resistant *Pseudomonas aeruginosa* [16], with a clear potentiation toward ciprofloxacin. DAAs may have augmented the activity of antimicrobials against biofilms by promoting biofilm dispersal. More recently, another Gram-positive pathogen emerged as a potential candidate for DAAs treatments: *E. faecalis* can cause life-threatening infections such as endocarditis, bacteremia, urinary tract infection and meningitis [17], and is especially problematic in hospitals where antibiotic resistance is prevalent. In this clinically relevant pathogen, DAAs were recently demonstrated to inhibit and disperse biofilms while restoring antibiotic sensitivity to the biofilm cells [18,19]. There is also an emerging

evidence for the potential role of DAAs in regulating biofilm formation of Gram-negative pathogens. While never tested for pathogenic *E. coli* strains involved in acute device related infections, DAAs directly inhibit adhesion to surfaces of a lab strain of *E. coli* [20]. In addition, the virulence of a prominent plant pathogen, *Xanthomonas citri* [21], is compromised in the presence of D-Leucine.

> "...a critical mass of labs and investigators must assess and validate new developments."

Recent technological and chemical breakthroughs are bridging the gap between the laboratory findings and the clinical applications for DAAs. For example, the development of noncytotoxic PUR scaffolds that allow a slow release of DAAs [15]. In a more recent study, a promising nanodevice for biofilm infections was introduced [22]. Under near infrared radiation (NIR), this nanodevice can release free DAAs, (D-Tyr) and ROS (·OH) in a specific spatiotemporal pattern, for combined biofilm dispersion and bacterial eradication.

The dispersing properties of DAAs were also demonstrated in industrial settings. On industrial occasions where biofilms are an issue, the term microbial fouling or biofouling is used. In industry, biofouling causes serious problems leading to energy and product losses [23]. DAAs were shown to combat biofouling in various settings. For example, D-Tyr triggered *P. aeruginosa* biofilm dispersal from membrane filters [24]. For *Desulfovibrio vulgaris*, a Gram-negative sulfur-reducing bacterium forming biofilms on carbon steel coupons, D-Tyr and D-Met effectively inhibited biofilm formation [25,26]. DAAs also inhibited biofilm formation in industrial water by direct interactions with exopolymeric substances [27]. In addition, DAAs enhanced the efficacy of THPS, a broad-spectrum biocide, in mitigation of *D. vulgaris* biofilms [27]. Most recently, DAAs were shown to augment the action of two additional industrial biocides against a biofilm consortium of sulfate reducing bacteria. The application of a DAAs cocktail that was previously optimized for *B. subtilis* [9] together with THPS reduced by a three to four order of magnitudes the biofilm cell counts compared with THPS alone, depending on the consortia composition [28]. Strikingly, just as in the original study [9], a mix of DAAs was extremely efficient in dispersing biofilm consortia, while the individual DAAs were less potent [28].

Biofilms are an optimal 'test-tube' for the development of novel therapeutics

The role of DAAs in biofilm inhibition and dispersal was originally described for *B. subtilis* and *S. aureus* [9,14]. Then, a handful of conflicting publications emerged, generating a discussion within the field [11,13]. Variation in results and conclusions at an early stage is not a unique to the study of DAAs. Another example to a difficult journey to consensus is establishing quorum-sensing as a target for biofilm interference in *P. aeruginosa* [29]. In 1998, Davies and colleagues suggested a role of the *P. aeruginosa las* quorum-sensing in biofilm formation [30]. In this first study, *lasI* mutants deficient in the synthesis of 3-oxododecanoyl-HSL formed biofilms that were flat, densely packed and homogenous relative to the highly structured, heterogeneous biofilms of the wild-type parent PAO1. Then, some conflicting reports emerged. Of note it was reported that a *lasI* mutant of PAO1 formed flat homogenous biofilms that were indistinguishable from the wild-type under their experimental conditions [29]. With time, the powerful premise of quorum-sensing inhibitors was established [29]. Just as in case of DAAs, the discrepancies in initial observations often reflect the limited methodology used to analyze the influence of small molecules on biofilm biology [29] and the inadequacy of relying on biofilm formation *per se* (e.g., 'all or none' analysis). Higher resolution single cell imaging techniques, as well as systemic transcriptional and translational profiling of the biofilm cells [10] are necessary for detection of functional differences in biofilms grown under laboratory conditions. Furthermore, it is clear that establishing a consistent and robust framework relying on multiple independent criteria for biofilm inhibition is essential, and several experimental conditions (e.g., growth media, temperature, inoculum condition) should be used [11]. If only a single condition is tested and the kinetics of biofilm development is not taken under consideration, false-negative results are likely. Similarly, the challenges of optimizing effective subtoxic concentrations may generate an understandable tendency to increase working concentrations toward nonspecific toxic concentrations [13]. Last and most importantly, a critical mass of labs and investigators must assess and validate new developments. While initial observations may vary, different groups utilizing new methodologies and consistent experimental framework will eventually achieve a 'quorum'. It is now clear that the role of DAAs as biofilm interfering agents, both in medicinal and industrial microbiology, has met this important criterion.

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The author has no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Review

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Future Medicinal Chemistry

The potential of combi-molecules with DNA-damaging function as anticancer agents

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DNA-damaging agents, such as methylating agents, chloroethylating agents and platinum-based agents, have been extensively used as anticancer drugs. However, the side effects, high toxicity, lack of selectivity and resistance severely limit their clinical applications. In recent years, a strategy combining a DNA-damaging agent with a bioactive molecule (e.g., enzyme inhibitors) or carrier (e.g., steroid hormone and DNA intercalators) to produce a new 'combi-molecule' with improved efficacy or selectivity has been attempted to overcome these drawbacks. The combi-molecule simultaneously acts on two targets and is expected to possess better potency than the parent compounds. Many studies have shown DNA-damaging combi-molecules exhibiting excellent anticancer activity *in vitro* and *in vivo*. This review focuses on the development of combi-molecules, which possess increased DNA-damaging potency, anticancer efficacy and tumor selectivity and reduced side reactions than the parent compounds. The future opportunities and challenges in the discovery of combi-molecules were also discussed.

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Keywords: anticancer activity • chemoresistance • combi-molecules • DNA-damaging agents • tumor targeting

DNA-damaging agents have become an important and valuable class of anticancer drugs in conventional clinical chemotherapy since the first introduction of poison gas 'mustard gas' in World War I [1,2]. As shown in Figure 1, typical DNA-damaging agents used for the treatment of cancer in clinic mainly include methylating agents, (e.g., temozolomide, dacarbazine and procarbazine), chloroethylating agents (e.g., nitrogen mustard, carmustine and nimustine) and platinum-based agents (e.g., cisplatin, carboplatin and oxaliplatin) [2,3]. They can undergo spontaneous decomposition or enzymatic hydrolysis to generate active electrophiles, which attack DNA, RNA or protein, resulting in the loss of normal physiological activity of these bio macromolecules [2,4]. Generally, alkylation of DNA produced by these anticancer agents is considered as the main cytotoxic lesion responsible for their antitumor activity [5,6]. If not repaired correctly, these lesions can inhibit strand separation during DNA replication and transcription or induce DNA strand breaks, leading to cell apoptosis [2,5,6]. Although having been employed as clinical chemotherapies for a relatively long time, these DNA-damaging agents were observed to exhibit prominent limitations, including inevitable drug resistance, serious side reactions, lack of selectivity and poor pharmacokinetic properties [7–9]. In fact, the limited efficacy of the chemotherapeutic alkylating agents is not surprising in view of the complex process of cancer biology, which involves multiple proteins, enzymes, signaling pathways or other biological mechanisms to bypass or abrogate the anticancer activity

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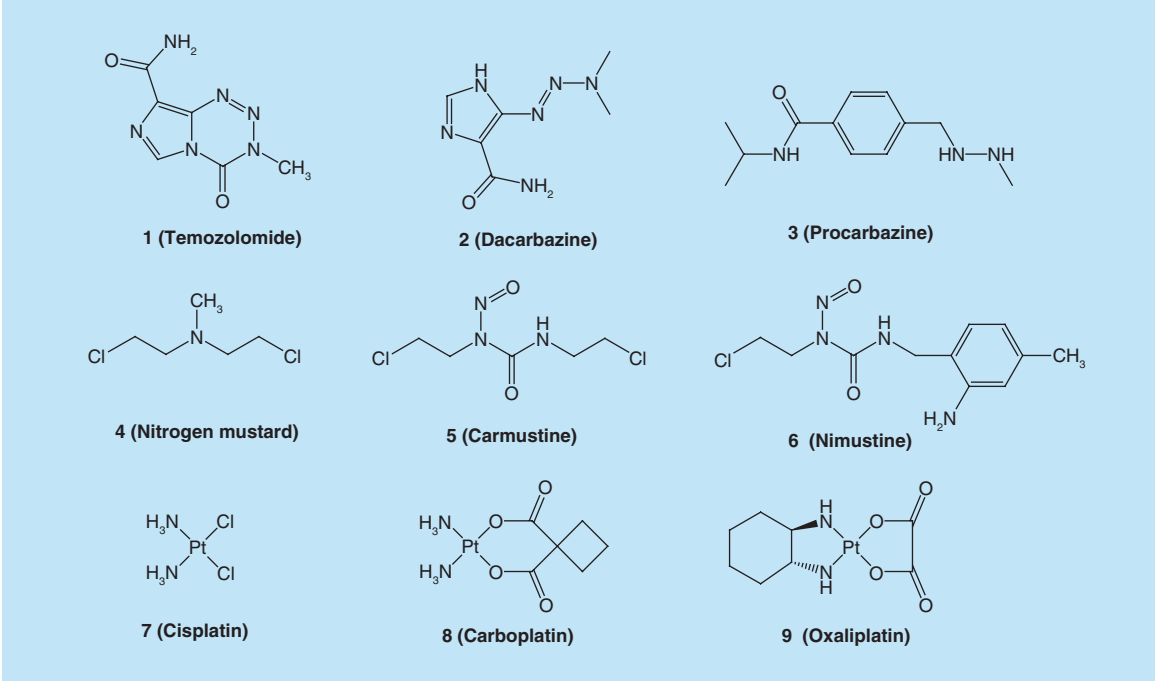


Figure 1. Structures of several clinically widely used DNA-damaging anticancer-alkylating agents.

induced by these alkylating agents [10]. In addition, because cancer is a complex and variable disease process, it is nearly impossible that a single monofunctional drug could provide desirable chemotherapeutic effects for most advanced cancers.

Generally, there are two main strategies that can be used to overcome the chemoresistance, reduce the side reactions and improve the chemotherapeutic effects. The first one is combination chemotherapy (drug cocktails), in which two or more different drugs are employed simultaneously in a specified treatment prescription. The rationality of the combination treatment is based on the employment of two or more drugs that act by different mechanisms, thereby reducing the possible development of resistance. At present, a large number of studies using combination chemotherapies were carried out in clinical trials, some of which exhibited promising anticancer effects [2,11–13]. The second one is the concept of 'combi-molecule' that incorporates two bioactive pharmacophores into a single molecule with dual mode of action [9–10,14–19]. This strategy can be employed to design a new type of drugs possessing better pharmacokinetic and pharmacodynamic properties, multiple mechanisms of action in a single molecule and less prone to resistance, when compared with the parent compounds. Several studies demonstrated that the combi-molecules exhibited superior anticancer activities and less cytotoxic effects to normal tissues compared with their respective parent drugs [20,21]. Within a combi-molecule, the two bioactive moieties can be connected directly or by a linking unit. Generally, directly connected combi-molecules linked by a special functional group of each molecule are enzymatically hydrolysable ester, carbamate or amide [10,16]. The combi-molecules containing a linking unit can be divided into cleavable and noncleavable types on the basis of the characteristics of the linkers. A cleavable combi-molecule is designed to either selectively release an active pharmacophore in targeted tissues or improve the anticancer activity of the parent compounds [22,23]. In contrast, the potency of a noncleavable combi-molecule is based on the maintenance of the biological activity or specific affinity to the targets of a single pharmacophore [18,19].

In this review, we described the development of the combi-molecules with DNA-damaging function and their potential anticancer chemotherapeutic effects over the past 15 years. The classification of the combi-molecules is based on the species of the pharmacophores connected to the DNA-damaging moiety, mainly including some bioactive molecules (e.g., enzyme inhibitors) and carriers (e.g., steroid hormone and DNA intercalating group). The opportunities and challenges in the development of combi-molecules as anticancer drugs were also discussed.

Development of DNA-damaging combi-molecules

The applications of conventional DNA-damaging agents as anticancer drugs are hampered by multiple factors, including high chemical reactivity, lack of selectivity, resistance, serious side effects, etc. The

complex disease process of cancer also suggests that single targeted chemotherapy may be ineffective for cancer treatment in most patients. Therefore, the concept of 'combi-molecule' emerges in response to this dilemma, which aims to act on multiple targets and destroy the self-rescuing mechanism of cancer cells. In this section, we present an overview of the combi-molecules with DNA-damaging function as potential anticancer agents.

EGFR TK inhibitor-linked DNA-damaging agents

Overexpression of certain growth factor receptors, such as EGFR and its closest homolog ErbB2 (also known as HER2), were observed in various human cancers including breast cancer, lung cancer, bladder cancer and colon carcinoma, which were associated with aggressive tumor progression and poor prognosis [24,25]. Given the crucial role of EGFR in carcinogenesis, it has become an important chemotherapeutic target in drug design for cancer treatment [25]. It has been demonstrated that blocking the tyrosine kinase (TK) activity of EGFR using small molecule inhibitors exhibited significant antitumor activity *in vitro* and *in vivo*. Gefitinib (**10**, Iressa®, AstraZeneca, UK) and erlotinib (**11**, Tarceva®, Roche, Switzerland) (Figure 2A & B) are two approved EGFR TK inhibitors in clinical cancer treatment and have exhibited a broad spectrum of antitumor activity against many human solid tumors, including lung, breast, prostate and colorectal cancers [26]. Although they are widely investigated in clinical oncology, most EGFR TK inhibitors do not induce apoptosis and only exert a reversible cytostatic effect, thereby requiring long-term repeated dose to induce tumor regression *in vivo*. This limitation of current EGFR TK inhibitors is due to the high intracellular ATP concentration, which represents a major obstacle of sustained EGF inhibition of EGF and stimulates signal transduction in tumor cells. The lack of sustained antitumor activity and the intrinsic and acquired resistance of EGFR TK inhibitors arouse interest in designing a novel antitumor strategy termed 'combi-targeting', which combines the EGFR TK inhibitor with a DNA-damaging moiety into one 'combi-molecule'. As outlined in Figure 3A, the combi-molecules (termed TZ-I) were designed to: inhibit the receptor TK on their own, and release another inhibitor (I) of the same receptor TK and a DNA-damaging agent (TZ) upon hydrolysis.

Over last decade, Jean-Claude's research group has synthesized a series of combi-molecules that merge the pharmacophore moiety of EGFR TK inhibitor with different DNA-alkylating groups (Supplementary Table 1) [21–23,27–47]. SMA41 (**12**) is the first TZ-I combi-molecule with mixed EGFR-DNA targeting properties, which undergoes degradation to generate SMA52 (**54**) as a competitive inhibitor occupying the ATP binding site of EGFR, and a 3-methyl-1,2,3-triazene as the precursor of methyldiazonium ion damaging DNA [22,31,34]. Significant DNA damage induced by SMA41 was observed in an alkaline comet assay. It is worth noting that the chimeric SMA41 molecule, which was designed to target high EGFR-expressing tumor cells, remains small enough to be able to interact with the EGFR on its own (IC_{50} = 0.2 μM). However, the released stable SMA52 was proven to be a weak EGFR TK inhibitor (IC_{50} = 1.0 μM), which might influence the overall antitumor effect of SMA41. In order to circumvent this problem, a less bulky chloro substituent was used to replace the methyl group in SMA41 to produce BJ2000 (**13**), a novel TZ-I combi-molecule with twofold stronger EGFR TK inhibitory activity (IC_{50} = 0.1 μM) than SMA41. BJ2000 can decompose into a 6-amino-4-anilinoquinazoline FD105 (**55**) with fivefold stronger potency against EGFR TK (IC_{50} = 0.2 μM) than SMA52 [27]. In addition, the DNA damage induced by BJ2000 provides an indirect evidence for the formation of methyldiazonium species. It is interesting that BJ2000 can selectively block the EGF-stimulated EGFR autophosphorylation by a partially irreversible mechanism. Therefore, the binary targeting effects of BJ2000 were converted into selectively blocking EGF or TGF-α induced proliferation in NIH3T3HER14 cells (NIH3T3 cells stably transfected with *EGFR* gene).

In order to enhance the potency and stability of the combi-targeting molecules, Jean-Claude's group put forward a novel strategy termed 'cascade release' that masked the combi-molecule into a prodrug with protective groups. Based on this strategy, a series of prodrug were designed to procedurally release antitumor species upon hydrolysis. Compounds **14** and RB24 (**15**) are synthesized as the first prototype of 'cascade release molecules' (CRM) [30]. Due to the little degradation of **14** under physiological conditions after long-time incubation (half-life >200 min), it was concluded that **14** was not suitable for being a CRM. On the contrary, RB24 is a CRM paradigm designed to degrade to ZR08 (**16**), RB10 (**56**) and a DNA-alkylating methyldiazonium ion in a progressive way, as shown in Figure 3A. *In vitro* growth inhibition assay indicated that RB24 (~10-fold) and ZR08 (~5-fold) exhibited superior antiproliferative activity when compared with the combination of temozolomide with AG478, a known EGFR TK inhibitor, in NIH3T3/neu cells (NIH3T3 cells stably transfected with ErbB2). Western blotting assays revealed that RB24 induced irreversible inhibition of EGFR autophosphorylation and blocked the downstream signaling pathway of EGFR [33]. The subsequent studies also demonstrated that RB24 induced significantly higher levels of DNA

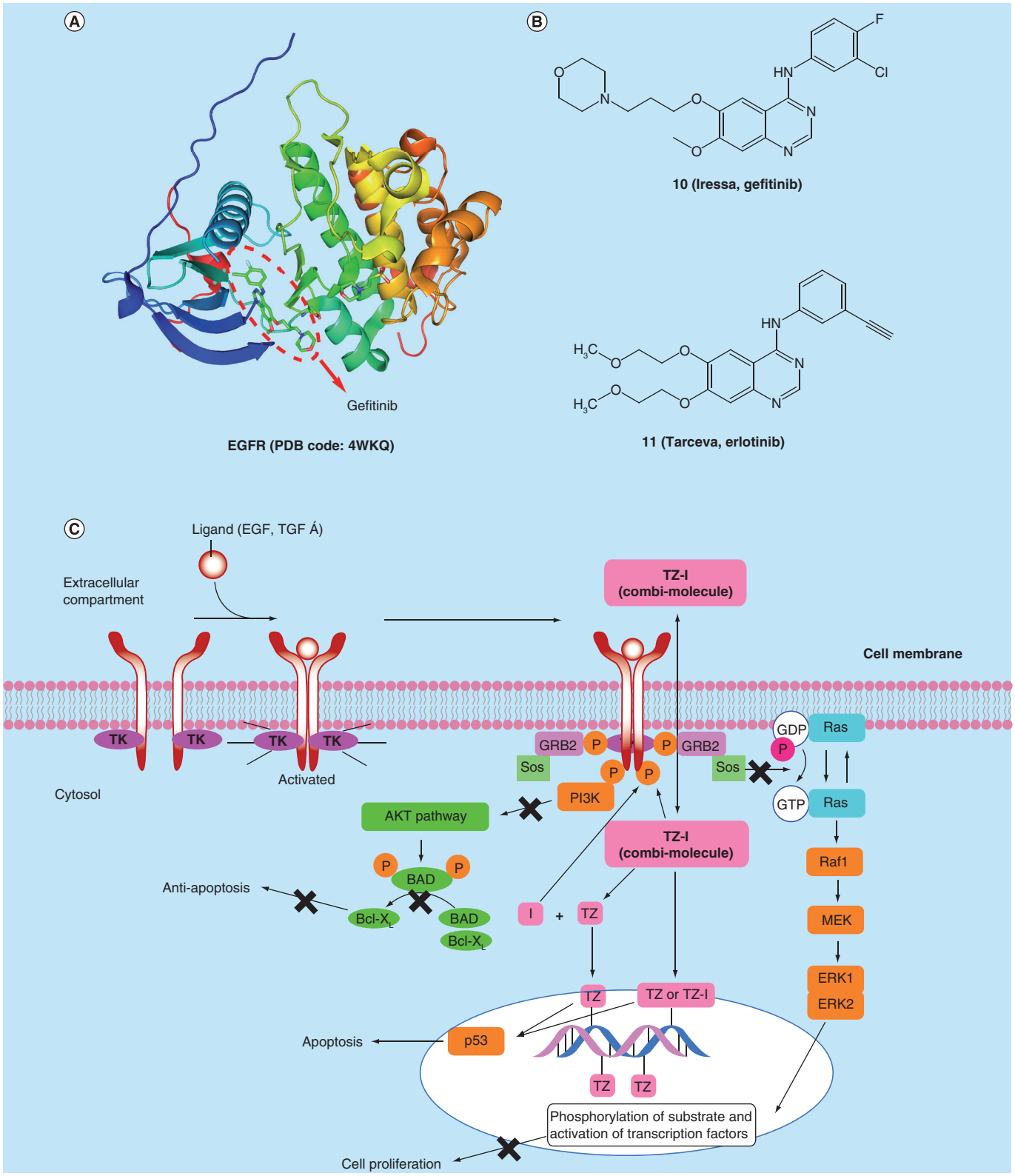


Figure 2. Overview of EGFR, its approved inhibitors and the mechanism of action of EGFR TK inhibitor-linked DNA-damaging combi-molecule. (A) The crystal structure of EGFR kinase domain containing gefitinib as ligand (PDB code: 4WKQ). **(B)** Molecular structures of two approved EGFR tyrosine kinase inhibitors, **10** (Iressa, gefitinib) and **11** (Tarceva, erlotinib). **(C)** Schematic representation of the intracellular distribution, and pathways activated or inhibited by the EGFR tyrosine kinase inhibitor-linked DNA-damaging combi-molecule (TZ-I).

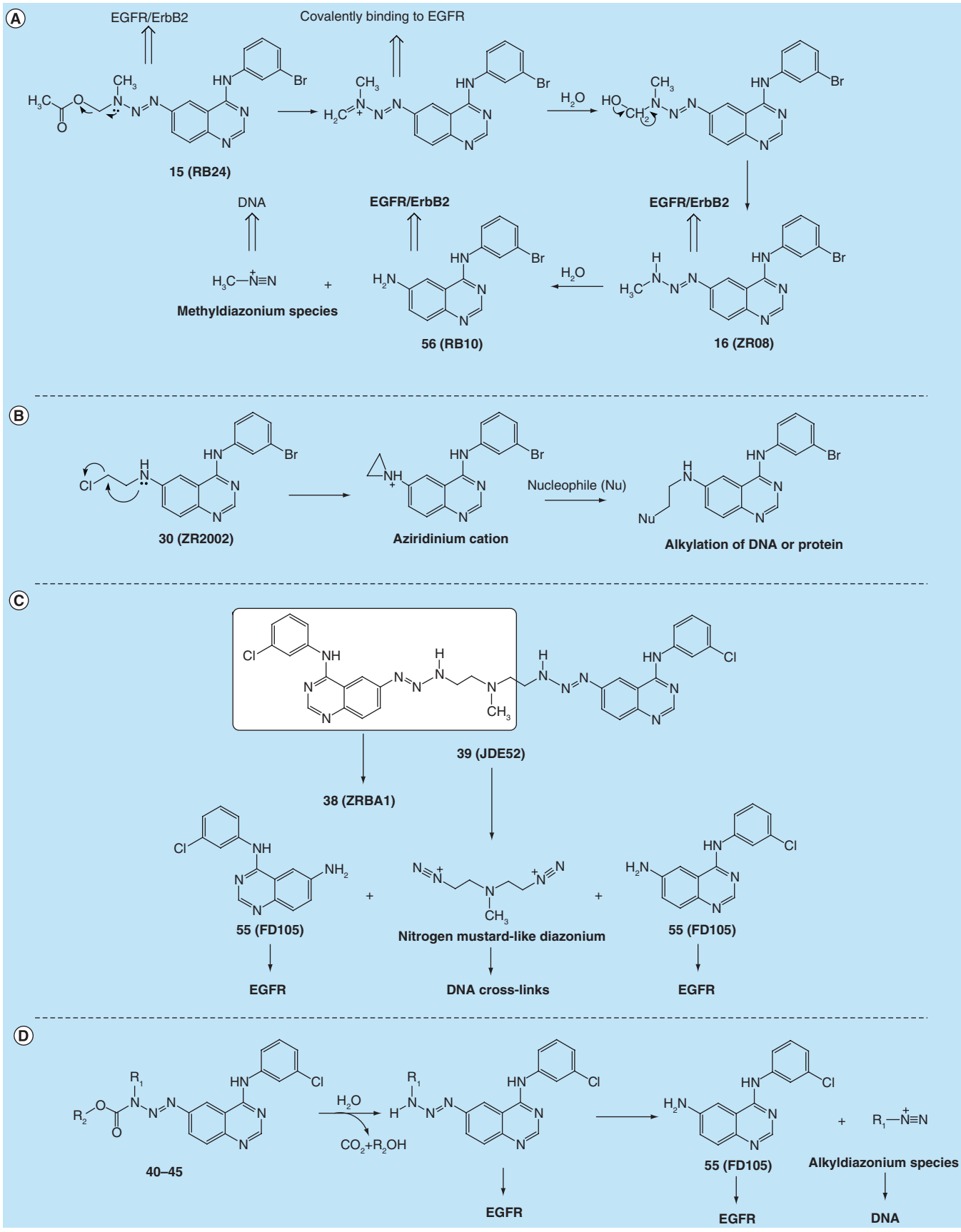
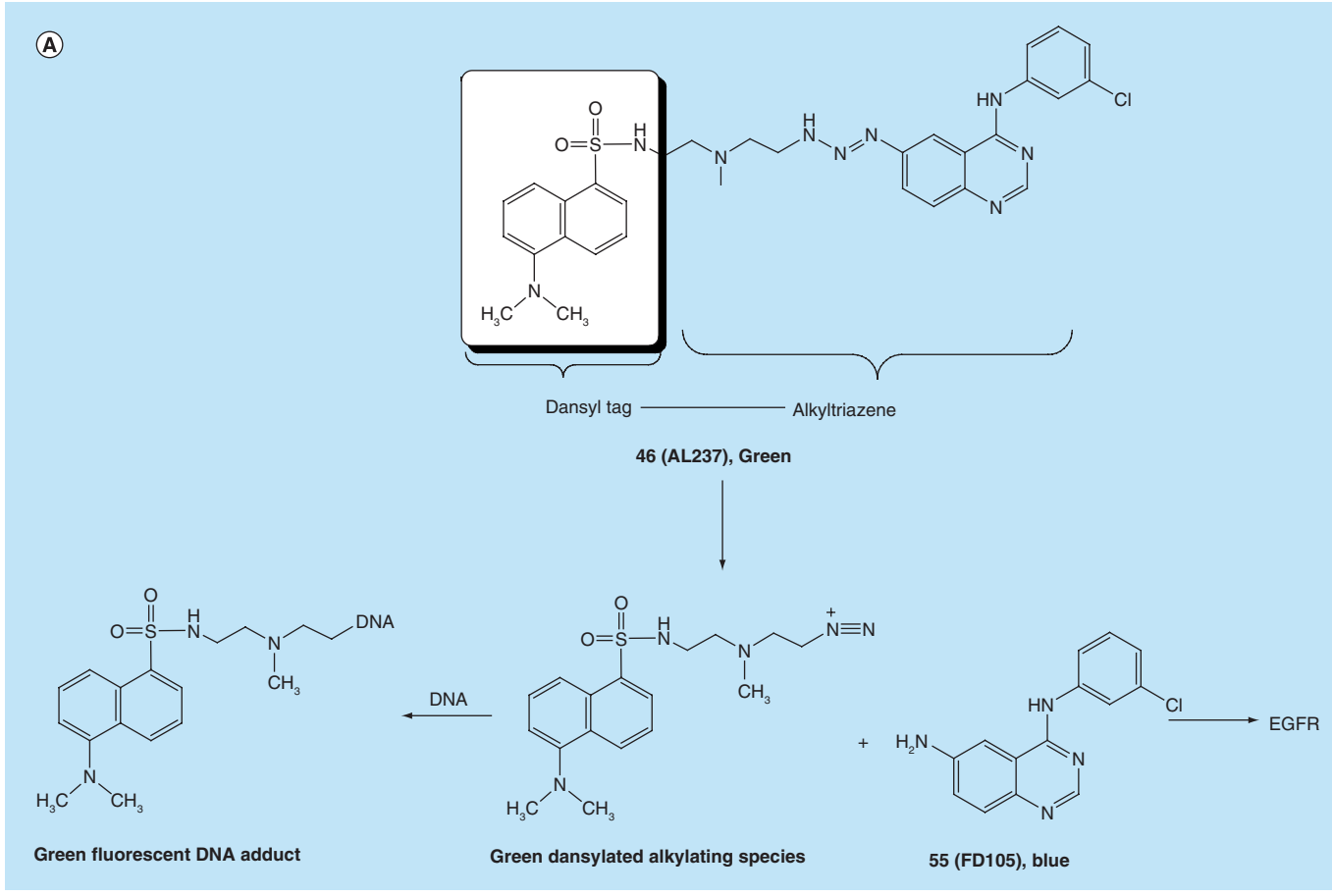

A
EGFR/ErbB2
Covalently binding to EGFR
15 (RB24)
DNA
EGFR/ErbB2
EGFR/ErbB2
Methyldiazonium species
56 (RB10)
16 (ZR08)
B
30 (ZR2002)
Aziridinium cation
Nucleophile (Nu)
Alkylation of DNA or protein
C
39 (JDE52)
38 (ZRBA1)
55 (FD105)
Nitrogen mustard-like diazonium
55 (FD105)
EGFR
DNA cross-links
EGFR
D
40–45
55 (FD105)
Alkyldiazonium species
EGFR
EGFR
DNA

Figure 3. EGFR TK inhibitor-linked DNA-damaging agents (cont. from previous page). **(A)** The decomposition mechanism and cellular targets of cascade release molecules RB24. **(B)** The proposed mechanism of action of ZR2002. **(C)** The mechanism of action of the bistriazene combi-molecule JDE52. Upon hydrolysis, JDE52 can decompose to generate two equivalents of FD105 as EGFR tyrosine kinase inhibitor and a nitrogen mustard-like diazonium as bifunctional DNA-damaging species. **(D)** The decomposition of monoalkyltriazenes (**40**~**45**) with carbamates and their cellular targets.

damage in EGFR and ErbB2 transfected cells than in wild type cells [21]. These results indicated that RB24 could selectively target the cancer cells overexpressing EGFR or ErbB2 through simultaneous induction of DNA damage and inhibition of EGFR TK.

Although the feasibility and superior potency of combi-molecules with dual targeting properties has been demonstrated, the substantial contribution of each component to their overall antitumor activity in some combi-molecules remains elusive. Rachid *et al.* [29] described a structure-function approach whereby a series of quinazoline-based 'combi-triazenes' (**12**, **13**, **16**~**29**) were designed to either abolish the EGFR-targeting ability of the quinazoline moiety or mask the DNA-damaging property of the triazene moiety. The compounds substituted with an *N*-methylanilino group (**23**, **26**, **27**) were almost deprived of EGFR TK inhibitory activity and exhibited a weak antiproliferative ability to NIH3T3/neu cells. On the other hand, since all dimethyltriazenes (**17**~**19**, **29**) maintain the EGFR TK affinity, the lack of DNA-damaging ability results in their decreased antiproliferative activity in A431 carcinoma of the vulva cells. In contrast, the monomethyltriazenes **12**, **13**, **16** and **28**, which possess the mixed EGFR/DNA targeting properties, were significantly more potent in this refractory tumor cell line than those of their mono-targeted counterparts. The results suggest that each component of combi-molecules plays a critical role in their overall antitumor activity.

Subsequently, Jean-Claude's group synthesized a new type of combi-molecules (**30**~**32**), which were designed to generate mixed EGFR/DNA targeting properties without requirement for hydrolytic cleavage [35,36,46]. ZR2002 (**30**) is the first prototype of combi-molecules that directly alkylate EGFR and DNA without hydrolysis (Figure 3B) [35]. It simulta-



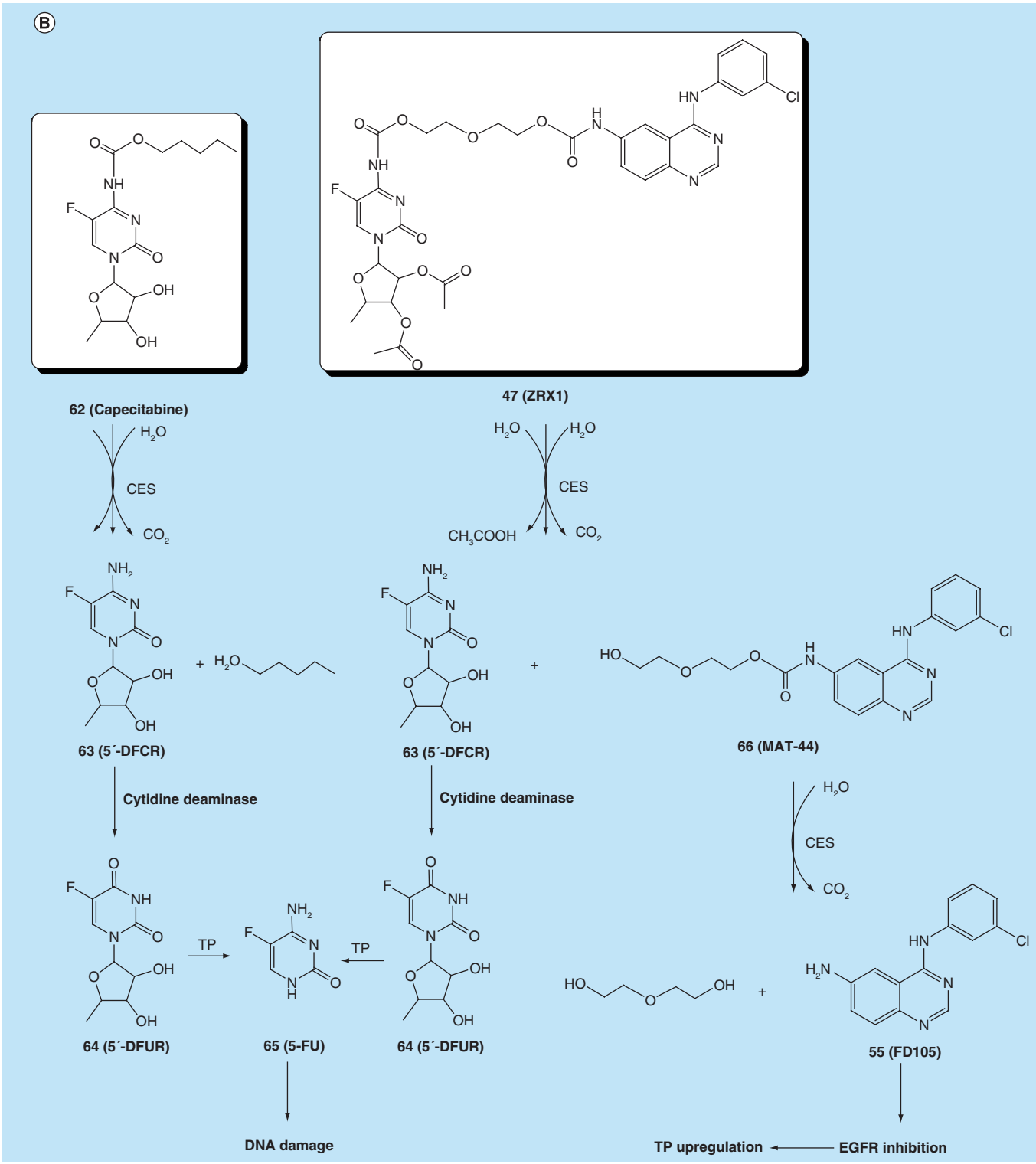


Figure 4. The proposed mechanism of the combi-molecules AL237 and ZRX1 (cont. from facing page). **(A)** The schematic representation of the distribution of a fluorescence-labeled combi-molecule AL237 designed to generate a blue EGFR tyrosine kinase inhibitor FD105 and a green DNA-damaging species. **(B)** The proposed mechanism of action of the EGFR inhibitor capecitabine-based combi-molecule ZRX1.

neously blocked both EGFR and ErbB2-mediated signaling and induced a significant DNA damage at concentration as low as 10 μM. Moreover, ZR2002 was observed to induce irreversible inhibition of EGF-stimulated autophosphorylation. The blockade of EGFR-mediated downstream signaling pathways by ZR2002 may downregulate DNA repair activities, thereby enhancing the cytotoxic effects of the DNA-damaging moiety. ZR2003 (**31**) and ZR2009 (**32**) are two isomers where the chloroethyl group is attached to the 6- and 7-position of quinazoline skeleton in ZR2003 and ZR2009, respectively [46]. In contrast to ZR2009, ZR2003 was more potent (~10-fold) in inhibiting EGFR TK, induced high levels of DNA damage, and exhibited significant stronger antiproliferative activity. These results suggested that the

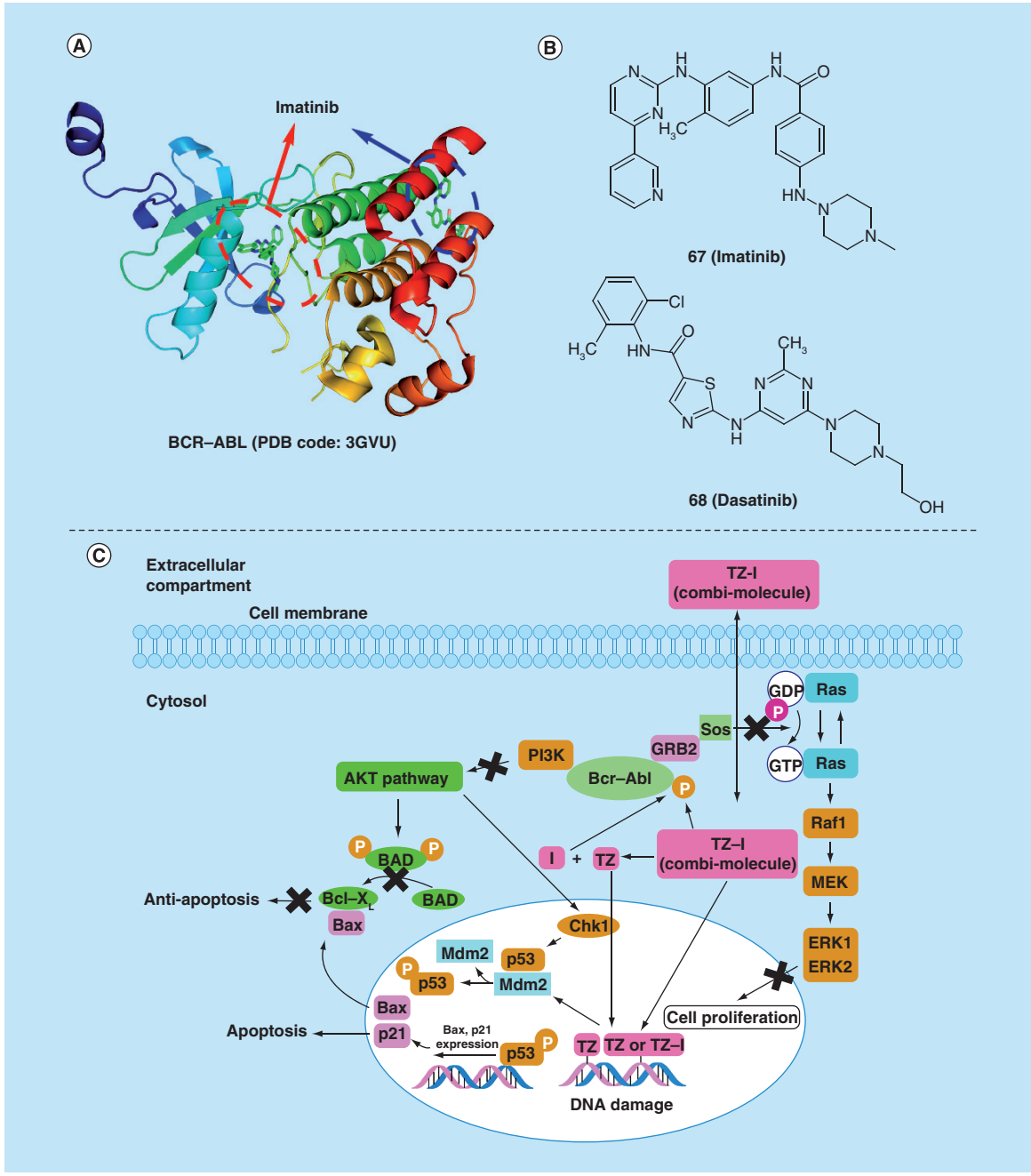


Figure 5. Overview of Bcr–Abl, its approved inhibitors and the mechanism of action of Bcr–Abl TK inhibitor-linked DNA-damaging combi-molecule. (A) The crystal structure of human ABL2 in complex with imatinib as ligand (PDB code: 3GVU). **(B)** Structures of two approved Bcr–Abl TK inhibitors, **67** (imatinib) and **68** (dasatinib). **(C)** Signaling pathways targeted by the dual mechanism of action of the combi-molecule (TZ-I) designed to simultaneously block the Bcr–Abl tyrosine kinase activity and damage DNA.

appendage of the chloroethyl group to the 6-position was optimal for DNA damage, and sustained the inhibition of EGFR TK. Interestingly, in addition to significant EGFR inhibitory potency, these compounds were found to possess obvious fluorescence property, which might be employed as nonimmunological small molecule probes for the detection of EGFR [36]. A linear correlation ($R = 0.7$; $p < 0.02$) between the levels of EGFR and single-cell fluorescence intensity was observed after cells being exposed to ZR2002 at low concentrations. Similarly, Rachid *et al.* [39] designed a series of combi-molecules (**33~37**) containing an EGFR TK inhibitory moiety and a 2-chloroethylaminoethyl group, which was conferred bifunctional alkylating ability. The novel nitrogen mustard-armed combi-molecules were demonstrated to selectively target EGFR overexperessing solid tumors. The most selective compound **33** induced irreversible inhibition of EGFR autophosphorylation, possessed similar cross-linking ability as nitrogen mustard (**4**) and arrested cells in mid-S phase.

Despite the potential activity *in vitro* of the combi-molecules described above, the antitumor activity *in vivo* is significantly hindered by their poor solubility and moderate alkylating ability due to DNA repair mediated by O^6-methylguanine (O^6-MG)-DNA methyltransferase (MGMT). To circumvent the O^6-MG lesion induced by methyldiazonium species being repaired by MGMT, Brahimi *et al.* [23] synthesized ZRBA1 (**38**), which contained a polar *N*,*N*-dimethylaminoethyl on the alkylating moiety to serve as a water soluble group and enable more potent alkylating ability. The alkylating species generated from ZRBA1 induced dimethylaminoethylguanine adducts that were poor substrates of MGMT. The *in vivo* antitumor study showed that ZRBA1 exhibited significantly ($p < 0.05$) superior potency compared with its parent molecule SMA41 against human MDA-MB-468 breast cancer xenograft model. It is noteworthy that there was no significant weight loss of the animals during the treatment with ZRBA1. Furthermore, Heravi *et al.* [47] demonstrated that ZRBA1 strengthened radiation response in MDA-MB-468 cells through delayed DNA damage repair process, which might be induced by the inhibition of EGFR-mediated downstream signaling. Even after 24 h, DNA breaks remained at a higher level in cells treated with both ZRBA1 and radiation than those treated with ZRBA1 or radiation alone. To improve the potency of ZRBA1, another bis-triazene combi-molecule JDE52 (**39**) was synthesized and programmed to release two equivalents of EGFR TK inhibitor FD105 and a more cytotoxic bifunctional DNA-damaging species [44], as shown in Figure 3C. The results suggested that JDE52 induced stronger blockade of EGFR phosphorylation and higher levels of DNA cross-links than ZRBA1.

Since most of the previously reported combi-triazenes were instable under physiological conditions, Rachid *et al.* [40] further designed and synthesized a series of monoalkyltriazenes (**40~45**) with carbamate moities, which underwent a rate limiting hydrolysis, to increase the bioavailability of combi-molecules (Figure 3D). Although none of these compounds showed greater EGFR TK inhibitory potency than Iressa (IC_{50} = 0.033 μM), all compounds were capable of inducing DNA damage except the most stable ZRL2 (**41**). Moreover, in cells exposed to the MGMT inhibitor O^6-benzylguanine (O^6-BG), ZRS1 (**45**) was more potent than Iressa, temozolomide or a combination of the two drugs [43]. This suggested that ZRS1 could be optimized to a lead structure with high stability and without significant loss of antitumor activity. To monitor the subcellular localization of the two bioactive products yielded from the EGFR/DNA binary targeting combi-molecules, Todorova *et al.* [41] designed a novel probe AL237 (**46**) by introducing a fluorescent dansyl tag on the 3-alkyl triazene moiety. AL237 can undergo decomposition to release a blue fluorescent EGFR TK inhibitor FD105 and a green fluorescent DNA-damaging species (Figure 4A). The special fluorescent feature can be used to image the biodistribution profile for the dual action mechanism of the combi-molecules, as well as their colocalization with corresponding cellular targets.

Capecitabine (**62**), which requires activation by carboxylesterase (CES) to form 5′-deoxy-5-flurocytidine ribose (**63**, 5′-DFCR), is an orally available antitumor prodrug of 5-fluorouracil (**65**, 5-FU) (Figure 4B). 5′-DFCR is further catalyzed by cytidine deaminase to give rise to 5′-deoxy-5-fluorouridine ribose (**64**, 5′-DFUR), which is finally converted to cytotoxic 5-FU through thymidine phosphorylasethymidine phosphorylase. Recently, Ait-Tihyaty *et al.* [45] reported a new EGFR inhibitor-capecitabine based combi-molecule ZRX1 (**47**), in which an acetylated 5′-DFCR moiety was linked to a quinazoline group of EGFR TK inhibitor through an alkyl dicarbamate spacer. ZRX1 was proven to generate a DNA-damaging 5-FU via intermediate 5′-DFCR and 5′-DFUR, and an EGFR inhibitor FD105 via intermediate MAT-44 (**66**) in the presence of CES (Figure 4B). Especially, ZRX1 can induce significant inhibition of EGFR autophosphorylation followed by an increase of thymidine phosphorylase expression upon CES activation, which further enhance its potency of DNA damage and apoptosis.

In addition to the combi-triazenes described above, the combi-molecules with DNA-alkylating nitrosourea moiety were also investigated. FD137 (**48**) is the first combi-nitrosourea possessing EGFR TK

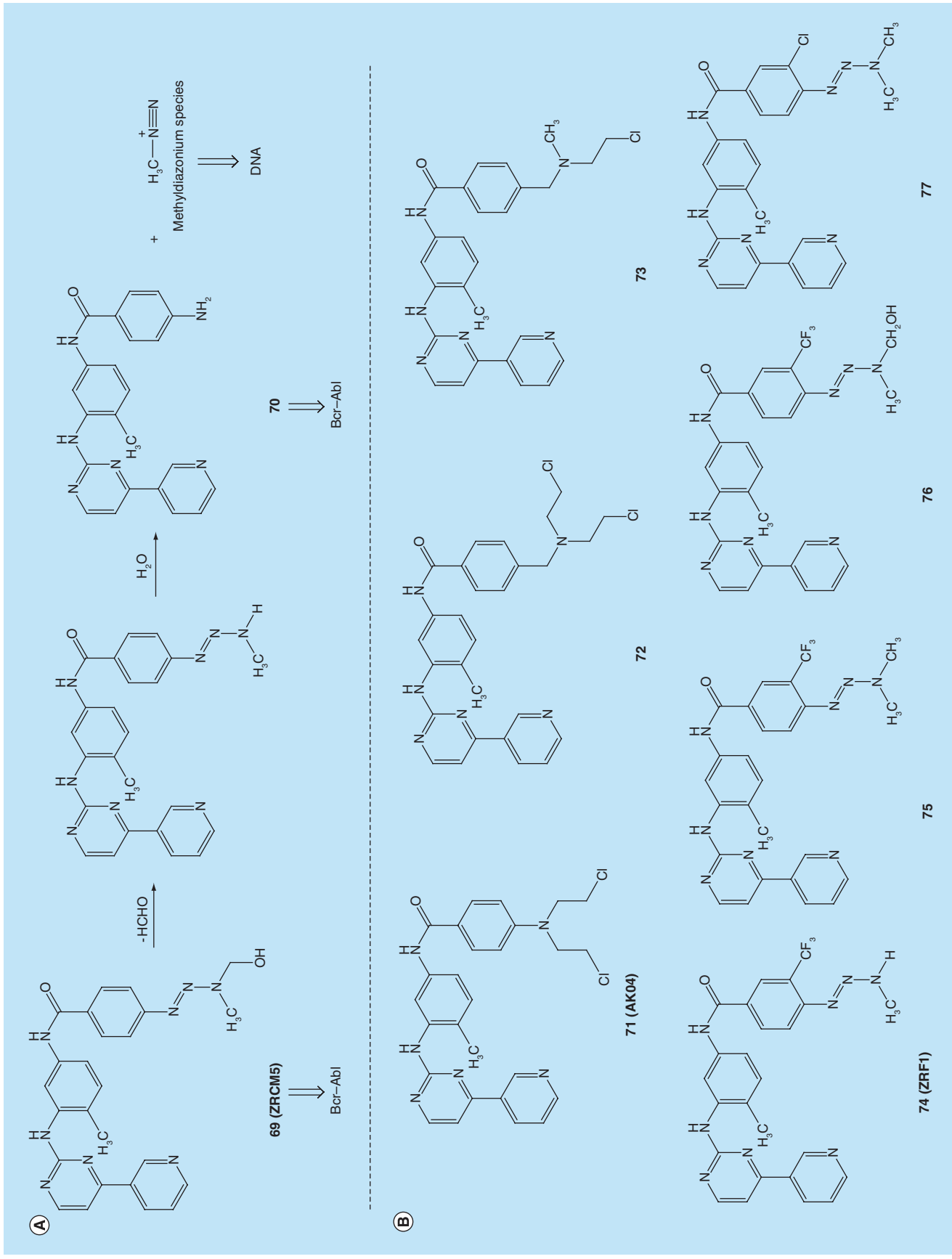

A
B
-HCHO
H_2O
Methyldiazonium species
DNA
69 (ZRCM5)
Bcr–Abl
70
Bcr–Abl
71 (AK04)
72
73
74 (ZRF1)
75
76
77

Figure 6. Bcr–Abl TK inhibitor-linked DNA-damaging agents (cont. from facing page). **(A)** The proposed mechanism of action of ZRCM5 (**69**). **(B)** Molecular structures of compounds **71**~**77**.

inhibitory activity ever reported, which releases the 1-chloroethyl-1-nitrosourea moiety to generate DNA-damaging 2-chloroethyldiazonium species, and the 4′-anilinoquinazoline moiety FD110 (**59**) to block the binding of ATP to EGFR TK domain [28]. The complex properties of FD137 were translated into a 55-fold greater antiproliferative activity than BCNU against the EGFR and MGMT overexpressing A431 cell line. Moreover, the inhibition of EGF-stimulated EGFR autophosphorylation induced by FD137 and FD110 leads to the blockade of *c-fos* gene expression in DU145 cells [32]. In order to optimize the potency of combi-nitrosoureas, Domarkas *et al.* [37] synthesized a series of combi-nitrosoureas (**49**~**53**) with variable substituents on the 3-position of ureido moiety and the 3-position of aniline portion. Among these compounds, JDA58 (**52**) was considered to be the optimal structure and was chosen for further investigation based on the stability ($t_{1/2}$ = ~21 h), EGFR TK inhibitory potency (IC_{50} = 0.2 μM) and selectivity. Qiu *et al.* [38] demonstrated that JDA58 hydrolyzed to JDA35 (**60**) in cell-free medium, induced significant levels of DNA damage in DU145 cells *in vitro* and *in vivo*, and showed potent antitumor activity in a DU145 xenograft model.

Bcr–Abl TK inhibitor-linked DNA-damaging agents

Bcr–Abl oncoprotein is a constitutively activated TK that plays a crucial role in the development of chronic myelogenous leukemia (CML) [48]. Blockade of the Bcr–Abl TK activity with imatinib (**67**, Novartis, Switzerland) and dasatinib (**68**, Bristol-Myers Squibb, USA) (Figure 5A & B), two potent inhibitors approved by US FDA for the treatment of CML, has been proven to exhibit significant antitumor activity [26,48,49]. Unfortunately, the developed resistance and relapse were observed in their clinical applications [26,50]. Moreover, it was demonstrated that Bcr–Abl could induce or activate DNA repair mechanism (e.g., Rad51), leading to resistance against cytotoxic drugs [51]. Therefore, a strategy that combines a Bcr–Abl TK inhibitor with a DNA-damaging agent into a single molecule (termed 'combi-molecule' TZ-I) is expected to have enhanced chemotherapeutic effect (Figure 5C).

ZRCM5 (**69**), the first prototype of combi-molecule designed to release a imatinib analog **70** for blocking Bcr–Abl TK activity and a methyldiazonium ion for damaging DNA (Figure 6A) [52,53]. Western blotting assay showed that ZRCM5 blocked Bcr–Abl autophosphorylation in a dose-dependent manner in promyeloblastic leukemia K562 cells. As expected, ZRCM5 was found to induce higher levels of DNA strand breaks in K562 cells than temozolomide, which might be due to the down-regulation of DNA repair enzymes responsible for restoring the cytotoxic lesions. The dual Bcr–Abl/DNA targeting properties were translated into approximately 74-fold more potent than temozolomide in the Bcr–Abl transfected cell line.

To further optimize this novel combi-molecules with mixed Bcr–Abl/DNA-targeting properties, Jean-Claude's group [54] synthesized a series of compounds **71**~**77**, in which **71**~**73** were noncleavable but **74**~**77** are cleavable combi-molecules (Figure 6B). Although AK04 (**71**) has significant Bcr–Abl TK inhibitory activity (IC_{50} = 0.22 μM), it exhibits moderate alkylating potency. Compounds **72** and **73** are both poor Bcr–Abl TK inhibitor and virtually inactive against K562 cells. Compounds **75** and **77** are deprived of DNA-alkylating ability and not considered to be combi-molecules. By comparing the cytotoxicity and Bcr–Abl TK inhibitory activity, ZRF1 (**74**) was selected as the optimal combi-molecule that induced approximately twofold Bcr–Abl TK inhibitory activity than imatinib and significantly higher levels of DNA damage in CML cells than temozolomide. More importantly, its superior potency over imatinib was more striking in Bcr–Abl positive cells co-expressing wild-type p53, which is available for transactivating apoptosis protein p21 and Bax (Figure 5C) [55].

MGMT inhibitor-linked DNA-damaging agents

The alkylation of O^6-position of guanine in DNA is the major source of antitumor activity of DNA-alkylating agents, such as temozolomide and carmustine, which can inhibit the double-strand separation or induce mismatch repair and ultimately results in cell apoptosis [2,3]. However, the alkyl lesions located at the O^6-position of guanine can be repaired by MGMT, also called O^6-alkylguanine-DNA alkyltransferase (Figure 7), through directly transferring the alkyl lesion groups to

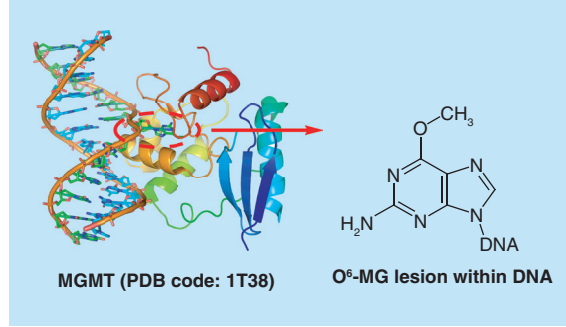
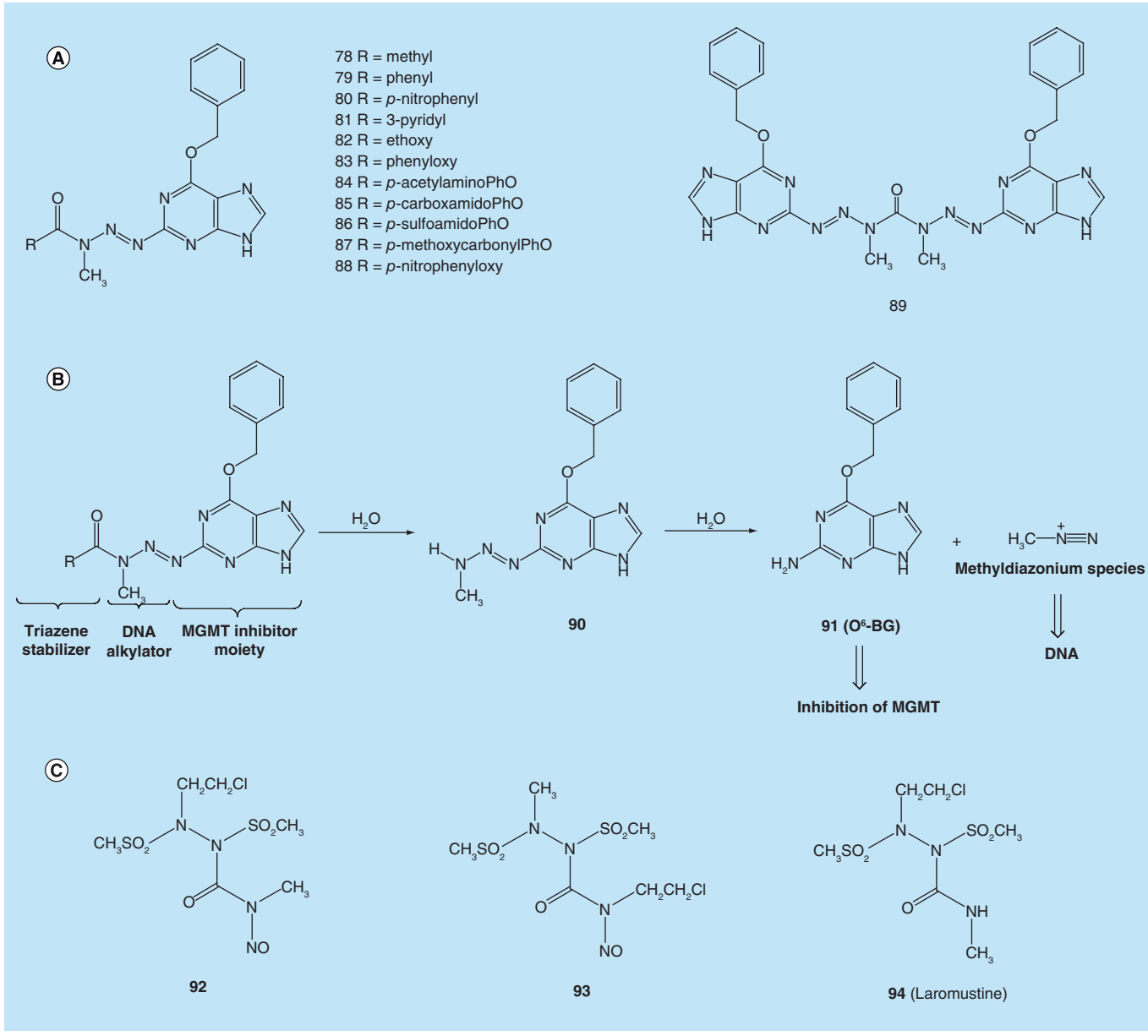


Figure 7. The crystal structure of human MGMT bound to DNA containing O^6-MG (PDB code: 1T38).
MGMT: O^6-methylguanin-DNA methyltransferase; O^6-MG: O^6-methylguanine.

Cys145 residue at the active centre of the enzyme. The repair restores normal DNA structure and leads to drug resistance [2,7]. An inverse relationship was observed between the MGMT content and the sensitivity of cells to guanine O^6-alkylating agents [56–59]. Since high levels of MGMT in tumor cells result in severe resistance to guanine O^6-alkylating agents, a series of MGMT inhibitors were synthesized as adjuvants to improve the chemotherapeutic effects [7,60]. O^6-benzylguanine (**91**, O^6-BG) is the first potent MGMT inhibitor that has entered clinical trials. O^6-BG acts as a pseudosubstrate of MGMT to form *S*-benzylcysteine at the active site to inactivate the enzyme [2]. Unfortunately, the combinations of O^6-BG with guanine O^6-alkylating agents only exhibited limited response in clinical trials [61,62].

Based on the concept of 'combi-molecule', Wanner *et al.* [63] designed and synthesized a set of methyltriazene prodrugs (**78**~**89**) containing the DNA methylating triazenes and the O^6-BG in one molecule (Figure 8A). As shown in Figure 8B, the combi-molecules undergo hydrolysis to yield a DNA-damaging methyldiazonium species and an MGMT inhibitor O^6-BG under physiological conditions. Among these compounds, *p*-nitrophenyloxy derivative **88** has an optimal half-life of approximately 23 min and was the most active (mean IC_{50} = 10 μM) toward NCI-60 human tumor cell line. The superiority of **88** is due to the MGMT depletion by O^6-BG released from the parent molecule and its favorable penetration property. The dimer compound **89** also showed slight



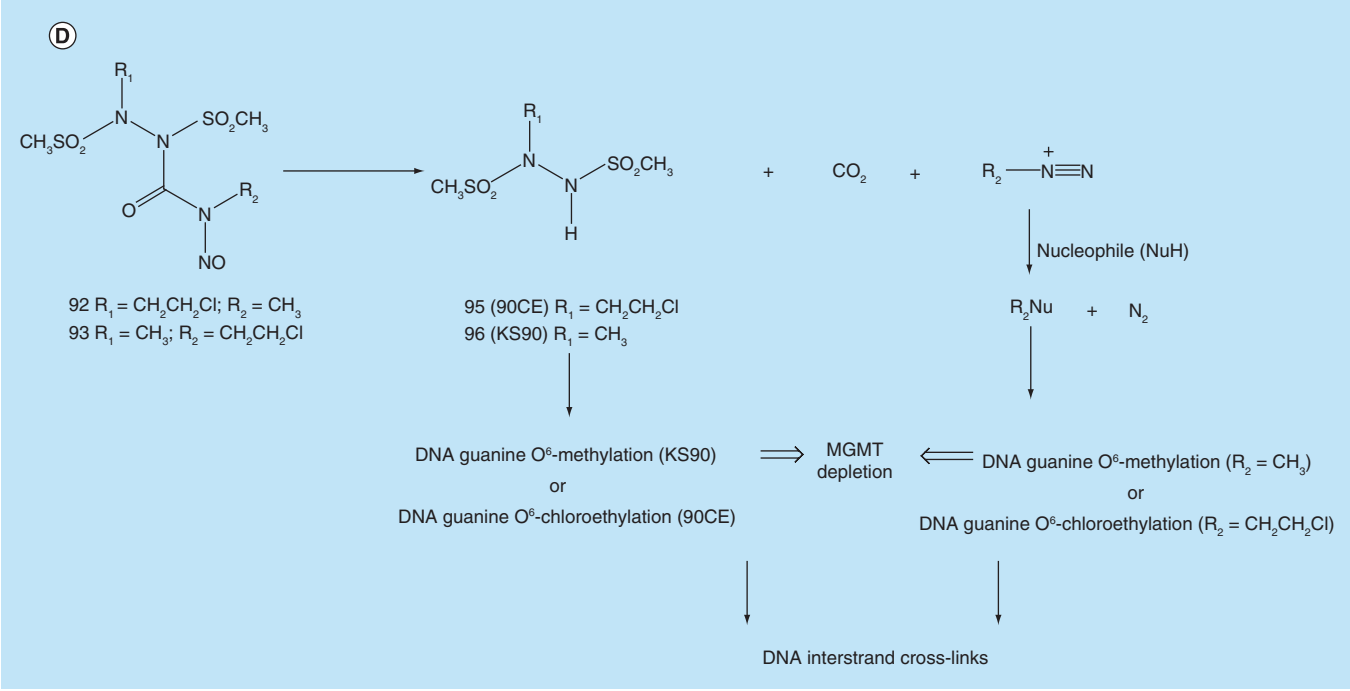


Figure 8. MGMT inhibitor-linked DNA-damaging agents (cont. from facing page). **(A)** Molecular structures of methyltriazene prodrugs (**78**~**89**) designed to simultaneously release a DNA methylating agent and an MGMT inhibitor O^6-BG and **(B)** Proposed hydrolytic pathway of this type of combi-molecules. **(C)** Molecular structures of compounds **92**~**94** and **(D)** the proposed decomposition mechanism and cellular targets of **92** and **93**.
MGMT: O^6-methylguanin-DNA methyltransferase; O^6-BG: O^6-benzylguanine.

potency against NCI-60 cells, but it was less active than **88**. Since the O^6-MG lesion within DNA was a native substrate of MGMT, Zhu *et al.* [64] synthesized chloroethylating and methylating dual function antitumor agents **92** and **93**, in which the methylating moiety was designed with the purpose of depleting the MGMT activity, thereby improving the sensitivity of cells to chloroethylating species inducing lethal DNA interstrand cross-links (Figure 8C & D). A clonogenic assay showed that compound **92** was significantly more cytotoxic than **93** and laromustine (**94**) in DU145 cells with high expression of MGMT. The high sensitivity of DU145 cells to **92** indicated that the MGMT-mediated resistance was obviously weakened after exposure to **92**, which was corroborated by the MGMT depletion assay that **92** can deplete MGMT activity more effectively than **93** in DU145 cells. DNA cross-linking studies revealed that **92** also induced higher level of DNA interstrand cross-links than **93**.

Recently, a novel combi-nitrosourea prodrug **97** was synthesized in our laboratory, which was designed to release a DNA cross-linking agent and **98–99** as more potent MGMT inhibitors than O^6-BG (Figure 9) [65]. The cytotoxic studies indicated that **97** exhibited higher cytotoxicity against MGMT high-expressing glioma cells compared with ACNU, BCNU and their respective combinations with O^6-BG. Using HPLC–ESI–MS/MS, higher levels of dG-dC cross-link were observed in **97**-treated SF763 glioma cells with high MGMT expression when compared with the groups treated by BCNU or ACNU alone. The results suggested that the superiority of **97** might result from the simultaneous releasing of a chloroethyldiozonium ion to induce DNA cross-link and O^6-BG analogs to inhibit the MGMT-mediated drug resistance in MGMT high-expressing cells.

Topoisomerase inhibitor-linked DNA-damaging agents

DNA topoisomerases are universal and present in archaebacteria, bacteria and eukaryotes [66,67]. Human DNA topoisomerases include two categories, type I and II, which alter the topology of DNA by catalyzing the transient breaking and rejoining of DNA single and double strand, respectively (Figure 10). These actions allow a second DNA duplex to pass through the break during replication and transcription [66]. Toposiomerases are involved in several important cellular processes including DNA metabolism, chromosome condensation and chromatid separation [66]. Therefore, topoisomerases have become attractive targets of a number of potent anticancer agents. Camptothecin (CPT, **100**) and its derivatives topotecan (**101**, GlaxoSmithKline, UK) and irinotecan (**102**, Pfizer,

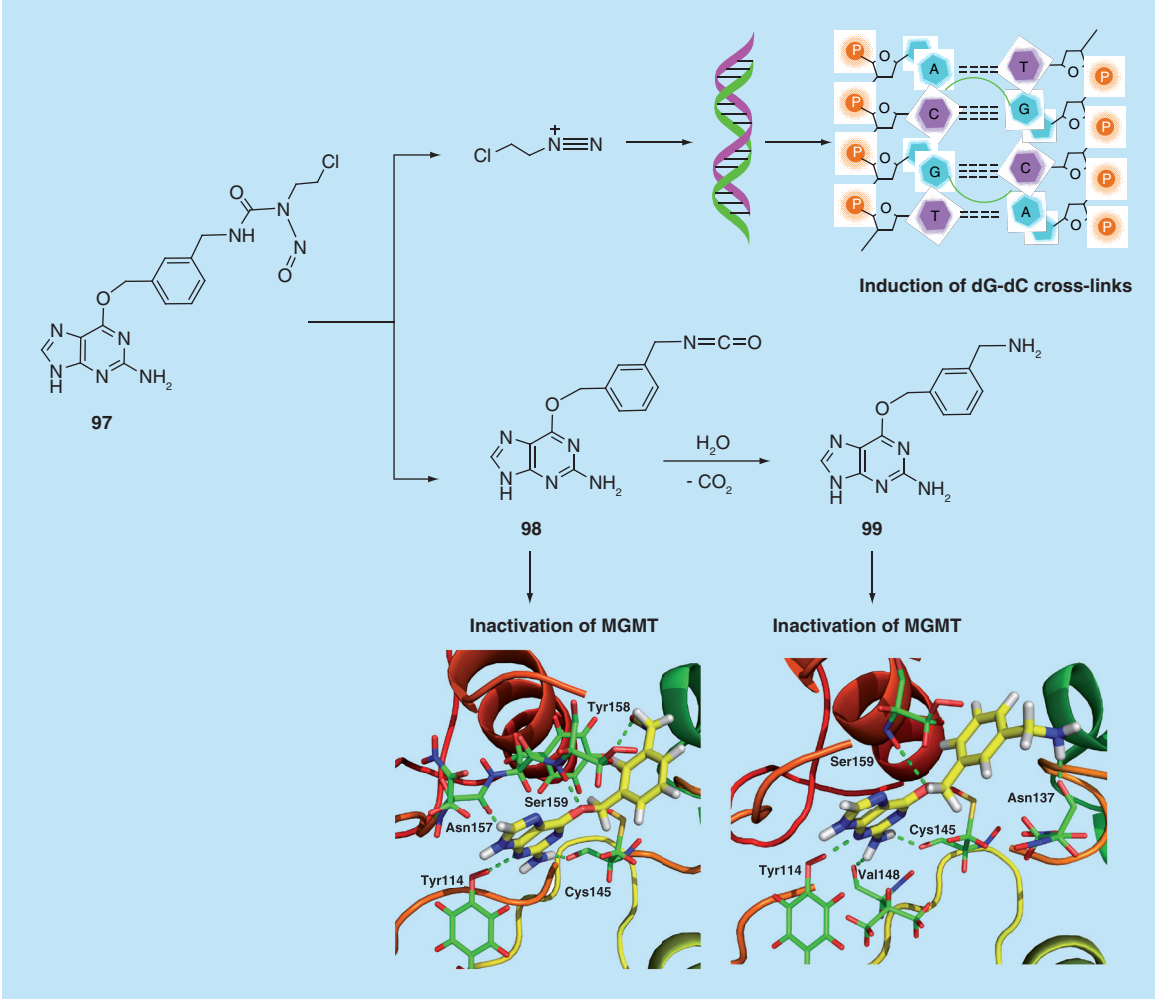


Figure 9. Schematic diagram depicting the decomposition pathway of combi-nitrosourea prodrug 97.

USA) are FDA approved topoisomerase I inhibitors as anticancer agents, which can form a ternary complex with the enzyme and DNA (Figure 11A) [67]. Cincinelli *et al.* [68] prepared two CPT–platinum complexes (**103** and **104**) with the purpose of exerting dual action and increase the drug–target interaction. The hydrophobicity of CPT can promote adequate cellular accumulation and nuclear localization of the Pt(II)-complex. Moreover, the incorporation of Pt could stabilize the CPT–DNA–enzyme ternary complex, thereby improving the drug–target interaction. The two compounds showed inhibitory activity toward the cell lines resistant to topotecan and cisplatin, and produced platinum–DNA adducts and topoisomerase I-mediated DNA damage. In particular, compound **104** exhibited a considerable antitumor activity *in vivo* against human H460 tumor xenograft. Recently, Kozerski's group [69,70] reported a new generation of CPT derivatives (**105–109**) with dual topoisomerase I inhibiting/DNA targeting properties (Figure 11A). In these compounds, the substituted aminomethyl groups were appended to the 9-position of CPT core as DNA-alkylating moieties. *In vitro* antiproliferative assays revealed that all compounds showed higher selectivity for cancer cells over normal cells compared with irinotecan, in which **109** was the most promising compound by exhibiting the weakest inhibition against normal cell growth. In addition, these CPT derivatives were able to covalently bind to the 2-NH_2 of guanine in DNA oligomer.

A-62176 (**110**), etoposide (**111**) and teniposide (**112**), which are topoisomerase II inhibitors, exert function through inhibiting the catalytic activity of topoisomerase II (e.g., A-62176) or stabilizing the topoisomerase II-DNA cleavage complex intermediate (e.g., etoposide and teniposide) (Figure 10D) [67,71]. On the basis of the action of A-62176, Kim *et al.* [72] designed a set of novel combi-molecules (**114~117**) (Figure 11B) combining the active pharmacophores of A-62176 and a DNA-alkylating agent psorospermin (**113**), which specially targeting the topoisomerase II–DNA complex site. The epoxydihydrofuran of psorospermin was

appended as a DNA-alkylating moiety to the pyridobenzophenoxazine ring of A-62176. Molecular modeling studies indicated that compounds **116** and **117** were the most promising combi-molecules and were further synthesized to evaluate their topoisomerase II-induced DNA-alkylating ability. The results showed that **116** possessed enhanced DNA-alkylating capacity than psorospermin regardless of the absence or presence of topoisomerase II, which might be due to the increased intrinsic binding interactions of **116** with DNA guanine. In contrast, chlorohydrin derivative **117** only exhibited moderate alkylating activity at pH 9.0. The antiproliferative studies also indicated that **116** and **117** exhibited significantly higher potency than psorospermin and A-62176 against ten cancer cell lines, in which **117** was slightly more active than **116**.

Delivering the classic alkylating antitumor drugs to DNA by attachment of a DNA binding carrier may reduce the undesired reactions of alkylating agents with other cellular components and improve DNA targeting efficacy [73]. Because of the DNA-binding affinity of topoisomerase inhibitors, the combinations of DNA-damaging agents with topoisomerases I or II inhibitor as combi-molecules provided possibility for acquiring novel chemotherapies with high targeting and antitumor efficacy. Bielawski *et al.* [74] synthesized three 2-chloroethylnitrosourea derivatives (**118**~**120**) of Hoechst 33258 as combi-molecules and examined their antiproliferative

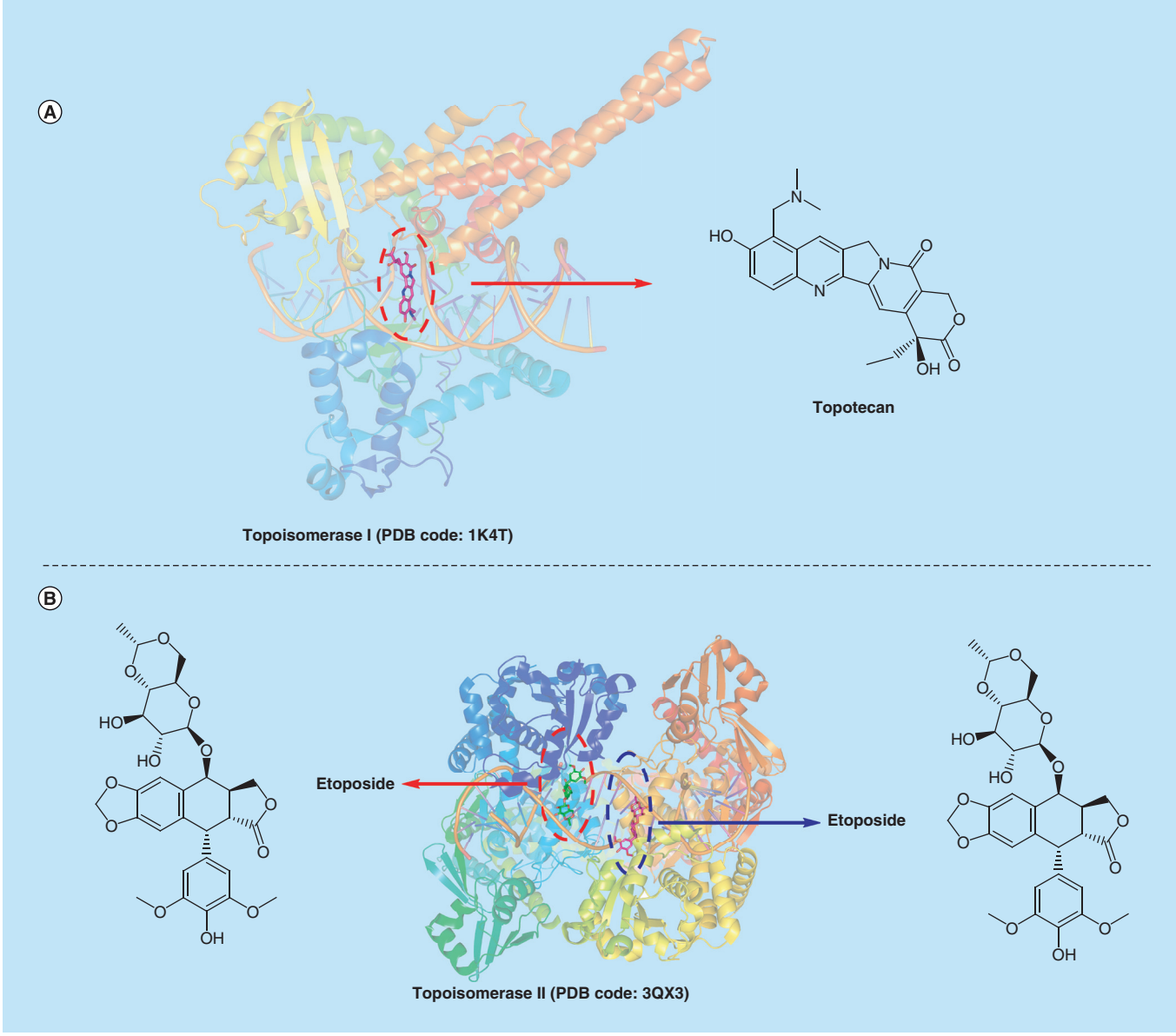
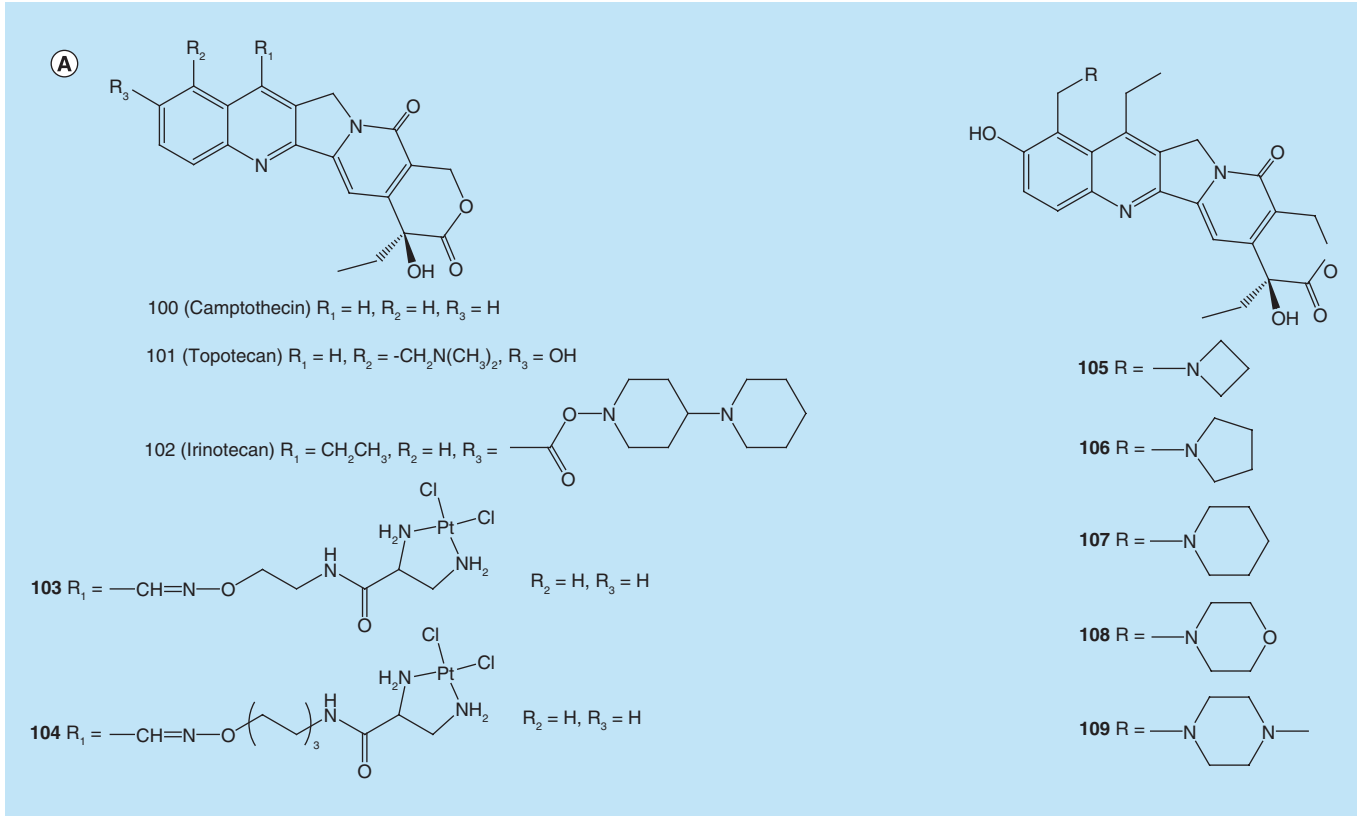


Figure 10. Interaction of topoisomerase and the approved inhibitors. (A) The crystal structure of human DNA topoisomerase I in complex with DNA and topotecan. **(B)** The crystal structure of human topoisomerase IIβ in complex with DNA and etoposide.

activity in MDA-MB-231 and MCF-7 breast cancer cells as well as their ability of inhibiting topoisomerases I and II *in vitro* (Figure 12A). Among these compounds, **118** was proven to be the most active with IC_{50} values of 8 ± 2 μM and 5 ± 2 μM for MDA-MB-231 and MCF-7 cell lines, respectively, which is ~20-fold more potent than Hoechst. The [^{3}H]thymidine incorporation and DNA binding assays gave the similar results as cytotoxic assay. All three compounds preferentially interact with adenine–thymidine base pair rather than guanine–cytosine base pair. Plasmid DNA relaxation assay indicated that they also had significant topoisomerase I and II inhibitory activity with IC_{50} values of 2~15 μM, in which **118** was the most potent topoisomerase I and II inhibitor with IC_{50} value of 2 μM. Yadav *et al.* [75] synthesized a series of combi-molecules (**121~127)** containing an etoposide analog epipodophyllotoxin as topoisomerase II inhibitor and a bifunctional N-mustard moiety as DNA or topoisomerase alkylating species (Figure 12B). The most potent combi-molecule **125** possessed a mean GI_{50} value of 0.7 μM in NCI-60 cell lines, which was 17- and 41-fold more potent than etoposide (GI_{50} = 12 μM) and N-mustard melphalan (GI_{50} = 29 μM), respectively. The other combi-molecules also exhibited lower GI_{50} values compared with etoposide. All of the combi-molecules tested were observed to target topoisomerase II as evidenced by a variety of *in vitro* or cell-based assay. A DNA cross-link assay was performed to demonstrated that **123** induced comparable level of DNA cross-link as that of chlorambucil. A comparing analysis indicated that the NCI-60 cell growth inhibition profiles were significantly correlated with the etoposide and N-mustard compounds derived from the combi-molecules.

Steroid-linked DNA-damaging agents

Steroid hormones, including their agonists and antagonists, are of interest in the prevention and treatment of various hormone-dependent cancers, such as breast cancer, ovarian cancer, prostate cancer and endometrial cancer [16,18,19]. The role of steroid hormones in cancer chemotherapy is mostly based on changing the hormonal balance of body and the corresponding hormone receptors [9]. It is anticipated that steroid hormones and their modified derivatives can be utilized as attractive biological vectors for the delivery of chemotherapeutic agents, because they have been demonstrated to localize in the receptor overexpressing tissues [9,76,77]. Naturally, the conjugation of a conventional chemotherapeutic DNA-damaging agent and a steroid skeleton moiety with high affinity toward its cognate receptor into a combi-molecule was supposed to be more selective and less toxic than the classic nontargeting chemotherapies. As expected, a series of combi-molecules containing the pharmacophores of estrogens, androgens and related modified steroids, which were designed to selectively deliver the DNA-damaging chemotherapeutic agents to the tumor tissues with



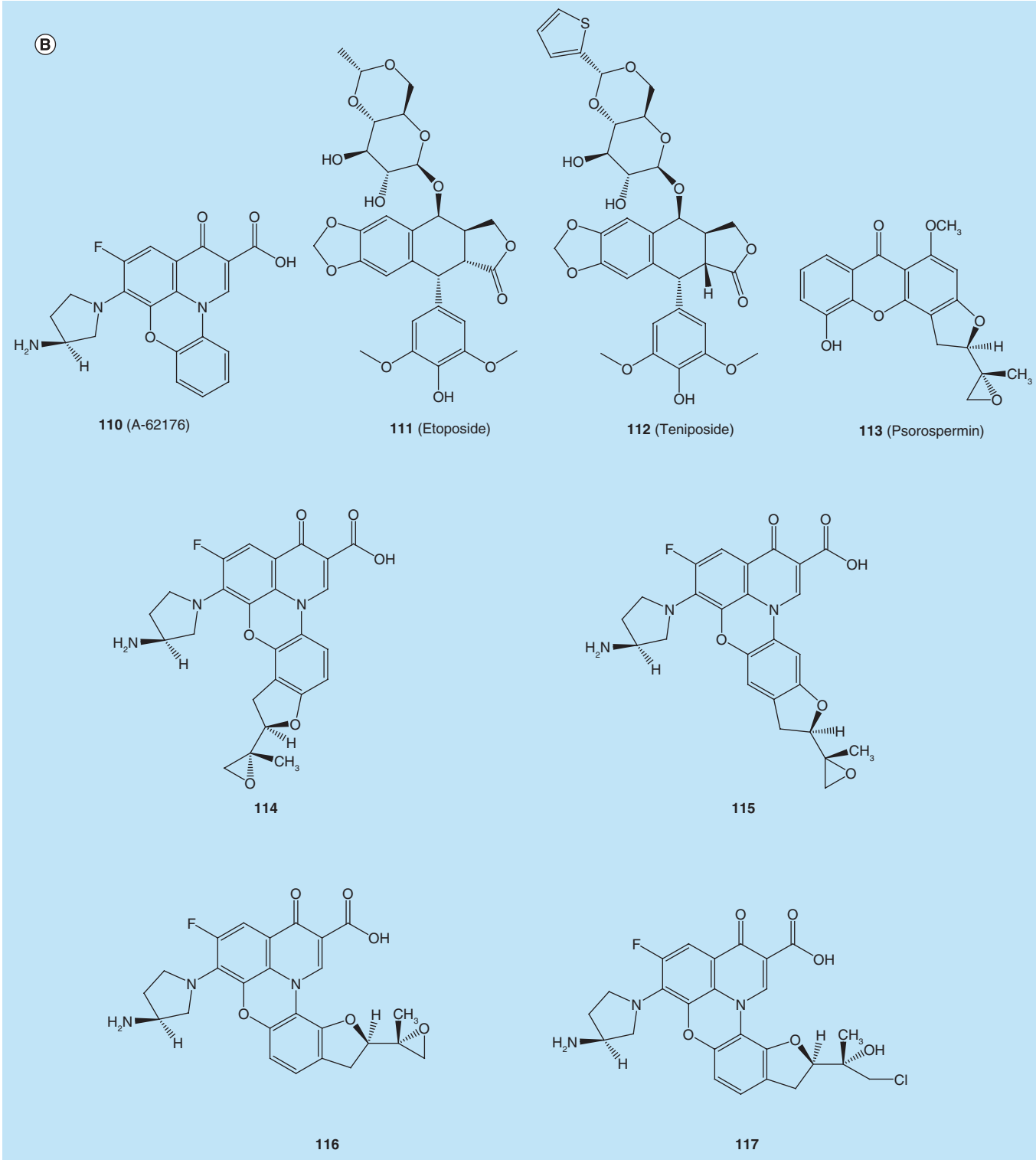
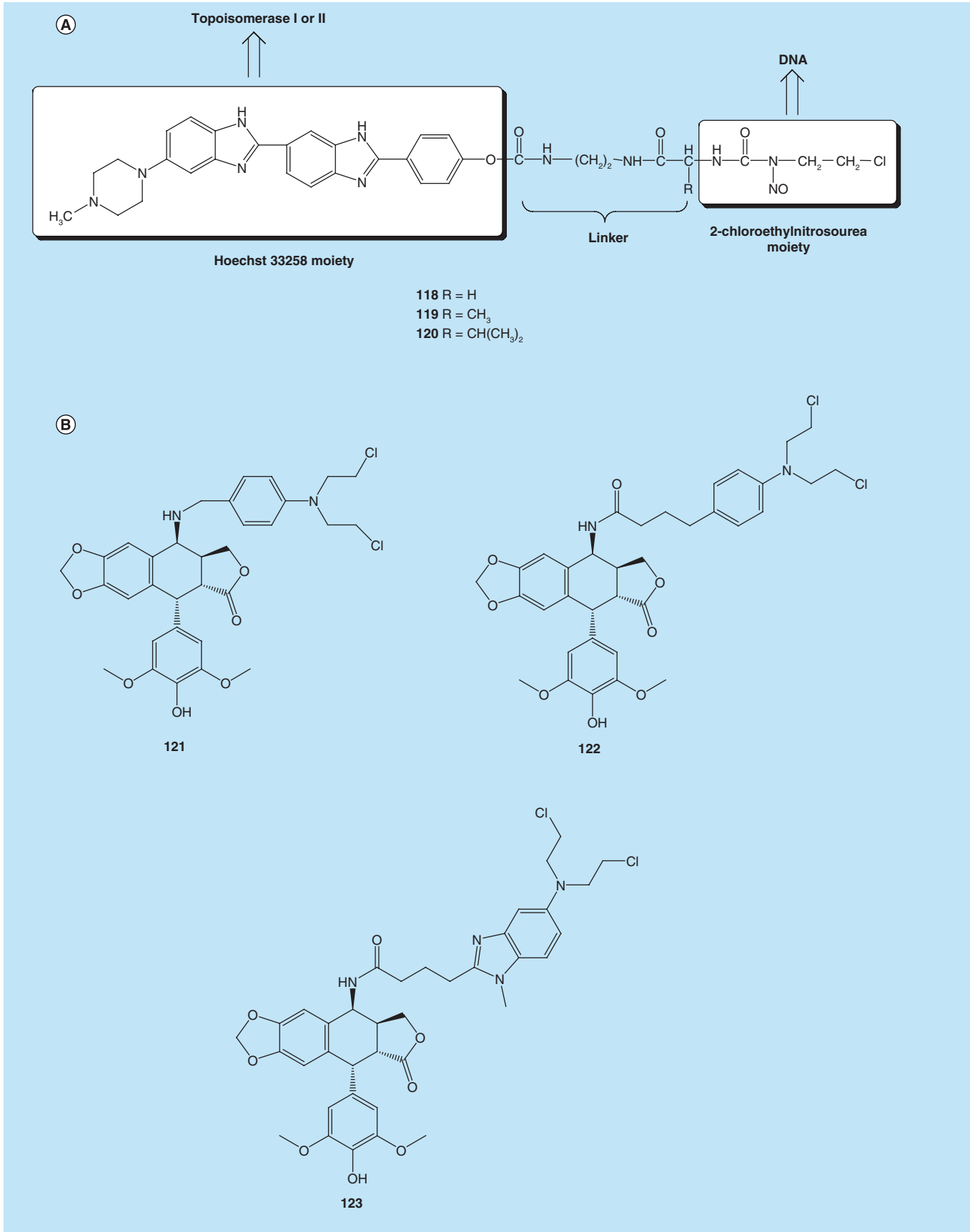


Figure 11. Topoisomerase I/II inhibitor-linked DNA-damaging agents. Molecular structures of **(A)** compounds **100**~**109** and **(B)** compounds **110**~**117**.

high levels of estrogen receptor (ER) or androgen receptor (AR), were demonstrated to target the hormone-dependent cancers and eliminate the known side reactions associated with conventional chemotherapies [9,16,18,19]. Estramustine (**128**, Pfizer), prednimustine (**129**) and phenesterin (**130**) are three clinically approved steroid–nitrogen mustard combi-molecules for treating prostate cancer, breast cancer and ovarian cancer, respectively


A
Topoisomerase I or II
DNA
Hoechst 33258 moiety
Linker
2-chloroethylnitrosourea moiety
118 R = H
119 R = CH3
120 R = CH(CH3)2
B
121
122
123

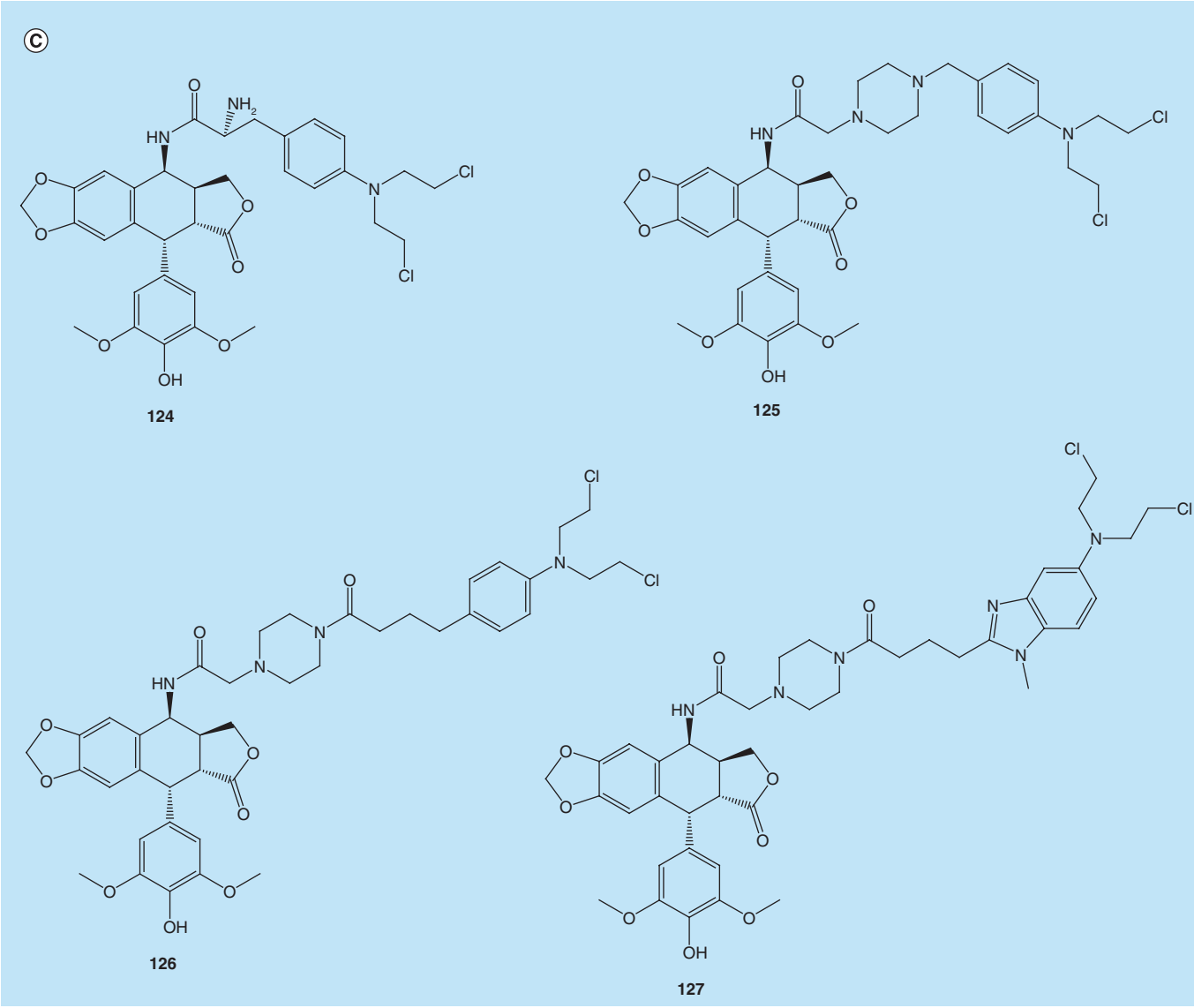


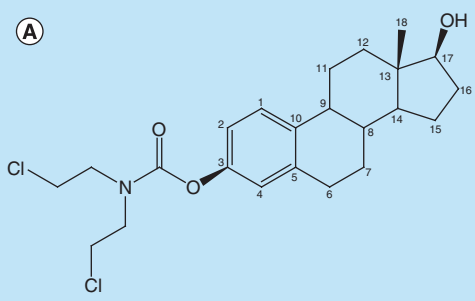
Figure 12. Topoisomerase inhibitor-linked chloroethylnitrosourea or N-mustard (cont. from facing page). Molecular structures of **(A)** combi-molecules (**118**~**120**) with a Hoechst 33258 moiety as Topoisomerase I or II inhibitor and a 2-chloroethylnitrosourea moiety as DNA-damaging agent and **(B)** combi-molecules (**121**~**127**) designed to contain an etoposide analog epipodophyllotoxin as Topoisomerase II inhibitor and a bifunctional N-mustard moiety as DNA or topoisomerase-alkylating species.

(Figure 13A) [9]. Over the past decades, a number of significant works have been performed for the development of steroid DNA-alkylating combi-molecules, in which the compounds possessing the properties of targeting ER and AR aroused great attention of chemists, pharmacists and clinicians. Therefore, in this review, the investigations on the steroid DNA-damaging combi-molecules targeting ER and AR in recent decade were summarized.

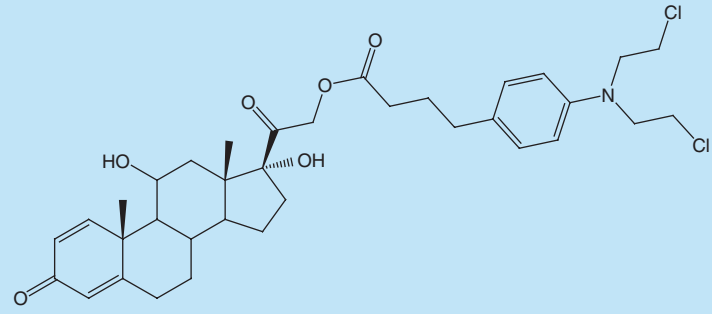
ER targeting steroid DNA-damaging combi-molecules

ER is usually overexpressed on the membrane and nucleus of breast cancer cells, thereby becoming a potentially biological target assisting in drug delivery [18]. This provides a basis for developing DNA-damaging combi-molecules with steroid estrogens as carriers, such as estradiol and its derivatives, to selectively target ER overexpressing cancer cells. Mitra *et al.* [78] designed and synthesized a steroid–nitrogen mustard **131**, in which the nitrogen mustard moiety was attached to the 7α-position of 17β-estradiol via an alkyl–amino–carbamate linker (Figure 13B). Compound **131** was proven to have high affinity for ER and form covalent DNA adducts. As shown in Figure 14, the DNA adduct induced by **131** has special binding affinity with ER, which not only suppresses the function of ER in cell growth or survival, but also camouflage the DNA adducts from being restored by DNA repair enzymes.

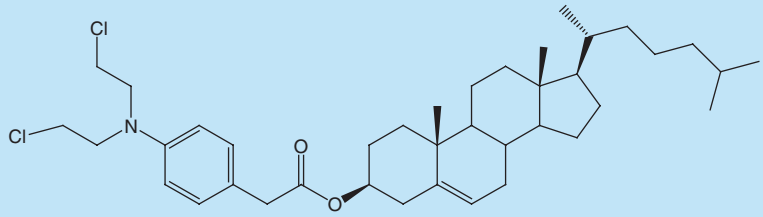
Clonal survival assays indicated that **131** showed selective toxicity toward ER-positive MCF-7 breast cancer cells compared with ER-negative MDA-MB-231 cells. To determine the influence of the linkers on the reaction with DNA and the binding of the resultant DNA adducts to ER, a set of combi-molecules containing a DNA-damaging *N*,*N*-bis-(2-chloroethyl)aniline linked to the 7α-position of 17β-estradiol were syn-



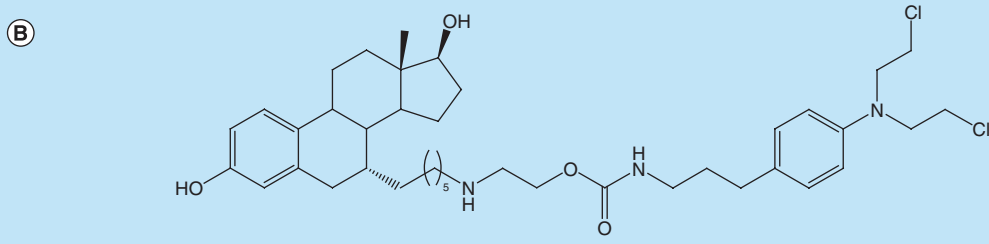
128 (Estramustine)



129 (Prednimustine)



130 (Phenesterin)



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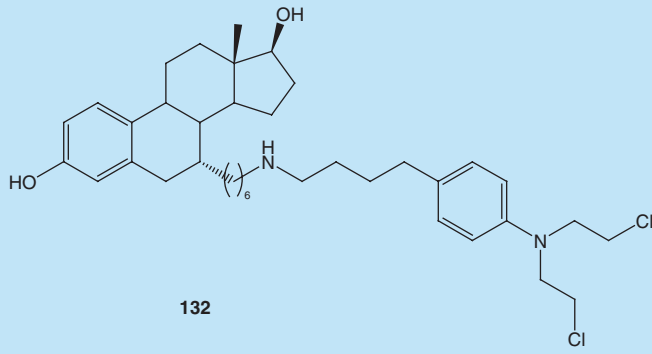

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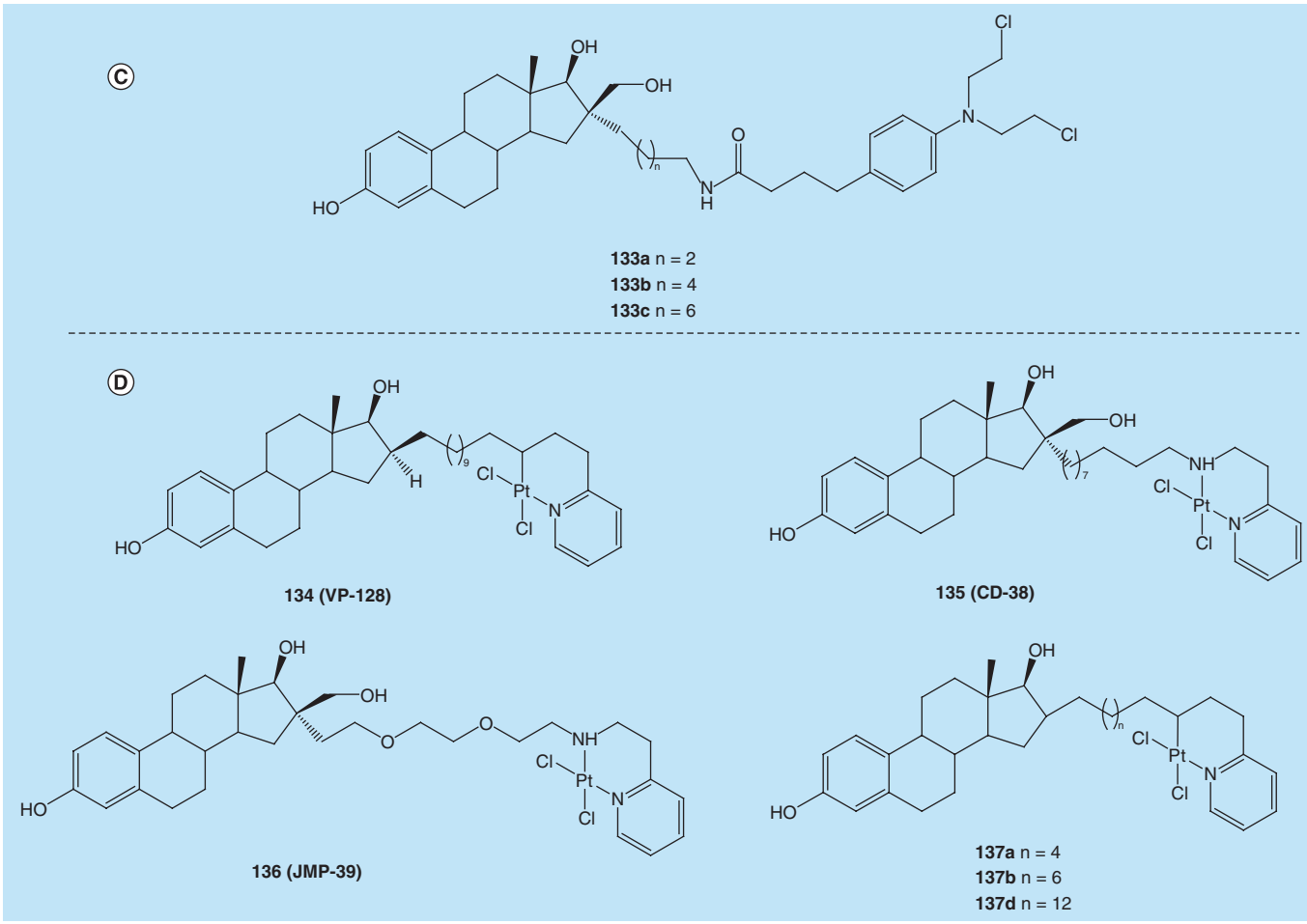


Figure 13. Steroid-linked DNA-damaging agents (cont. from facing page). (A) Three clinically approved steroid DNA-alkylating combi-molecules, **128** (estramustine), **129** (prednimustine) and **130** (phenesterin). **(B)** Structures of steroid–nitrogen mustard combi-molecules (**131** and **132**) in which the nitrogen mustard moiety was attached to the 7α-position of 17β-estradiol via different linkers. **(C)** Structures of estradiol–chlorambucil combi-molecules (**133a**~**c**) the chlorambucil moiety was linked to the 16α-position of estradiol nucleus with alkyl linkers with varying lengths. **(D)** Molecular structures of estradiol-linked cisplatin derivatives, VP-128 (**134**), CD-38 (**135**) JMP-39 (**136**) and **137**.

thesized, and their affinity for ER and selective cytotoxicity against ER-positive breast cancer cells were evaluated [79]. All the new compounds were capable of forming DNA adducts and had relative binding affinity (RBA) for ER ranging from 6 to 40% compared with estradiol (RBA = 100%). Compound **132** containing a single amino group in the linker exhibited comparable RBA (40%) to that of **131** (RBA = 46%). Similar to **131**, **132** also possessed selective cytotoxicity against ER-positive MCF-7 cells rather than ER-negative MDA-MB-231 cells. In order to develop alternative steroid–nitrogen mustard combi-molecules as tumor targeting chemotherapy, Gupta *et al.* [80] synthesized a series of estradiol–chlorambucil combi-molecules (**133a**~**c**), where the chlorambucil moiety was linked to the 16α-position of estradiol nucleus by alkyl linkers with varying lengths (Figure 13C). MTT assays showed that the newly synthesized compounds had moderate to significant cytotoxic activity in ER-positive MCF-7 and ER-negative MDA-MB-436 and MDA-MB-486 breast cancer cell lines. The combi-molecule **133b** with six carbon atoms spacer was found to be the most active in hormone-dependent MCF-7 cells, while **133a** with four carbon atoms spacer was more active toward hormone-independent cell lines, e.g., MDA-MB-436.

Platinum-based agents, such as cisplatin, carboplatin and oxaliplatin, have been widely used for the treatment of various cancers, including breast, ovarian, bladder and testicular cancers [81]. The antitumor activity of chemotherapeutic platinum complexes mainly arises from their ability to damage DNA guanine by inducing the formation of DNA intrastrand and interstrand crosslinks [2]. Although these compounds are very active, their clinical applications are limited because of the nonspecificity to tumors, which leads to severe side effects, particularly nephrotoxicity, neurotoxicity and triggering of chemore-

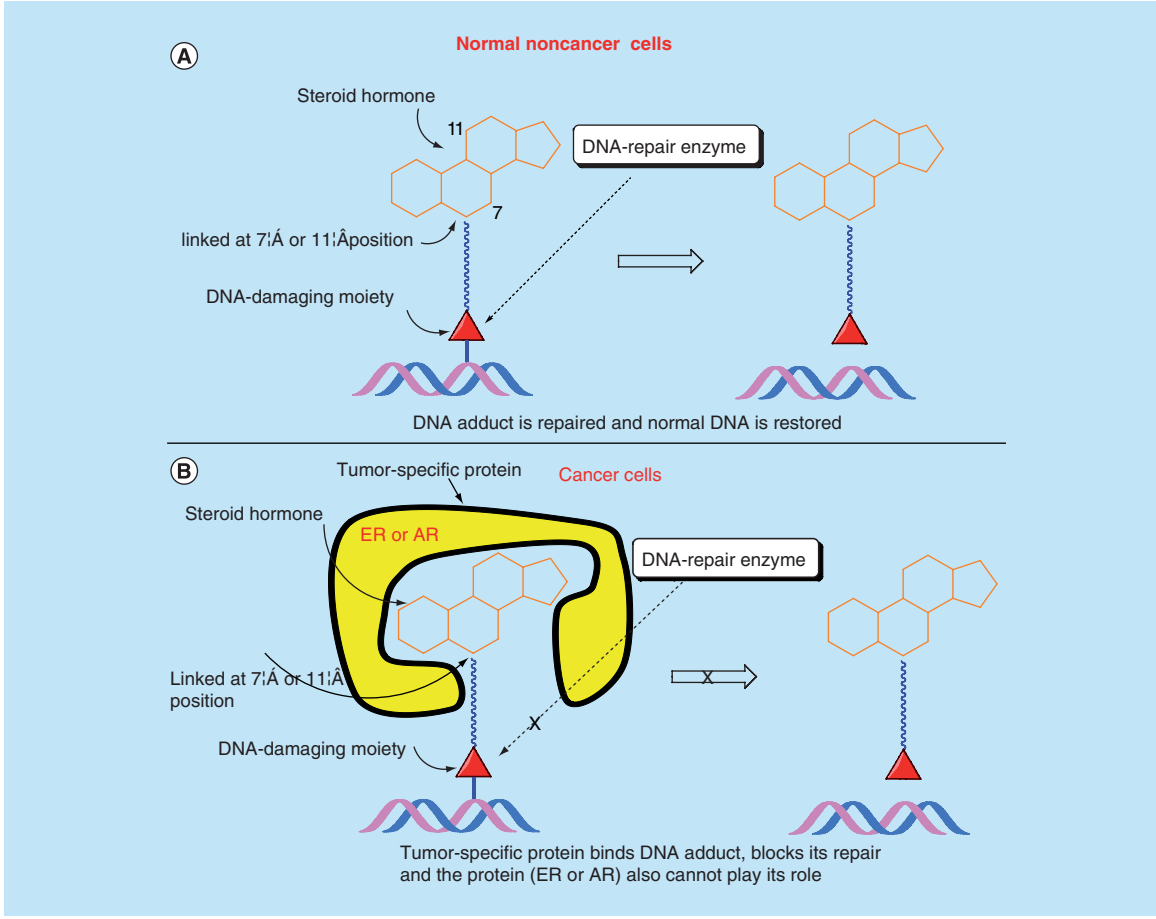
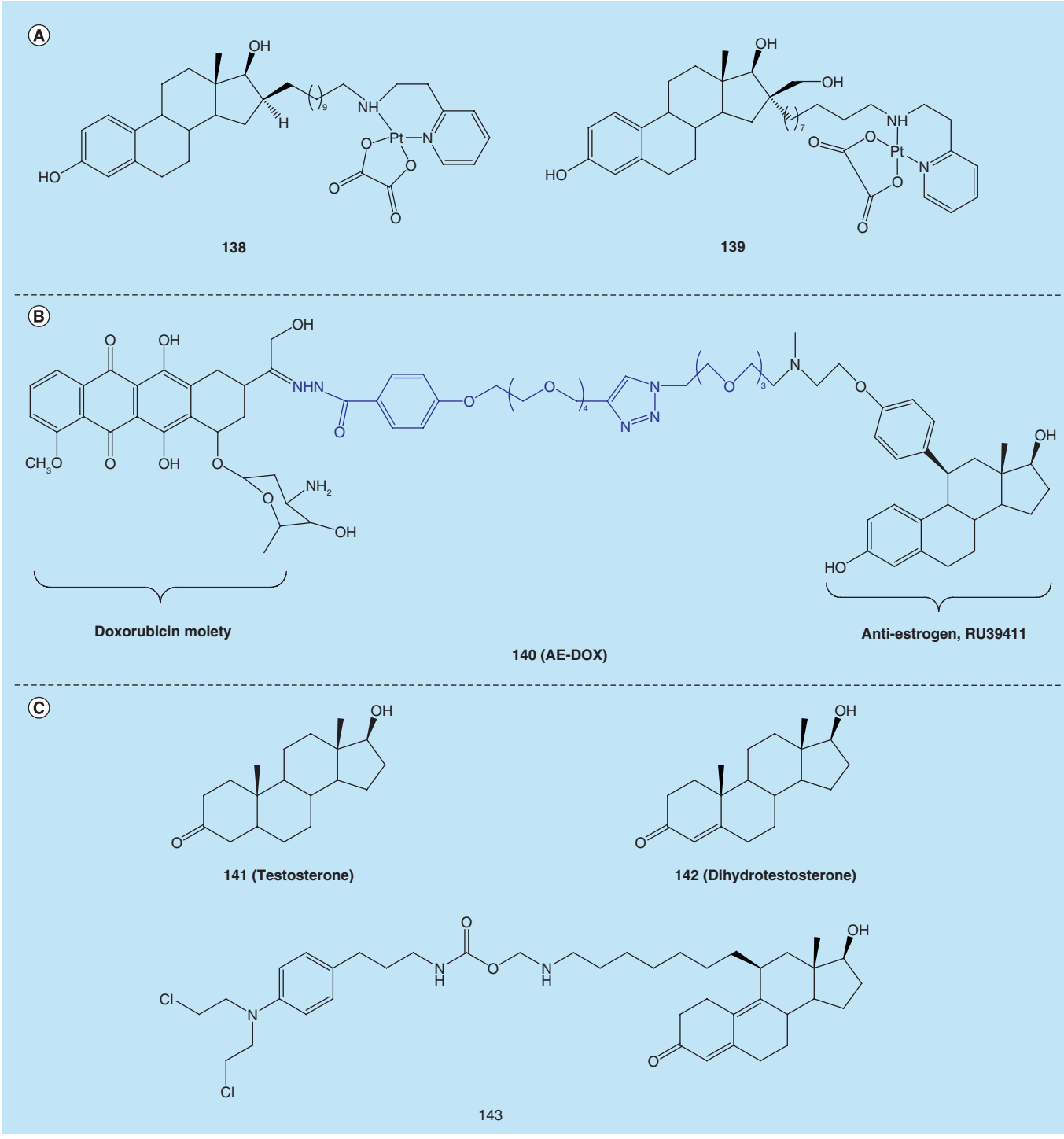


Figure 14. Schematic diagram depicts the targeting mechanism of action of active steroid-linked DNA-damaging combi-molecules that form DNA adducts which can interact with tumor specific protein (estrogen receptor or androgen receptor) in cancer cells. (A) In normal noncancer cells that do not express the target protein at high levels, the DNA adducts formed by active steroid-linked DNA-damaging combi-molecules would be repaired by DNA-repair enzymes, while **(B)** in cancer cells with overexpression of the target protein, the DNA adducts formed by active steroid-linked DNA-damaging combi-molecules would not be repaired by DNA-repair enzymes due to the binding with target protein, leading to accumulation of DNA lesions and loss of the normal function of target protein.
AR: Androgen receptor; ER: Estrogen receptor.

sistance [82]. To improve the efficacy and minimize the toxic side effects, there is an increasing need to selectively deliver the platinum anticancer agents to specific tumor sites. Bérubé's group carried out extensive investigations on the development of platinum–estradiol combi-molecules targeting the ER-positive breast cancer cells [15,83–88]. As shown in Figure 13D, VP-128 (**134**), CD-38 (**135**) and JMP-39 (**136**) are three potent compounds with better *in vitro* biological activity than cisplatin against breast cancer cell lines and with higher affinity for ERα, DNA and RNA [83–86]. Especially, VP-128 (IC_{50} = 0.35 nM) showed high affinity for the ERα even better than 17β-estradiol itself (IC_{50} = 4.79 nM). Moreover, VP-128 exhibited a better tumor regression than cisplatin on an ER-positive MCF-7 human breast cancer xenograft mice model [84]. In order to investigate the effect of the length of linker on the biological activity of estradiol–cisplatin combi-molecules, several VP-128 analogs (**137a~c**) bearing 6, 8 and 14 carbon atoms in the alkyl chain were synthesized for evaluating their RBA for ER and cytocidal activity on several breast cancer cells [86]. The results showed that the length of the alkyl linker had little influence on the biological activity, and **137a~c** exhibited higher cytotoxicity to ER-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cell lines than cisplatin. *In vivo* studies indicated that the estradiol–cisplatin combi-molecules were specific toward hormone-dependent breast and ovarian cancers.

Inspired by the potency of **134–136**, Saha *et al.* [88] further synthesized and assessed the biological activity of two series of estradiol-linked carboplatin and oxaliplatin combi-molecules. Most of these compounds were observed with antiproliferative activity on MCF-7 and MDA-MB-231 breast cancer cells in micromo-

lar range and were more active than carboplatin and oxaliplatin. The oxaliplatin derivatives **138** and **139** (Figure 15A) had the highest cytotoxicity among all the newly prepared compounds and showed higher affinity for ERα than their cisplatin counterparts (**134** and **135)** and 17β-estradiol. Thus, **138** and **139** will be promising alternative compounds to be studied *in vivo* for targeting estrogen-dependent tumor tissues. For seeking novel agents targeting ER-positive breast cancer cells, Dao *et al.* [89] chose the clinically effective anticancer alkylating agent doxorubicin as a cytotoxic moiety to prepare the anti-estrogen–doxorubicin (AE-DOX) combi-molecule **140**, in which the anti-estrogen RU39411 moiety was linked to the doxorubicin moiety through the linkage at the 11β-position and acted as an ER targeting component (Figure 15B). Cytotoxic-



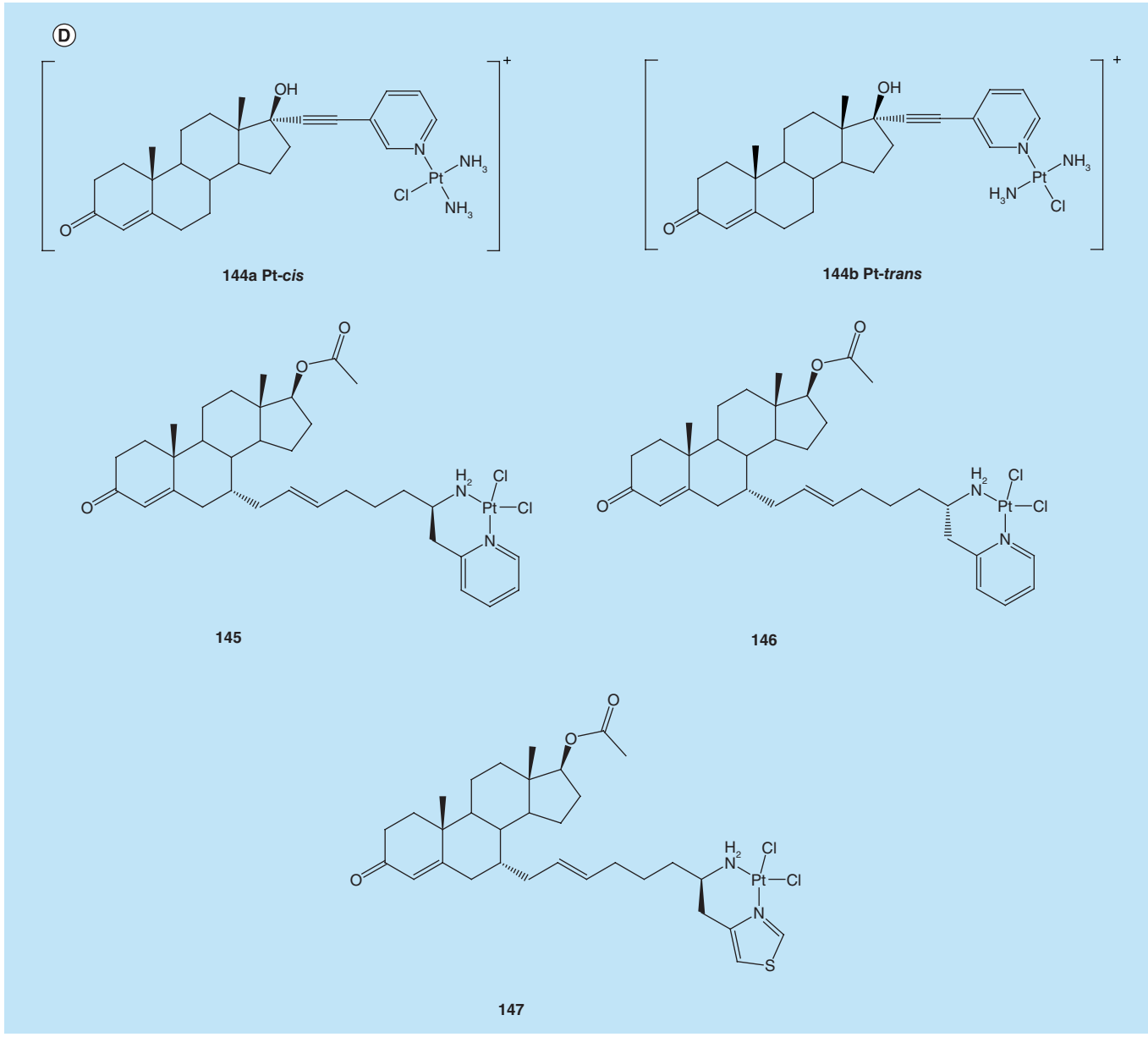


Figure 15. Some novel steroid-linked DNA-damaging combi-molecules (cont. from previous page). (A) Structures of two potent estradiol-linked carboplatin derivatives **138** and **139**. **(B)** Structure of AE-DOX combi-molecule **140** where the alkylating doxorubicin moiety is attached to the 11β-position of estradiol. **(C)** Molecular structures of testosterone (**141**), dihydrotestosterone (**142**), and steroid-nitrogen mustard combi-molecule **143**. **(D)** Chemical structures of combi-molecules **144**~**147** by merging the steroid testosterone skeleton and different platinum complexes linked at 17α or 7β-position.
AE-DOX: Anti-estrogen-doxorubicin.

ity assays revealed that **140** (IC_{50} = 0.011 μM) showed approximately 50-fold higher cytotoxicity toward ER-positive MCF-7 cells compared with free doxorubicin (IC_{50} = 0.602 μM), while no difference was observed for ER-negative MDA-MB-231 cells.

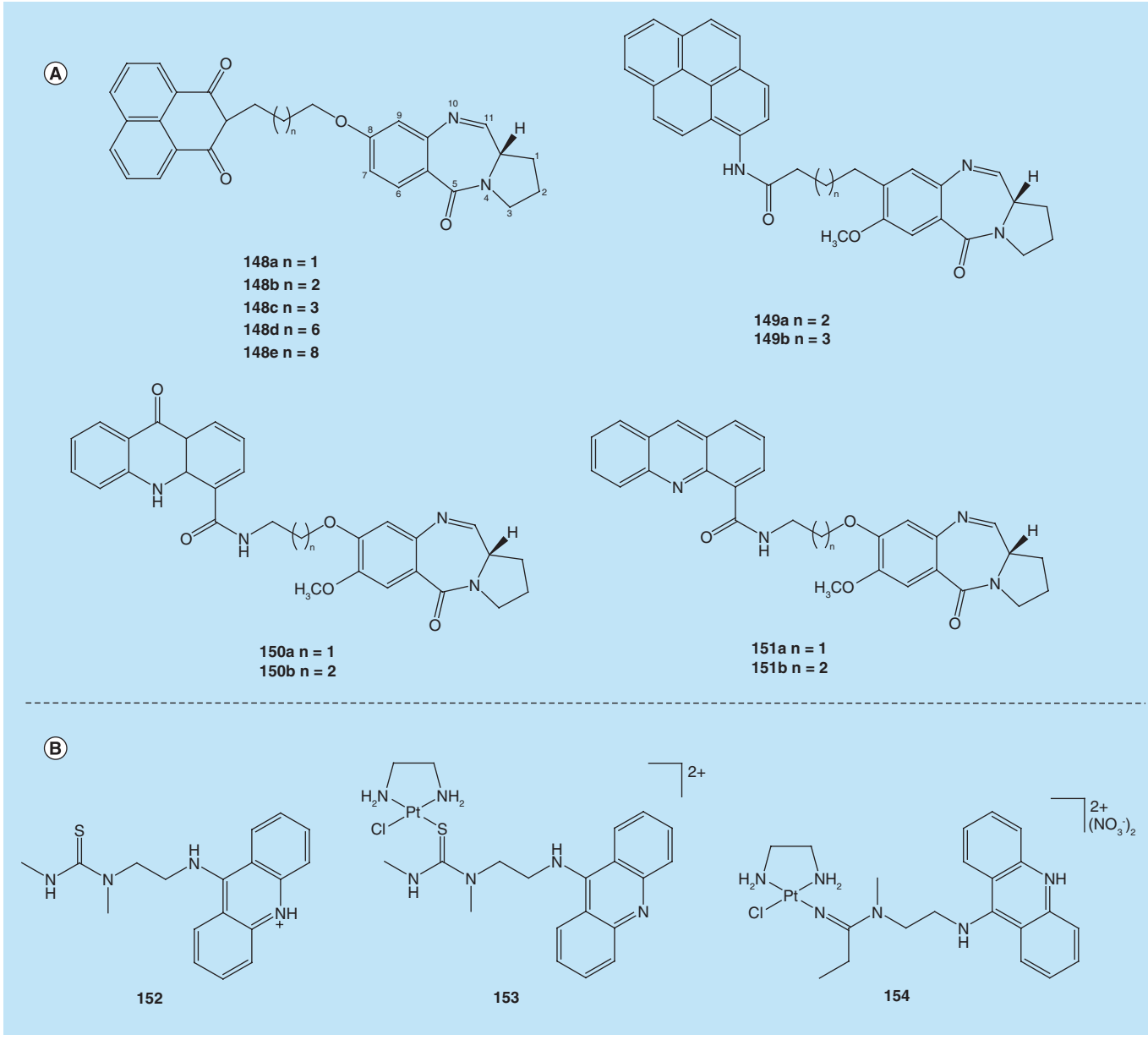
AR Targeting steroid DNA-damaging combi-molecules

Similar to the ER overexpression in most breast cancer tissues, the high expression of AR in malignant tissue is often associated with the progression of prostate cancer, so AR becomes an important chemotherapeutic target that can be exploited for prostate cancer drug development [19]. The combination of an AR-targeting moiety, such as testosterone (**141**) and dihydrotestosterone (**142**) (Figure 14C), with a DNA-damaging agent is a promising strategy for the development of novel chemotherapies selectively targeting AR overexpressing tumor cells.

Marquis *et al.* [90] designed and synthesized a bifunctional steroid–nitrogen mustard combi-mole-

cule (**143**) that specifically targeted the prostate cancer cells (Figure 15C). The DNA-damaging *N,N*-bis-2-chloroethylaniline moiety was linked to a steroid ligand (11β-substituted estra$\Delta^{4(5),9(10)}$-3-one) targeting AR, which allowed the combi-molecule to produce DNA adducts interacting with AR. The DNA adducts in the AR–DNA adduct complexes was supposed to be camouflaged from DNA repair enzymes, which meanwhile disordered the function of AR in normal cell growth (Figure 14). It was demonstrated that **143** induced apoptosis in AR-positive LNCaP prostate cancer cells and exhibited antitumor activity against LNCaP cells in a mouse xenograft model. Recently, Huxley *et al.* [91] reported the first type of steroid-platinum combi-molecules targeting tumor cells with high level of AR. Eight potential compounds were synthesized, in which Pt-*cis* **144a** (IC_{50} = 15.9 μM) possessed more potent cytotoxicity than the Pt-*trans* isomer **144b** (IC_{50} = 63 μM) and cisplatin (IC_{50} = 32 μM) in AR positive T47D cancer cells (Figure 15D). Besides, **144a** exhibited potent activity against cisplatin-resistant A2780 cells. Cellular uptake assay indicated that **144a** could be selectively transported across the membrane of the targeting cells with enhanced efficiency.

In order to improve the selectivity and pharmaceutical properties of the anticancer drugs toward prostate cancer tumor, Fortin *et al.* [92] designed a set of new combi-molecules by merging 17β-acetyltestosterone with different platinum complexes at



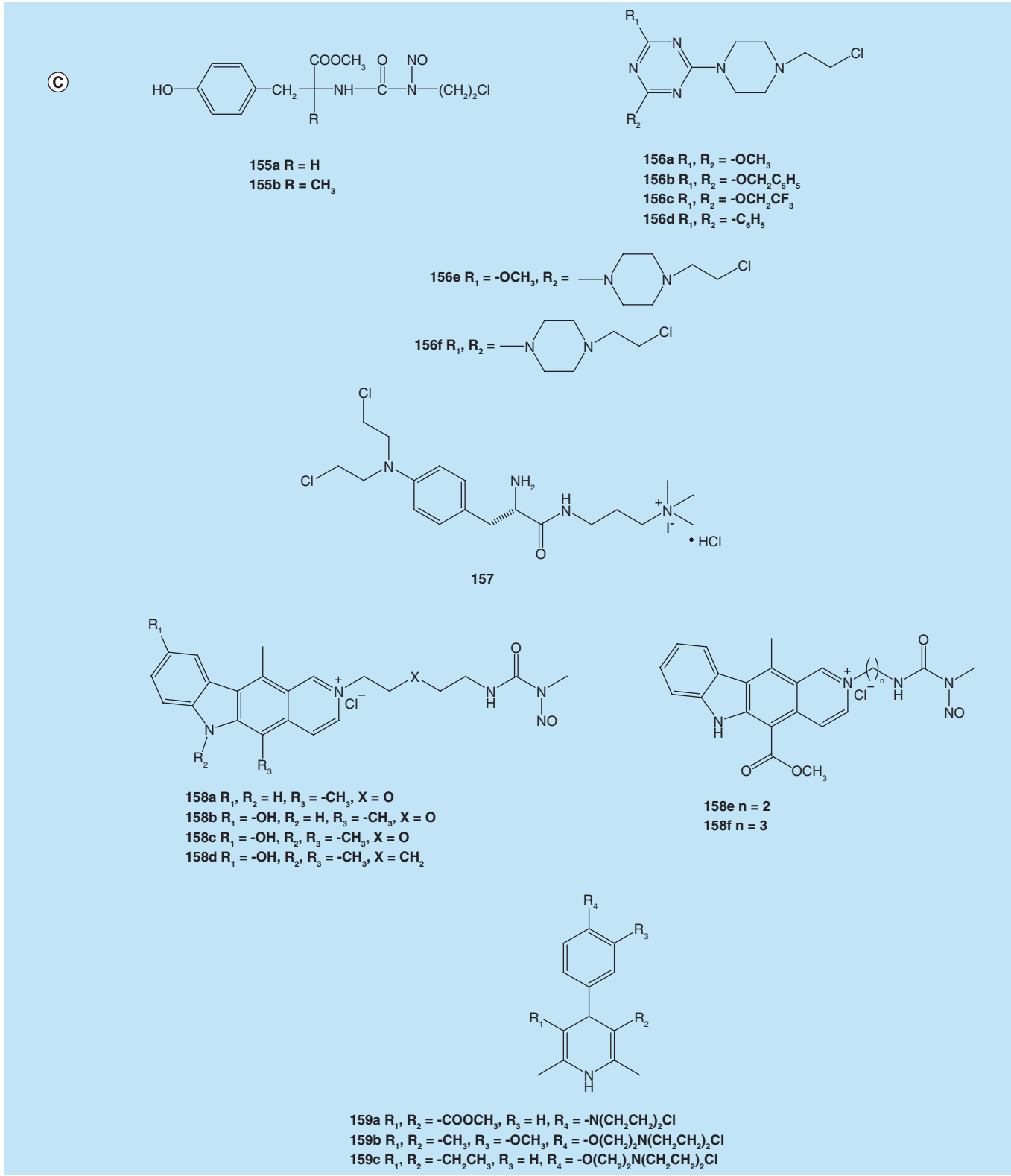


Figure 16. A series of DNA intercalating agent or other bioactive group-linked combi-molecules (cont. from previous page). Molecular structures of **(A)** a series of C8-linked PBDs–DNA intercalating agents combi-molecules (**148**~**152**), **(B)** platinum-free DNA intercalator **152** and platinum–acridine combi-molecules **153** and **154** and **(C)** combi-molecules based on the combinations of the tyrosine (**155**), 1,3,5-triazine skeleton (**156**), quaternary ammonium (**157**), ellipticine (**158**), 1,4-dihydropyridines (**159**) skeletons with a DNA-damaging anticancer agent.

the 7α-position (Figure 15D) to simultaneously target AR-mediated signal pathway and DNA. Antiproliferative activity assay showed that all platinum complexes had relatively lower IC_{50} ranging from 1.4 to 28 μM in three prostate cancer cell lines, LNCaP (AR+), PC3 (AR-) and DU145 (AR-). The combi-molecules bearing a pyridinyl (compound **145** and **146**) and thiazolyl (compound **147**) side chain displayed the highest activity with IC_{50} ranging from 1.4 to 3.6 μM, which were more potent than that of cisplatin with IC_{50} between 2.1 and 6.7 μM. In addition, compounds **145–147** mainly arrested the cell cycle in the S-phase and induced DNA double-strand breaks by histone H2AX phosphorylation (γH2AX) in PC3 cells. *In vivo* chick chorioallantoic membrane tumor assay indicated that **145–147** dramatically decreased the grafted HT-1080 fibrosarcoma tumor growth by 33, 21 and 30%, respectively. Importantly, **145–147** exhibited low toxicity, suggesting that these new combi-molecules might be promising anticancer agents specifically target prostate cancer.

DNA intercalating agent-linked combi-molecules

DNA intercalating agents, such as acridine, acridone and naphthalimide, can intercalate into DNA helix and result in considerable anticancer activity. Therefore, the incorporation of DNA intercalating moiety and other DNA-damaging agents may provide a method for discovering potential combi-molecules with increased ability of targeting and acting on DNA. Based on the antitumor activity of naturally occurring pyrrolo[2,1-c] [1,4]benzodiazepines (PBDs) isolated from *Streptomyces* species, Kamal's [93–95] group synthesized a series of C8-linked PBDs–DNA intercalating agents' combi-molecules (**148–151**) and evaluated their DNA binding ability and antitumor activity (Figure 16A). The *in vitro* study on the antitumor activity showed that among the five C8-linked PBD-naphthalimide combi-molecules (**148a–e**), **148b** and **148c** exhibited significant higher cytotoxicity than the other three compounds, and their cytotoxicity were related to the length of the alkyl linkers. Interestingly, **148b** was more potent against colon and renal cancers, while **148c** was more potent against colon and melanoma cancers [93], compared with the other **148** compounds. For the pyrene-linked PBD combi-molecules (**149a** and **149b**), **149a** displayed higher anticancer activity in NCI-60 cells than **149b**, and increased the melting temperature of calf thymus DNA by 6.5°C after an 18 h incubation at 37°C. This result suggested that **149a** has significant DNA binding ability, which enhanced the alkylating activity of PBD moiety [94]. The C8-linked PBD–acridone/acridine combi-molecules (**150a~b** and **151a~b**) also exhibited high DNA binding ability. Particularly, combi-molecule **150b**, which induced a 12.5°C increase of the melting temperature of calf thymus DNA, showed promising anticancer activity *in vitro* against non-small-cell lung cancer NCI-H23 cells (LC_{50} = 0.07 μM), melanoma M14 and UACC-62 cells (both LC_{50} < 0.01 μM), and renal cancer A498 cells (LC_{50} = 0.05 μM) [95].

Bierbach's group [20] reported that a new platinum–acridine combi-molecule **153** (Figure 16B), which showed potential cytotoxicity against two glioblastoma SNB19 (IC_{50} = 0.37 μM) and U87 (IC_{50} = 0.75 μM) cell lines observed in clonogenic survival assays. The potency of **153** was 6.7- and 2.5-fold higher than that of the platinum-free DNA intercalator **152** in SNB19 and U87 cells, respectively. It is interesting that no influence of **153** on the viability of normal cells was observed in a trypan blue exclusion assay. Another study indicated that **153** could bind to guanine N7 position in the major groove of DNA, forming a monofunctional DNA adduct [96]. Subsequently, Bierbach and coworkers [97] designed and synthesized another platinum-acridine combi-molecule **154**, which also induced monofunctional DNA adducts as **153**. In comparison with cisplatin, **154** showed 40–200-fold higher cytotoxicity in three non-small-cell lung cancer cells (NCI-H460, NCI-H522 and NCI-H1435) with IC_{50} values at nanomolar range [98]. Cell cycle analysis suggested that **154** induced a pronounced S phase arrest, which was different from the G2/M phase arrest observed for cisplatin. *In vivo* antitumor study showed that **154** exhibited tumor growth delay by 40% in NCI-H460 cells grafted mouse xenografts at a dose of 0.5 mg/kg [97]. The superiority of **154** may be due to the fact that the DNA adducts are difficult to be recognized by the nucleotide excision repair system.

Other bioactive group-linked DNA-damaging agents

In addition to the combi-molecules described above, there are also a number of other bioactive group-linked DNA-damaging agents with potential antitumor activity (Figure 16C). Compounds **155a** and **155b** are two combi-nitrosoureas containing tyrosine derivatives as carriers of cytotoxic nitrosourea moiety to selectively target melanoma cells, because tyrosine was the biosynthetic precursor of melanin [99]. These two combi-nitrosoureas were proven to possess high alkylating and low carbamoylating activity compared with clinically used lomustine. Compared with lomustine, **155a** and **155b** exhibit more significant preference to inhibit B16 melanoma cells rather than moloney lymphoma YAC-1 cells, which suggests that **155a** and **155b** may have higher selectivity to melanoma than lomustine. The introducing of tyrosine derivatives into nitrosoureas or other DNA-damaging

agents paved a new way for developing anti-melanoma drugs with high selectivity and low toxicity. Due to the inhibitory activity of 1,3,5-triazine derivatives against a variety of targets involved in cell proliferation, including glutathione *S*-transferase, topoisomerase and ER, Kolesinska *et al.* [100] designed and synthesized a series of combi-molecules (**156a~f**) of nitrogen mustards attached to 1,3,5-triazine as potential chemotherapies. Compound **156f** showed the most potent cytotoxicity against MCF-7 breast cancer cells with IC_{50} of 18.7 μM, which was more active than that of chlorambucil (IC_{50} = 29.14 μM). The potent cytotoxicity of **156f** was consistent with its high DNA-alkylating activity observed in an *in vitro* assay. Proteoglycans is a component of extracellular matrix of chondrosarcoma. Based on the affinity of positively charged quaternary ammonium for proteoglycans, a quaternary ammonium–nitrogen mustard combi-molecule (**157**) was designed for selectively delivering DNA-alkylating agents to the cartilage tumor tissues [101]. Compound **157** exhibited inhibitory activity toward human HEMC-SS chondrosarcoma and Saos-2 osteosarcoma cells at micromolar concentrations *in vitro*, and significant antitumor activity in an orthotopic Swarm rat model of chondrosarcoma. Ellipticine and its derivatives possess versatile bioactivity, such as p53 inhibition, DNA intercalation, energy metabolism disruption and topoisomerase inhibition. Based on the bioactivity of ellipticine, Mori *et al.* [102] designed and prepared a series of novel ellipticine-nitrosourea combi-molecules (**158a~f**) linked by an oxydiethylene unit at the N2-position of ellipticine and evaluated their antitumor activity against human cervical cancer HeLa S-3 cells. All compounds showed cytotoxicity at micromolar concentration range, in which **158f** displayed the highest antitumor activity with IC_{50} of 1.3 μM and was more potent than ellipticine (IC_{50} = 2.1 μM).

It is well known that P-glycoprotein is an important protein of cell membrane that pumps foreign substances out of cells. Thus, anticancer agents can be prevented from entering into cancer cells. Since 1,4-dihydropyridines (1,4-DHPs) were identified as potential P-glycoprotein inhibitors and multidrug resistant antagonist, Singh *et al.* [103] prepared a series of 1,4-DHPs-nitrogen mustard combi-molecules and assessed their cytotoxicity against four human cancer cell lines (A549, COLO-205, U87 and IMR-32). It was found that most of these compounds possessed moderate to significant antitumor activity, and **159a~c** exhibited the most potent cytotoxicity.

Conclusion

The drawbacks of conventional chemotherapies, such as limited efficacy, drug resistance and nontargeting activity, prompt the discovery of novel strategies for cancer treatment. The concept of 'combi-molecule', which incorporates two pharmacophores into a single molecule to achieve dual mode of action, has attracted significant attention and is being explored to acquire desired anticancer effects. In this review, we summarized the 'combi-molecules' reported in recent years. Via direct linking or a linker unit, two pharmacophores with distinct bioactivity are connected in a 'combi-molecule', in which one pharmacophore was DNA-damaging agent, and the other pharmacophore was another bioactive molecule or a carrier, including inhibitors of specific enzymes, agonist or antagonist of receptor mediating cell growth or molecules with high affinity toward DNA or tumor specific proteins. Principally, there are three merits of the combi-molecules that can be naturally concluded from these investigations. First, the uptake of DNA-damaging agents released from the combi-molecules by tumor tissues can be enhanced due to the biological properties of the other bioactive component. Second, less probability of developing resistance can be expected due to the dual function of different targets. Thirdly, the pharmacokinetic and pharmacodynamics properties can be better predicted when compared with the combination chemotherapy of the parent molecules.

Future perspective

In the recent two decades, quite a number of combi-molecules with dual targeting properties were developed and were demonstrated anticancer activities both *in vitro* and *in vivo*. However, there also has been a great deal of failed attempts to develop novel combi-molecules, which means that great challenges are still existing in the discovery of such therapies. Except for a considerate design of the chemical structure, the obtained combi-molecules must be confirmed to comply with the following criteria. First, the activity of each component in the combi-molecule must not be significantly reduced, such as the inhibitory activity against target protein and DNA-damaging ability. Second, the chemotherapeutic properties of the parent compounds should not be changed. Third, the entire combi-molecule must have desirable biological properties with respect to solubility, stability, selectivity, bioavailability and efficacy *in vivo*. Future combi-targeting DNA-alkylating agents can be focused on further exploration of novel molecules targeting crucial processes in DNA repair, cell-cycle control, drug metabolism and delivery. The pharmacodynamics and pharmacokinetics properties of these 'combi-molecules' also should be optimized to satisfy the requirements in further clinical application. Considering the complexity and stringency of drug approval procedures for anticancer agents, the clinical application of novel combi-molecules as anticancer drugs will go through a more complex awnd time-consuming process. Fortu-

nately, along with the mechanism of cancer genesis and progression being understood detailedly and the development of cancer precision therapeutics, the potential of combi-molecules with DNA-damaging function as anticancer drugs should be gradually recognized in clinical treatments of cancer.

Financial & competing interests disclosure

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Supplementary data

To view the supplementary data that accompany this paper, please visit the journal website at: www.future-science.com/doi/full/10.4155/fmc-2016-0229

Executive summary

Combi-molecules bearing DNA-damaging activity

- DNA-damaging agents, such as temozolomide, carmustine and cisplatin, were widely used anticancer drugs in clinics, which act through alkylating DNA thus inhibiting DNA replication and transcription or inducing mismatch repair, and ultimately result in cell apoptosis.
- The undesired properties of conventional DNA-damaging agents, such as high toxicity, lack of targeting activity and drug resistance, prompt the development of dual functional 'combi-molecules' as anticancer drugs containing a DNA-damaging moiety and another bioactive pharmocophore.

EGFR TK inhibitor-linked DNA-damaging agents

- Overexpression of EGFR were observed in various human cancers including breast cancer, lung cancer, bladder cancer and colon carcinoma, which were associated with aggressive tumor progression and poor prognosis.
- The combi-molecules that merge the pharmacophore moiety of EGFR tyrosine kinase (TK) inhibitor with different DNA-alkylating groups were observed to possess selective antiproliferative activities against tumor cells with high EGFR expression.

Bcr–Abl TK inhibitor-linked DNA-damaging agents

- Bcr–Abl oncoprotein is a constitutively activated TK that plays a crucial role in the development of chronic myelogenous leukemia.
- The strategy that combines a Bcr–Abl TK inhibitor with a DNA-damaging agent into a combi-molecule was demonstrated to have enhanced chemotherapeutic effect in Bcr–Abl-transfected cell lines.

MGMT inhibitor-linked DNA-damaging agents

- MGMT can repair the alkyl lesions located at the O^6-position of guanine induced by DNA-alkylating agents and finally results in drug resistance.
- The MGMT inhibitor-linked DNA-damaging agents can be employed to overcome the resistance induced by MGMT and therefore enhance the potency of the DNA-damaging moieties.

Topoisomerase inhibitor-linked DNA-damaging agents

- Toposiomerases are attractive anticancer target, because it can alter the topology of DNA by catalyzing transient breaking and rejoining of DNA double-strands involved in several important cellular processes.
- The combi-molecules with dual topoisomerase I/II-inhibiting and DNA-targeting functions were proven to have higher potency than the parent topoisomerase inhibitor or DNA-damaging agents.

Steroid-linked DNA-damaging agents

- Steroid hormones and their modified derivatives can be utilized as attractive biological vectors for the delivery of chemotherapeutic agents.
- The steroid-linked DNA-damaging agents target the hormone-dependent cancers, such as breast cancer and prostate cancer, and eliminate the known side reactions associated with conventional chemotherapies.

DNA intercalating agent-linked combi-molecules

- DNA-intercalating agents, such as acridine, acridone and naphthalimide, can intercalate into DNA helix and exert anticancer activity.
- The combi-molecules incorporating a DNA-intercalating moiety and a DNA-damaging agent exhibit increased ability of targeting and acting on DNA.

Other bioactive group-linked DNA-damaging agents

- Some compounds with biological activity, such as tyrosine, 1,3,5-triazine, quaternary ammonium, ellipticine and 1,4-dihydropyridines, can also be employed as bioactive pharmacophores in combi-molecules by merging with a DNA-damaging agent to achieve increased anticancer potency.

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