COMMENTARY

Perspectives in the development of hybrid bifunctional antitumor agents.

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Running title: Hybrid bifunctional antitumor agents

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Abstract

In spite of the development of a large number of novel target-specific antitumor agents, the single-agent therapy is in general not able to provide an effective durable control of the malignant process. The limited efficacy of the available agents (both conventional cytotoxic and novel target-specific) reflects not only the expression of defence mechanisms, but also the complexity of tumor cell alterations and the redundancy of survival pathways, thus resulting in tumor cell ability to survive under stress conditions. A well-established strategy to improve the efficacy of antitumor therapy is the rational design of drug combinations aimed at achieving synergistic effects and overcoming drug resistance. An alternative strategy could be the use of agents designed to inhibit simultaneously multiple cellular targets relevant to tumor growth/survival. Among these novel agents are hybrid bifunctional drugs, i.e. compounds resulting by conjugation of different drugs or containing the pharmocophores of different drugs. This strategy has been pursued using various conventional or target-specific agents (with DNA damaging agents and histone deacetylase inhibitors as the most exploited compounds). A critical overview of the most representative compounds is provided with emphasis on the HDAC inhibitor-based hybrid agents. In spite of some promising results, the actual pharmacological advantages of the hybrid agents remain to be defined. This commentary summarizes the recent advances in this field and highlights the pharmacological basis for a rational design of hybrid bifunctional agents.

Keywords: dual action, hybrids, bifunctional antitumor agents, drugs design, multitarget compounds.

1.Introduction

Drug resistance of tumor cells is recognized as being a major obstacle to effective cancer treatment [1]. This phenomenon has been ascribed to many mechanisms operating at various cell levels, specifically defence mechanisms, activation of DNA repair mechanisms and modulation of survival/cell death pathways [2]. The heterogeneity of cancer cells and their ability to activate compensatory pathways in response to drug treatment likely account for the intrinsic insensitivity and the frequent development of drug resistance [3]. To overcome these limitations, a well-established approach in cancer therapy for advanced disease is the use of combination of agents with different mechanism of action and non-overlapping toxicity profile [4]. This strategy is based on the compelling evidence that single-agent therapy is not able to provide an effective and durable control of the malignant process. The complex alterations of tumor cells and the redundancy of survival-related pathways contribute to tumor cell survival under stress conditions. For these reasons, the outcome of treatment with both conventional cytotoxic drugs or novel targeted agents is often a cytostatic cellular response rather than induction of cell death.

The *in vivo* combination of agents that exhibit synergistic interaction in cell culture can be less effective than expected due to differential pharmacokinetic behaviour of each drug and to the difficulty to afford optimal concentrations for the required time. Other shortcomings of drug combinations are unpredictable drug-drug interactions and possible enhancement of adverse effects. To avoid problems related to the pharmacokinetic behaviour of the combined agents and to better exploit the expected synergistic interactions, an alternative strategy is the development of hybrid bifunctional agents that may be able to inhibit simultaneously multiple targets involved in tumor cell defence and survival.

The interest in the strategy of multiple target inhibition is also supported by evidence of improved efficacy of dual (or multiple)-action inhibitors, as highlighted by novel kinase inhibitors active against various targets or receptors [5,6]. The multiple inhibitions of related pathways may have

convergent effects on these pathways, thus enhancing the potency of compounds. Given the structural homology of related kinases, some receptor kinase inhibitors are effective against multiple targets, other (irreversible inhibitors) are active against different kinases by interacting with cysteine residues [6]. The inhibitors of closely related targets containing a single pharmacophore have been extensively studied and the results have been already discussed in recent reviews [6,7].

The development of hybrid molecules represents a different strategy, because these novel agents are conceived as: a) molecules containing the pharmacophores of different drugs or b) molecules resulting by combination of entire drugs, usually connected through a linking arm (Figure 1). The rationale of this approach is based on the expected synergistic interaction of the two pharmacologically active components that could be favoured by optimal pharmacodynamic conditions.

Figure 1

The focus of this commentary is to summarize the most recent advances in the development of hybrid bifunctional compounds as antitumor agents, with particular emphasis on the critical aspects of drug design, the potential drawbacks and future directions of this approach.

The commentary is divided into sections according to the mechanism of action of the hybrid constituting drugs.

2. Hybrid compounds incorporating cytotoxic agents

Hybrid molecules of cytotoxic agents have been designed by overlapping structural motifs of compounds known to interact with the same target or by merging pharmacophore moieties to optimize or enhance interaction with the putative target. A number of molecules of this type have been described in previous reviews [8,9]. Since DNA topoisomerases I and II (topo I and II) are

recognized as the primary targets of well-established antitumor drugs, hybrid compounds containing topo I/topo II inhibitors have been explored. Hybrid agents, designed as topo II inhibitors, have been obtained by combining the chemical features of known inhibitors (e.g., DNA intercalating agents such as ametantrone and amsacrine) in the attempt to optimize the interaction of the drug with the DNA-enzyme complex (**1**, Figure 2) [10]. Although these studies may provide valuable information concerning the drug interaction in the enzyme-DNA ternary complex, the pharmacological activity of these hybrids was not investigated.

Other efforts in this field have been directed to increase the therapeutic potential of multifunctional DNA damaging agents, i.e. agents capable to induce genotoxic damage through distinct mechanisms. The compound Alchemix (**2**) is an anthraquinone inducing topo II-mediated DNA damage. Due to the incorporation of an alkylating function, the genotoxic stress is expected to be more persistent and less susceptible to repair [11]. The induction of irreparable damage is consistent with the activity of Alchemix against anthracycline-resistant and platinum-resistant ovarian carcinoma models and the induction of cell death via activation of a p53-independent apoptotic pathway [12]. This compound, characterized by intercalating and alkylating properties, preferentially induces the formation of a covalently bound topo II alpha-drug-DNA ternary complex, but does not stabilize a topo II beta-DNA complex. The formation of an irreversible topo II-DNA-drug complex may result in a persistent inhibition of the target enzyme function. In spite of some promising features, including the ability to overcome resistance mechanisms related to recognition by efflux transport systems and to DNA damage response, the therapeutic value of the hybrid compound and the actual advantages over conventional cytotoxic agents was not demonstrated at least in terms of efficacy and tolerability.

Other topoisomerase inhibitors, indolizino[6,7-b] indoles (**3**), described as DNA cross-linking agents, are expected to damage DNA through multiple mechanisms. [13]. These agents were designed as hybrid molecules of β-carboline (topoisomerase inhibiting moiety) and bis(hydroxymethyl)pyrrole (DNA cross-linking moiety). The compounds have been reported to

exhibit antitumor activity comparable to irinotecan (a camptothecin (CPT) in clinical use) and efficacy against doxorubicin-resistant cells.

Other attempts to develop multifunctional DNA damaging agents have been performed using topoisomerase inhibitors (intercalating agents or CPTs) as ligands for platinum compounds. Platinum complexes (e.g.,cisplatin, oxaliplatin) are well-established antitumor agents effective in the treatment of several solid tumors. The antitumor activity of platinum compounds is ascribed to their ability to covalently bind DNA, thus forming stable cross-links. Platinum compounds are used in combination with various topoisomerase inhibitors, but the efficacy of this approach is impaired by considerable toxicity. In the attempt to overcome this limitation and to exploit the synergistic interaction, topoisomerase inhibitor-linked platinum complexes have been envisaged. The first example of these efforts was the preparation of platinum(II)-doxorubicin complex, where platinum was coordinated via the amino group of daunosamine [14]. The complex exhibits promising features, including efficacy against doxorubicin-resistant tumors and tolerability profile comparable to doxorubicin itself.

A recent study has reported the synthesis and preclinical evaluation of platinum-CPT complexes with different Pt-containing linkers at position 7 of the CPT core (**4**) [15]. The available evidence supports the critical role of the linker length to achieve optimal drug interaction within the ternary complex. Compound **4**, effective as both platinating agent and topo I inhibitor, exhibits a good profile of activity and tolerability. Pt-mediated stabilization of this interaction could represent a potential advantage over parent CPTs, because it should enhance the formation of lethal lesions. Indeed, it is well known that the activity of CPTs is related to their ability to stabilize the topo I-DNA complex, thus resulting in the formation of double-strand DNA breaks during DNA replication. The reversibility of drug interaction in the ternary complex may be a limitation of CPT efficacy in the treatment of slowly growing tumors. Recently, other Authors have described hybrid compounds which incorporate intercalating moieties and other DNA damaging functions (**5**) [16].

Enhanced potency of these cytotoxic hybrid molecules is expected, but the therapeutic value, in particular the therapeutic index, remains unknown. Unfortunately, in spite of some promising features, relevant limitations related to lack of selectivity, formulation problems and intellectual property protection, represent obstacles to further development of these cytotoxic agents.

Figure 2

3. Histone deacetylase inhibitor-based bifunctional agents

Several lines of evidence support the potential of target-specific agents to sensitize the activity of conventional cytotoxic drugs, through inhibition of survival signals and synergistic activation of cell death pathways [2]. Moreover, rationally designed combinations of target-specific agents may exhibit synergistic effects when their action converge to inhibit pathways of survival. Among target-specific agents, inhibitors of histone deacetylases (HDAC) have been extensively employed to exploit possible synergistic interactions. Indeed, aberrant silencing of various tumor suppressor genes has been related to changes in DNA methylation and histone acetylation [17,18]. A persistent inhibition of HDACs results in growth arrest and promotes apoptosis. In addition, modulation of specific HDAC activity may alter the function of proteins implicated in regulatory processes, including tubulin, Hsp90 and trascriptional factors (e.g., p53) [19]. Therefore, based on the critical role that the "epigenetic" changes exert when associated with the malignant behavior, HDAC have been recognized as relevant targets of modulation.

HDAC inhibitors (HDACi) have been employed in a number of efforts to generate bifunctional molecules and reports on HDACi containing hybrid agents are increasing exponentially. Both cytotoxic agents (specfically, topoisomerase inhibitors and antimicrotubule agents) or novel target-specific agents have been employed for incorporation in the hybrid molecules. Although this division may be questionable, as several cytotoxic compounds are themselves target-specific, the

large difference in the relative potency of agents belonging to these classes may have important pharmacological implications in the design of hybrid molecules.

The classic pharmacophore of HDACi consists of a zinc-binding group (ZBG), a hydrophobic linker, and a recognition cap [20]. The approach followed to design most of the HDACi-containing hybrid compounds is based on the connection through a suitable spacer of the ZBG (i.e. hydroxamic acid or *o*-aminobenzamide) to the pharmacophore of another agent (either a cytotoxic agent or a target-specific agent), which represents at the same time the capping group (Figure 3). The conjugated molecules are predicted to maintain the key interactions with the two biological targets, as they have a hydroxamate group essential for HDAC zinc chelation and the key structural elements of the second agent for binding with its specific target.

Figure 3

3.1 Hybrid compounds incorporating cytotoxic moieties and HDAC inhibitors

HDAC inhibitors are known to sensitize tumor cells toward various cytotoxic agents, including DNA damaging agents and antimicrotubule agents, resulting in synergistic induction of cell death and improvement of therapeutic efficacy [21]. Given the pleiotropic effects of HDACi, the mechanism of the synergism likely involves modulation of diverse pathways and reversal of epigenetic alterations associated with drug resistance. Based on the evidence of enhancement of activity of DNA damaging agents when combined with HDAC inhibitors, hybrids were designed with particular attention to topoisomerase poisons. The rationale for this approach is also supported by the observation that the isoforms HDAC1 and HDAC2 and topo II co-localize in functional complexes implicated in drug induction of apoptosis [22, 23]. An additional advantage of a hybrid molecule with DNA binding affinity (e.g., DNA intercalating moieties) could be an increased accessibility to DNA within chromatin following hyperacetylation of histones.

The hybrid molecule should be designed to allow a favourable interaction with the enzyme active site (enzyme pocket). Several topo II inhibitors are characterized by a polycyclic ring (intercalating moiety), which could provide a favourable interaction with the surface recognition region of HDAC. However, in the case of non-cleavable linkers between the two moieties, the immobilization of the HDAC inhibitory function does not ensure a concomitant interaction with the target enzyme when the cytotoxic moiety is bound in the DNA-topoisomerase complex.

The HDAC inhibitor SAHA (vorinostat) was extensively used for the generation of hybrid molecules incorporating intercalating structures. WJ35435 (**6**, Figure 3), hybrid between SAHA and an acridine derivative known as a dual topo I/II inhibitor (DACA), is described as a potent HDAC inhibitor with preferential inhibition of HDAC1 and HDAC6 and selective inhibition of topo I [24]. Preclinical studies show antitumor activity against a model of human prostate carcinoma PC3, but the available data on biological evaluation does not allow a definitive conclusion on the therapeutic interest of the hybrid compound.

Based on the therapeutic relevance of anthracyclines and on the synergistic antitumor effects of topo II and HDAC inhibitors, derivatives of daunorubicin linked to SAHA were designed as bifunctional agents [25]. Compounds of this series (**7**) exhibit cytotoxic activity comparable to daunorubicin, in spite of the *in vitro* inhibitory activity towards HDAC1 and HDAC6 similar to SAHA. Thus, the contribution of dual action on the overall activity of the conjugate remains unclear. As emphasized by the Authors, the evidence of the dual action is based on validation experiments of target inhibition, performed under different conditions for each target. Thus, we cannot rule out the possibility that the predominant effect responsible for bioactivity at a cellular level is actually due to the most potent compound (i.e., daunorubicin).

The same Authors reported a similar approach using CPT as the cytotoxic component of the hybrid molecule [26]. In this conjugate the hydroxamic acid moiety was linked to the 10-hydroxy group of CPT through a spacer (**8**). The reported compounds exhibited antiproliferative activity comparable to that of SAHA, but a loss of potency as compared to SN38 (7-ethyl-10-hydroxycamptothecin),

used as a standard CPT with a free 10-OH group. Indeed, irinotecan (the prodrug of SN38), which contains a bulky substituent at position 10, is characterized by a substantially reduced cytotoxic potency as compared to its metabolite. Thus, the limited success of this conjugate emphasizes the critical impact of the optimal drug design.

Podophyllotoxins represent a class of clinical useful nonintercalating topo II inhibitors. In a study of novel HDACi-podophyllotoxin hybrid agents, [27] the aromatic capping group, linker length and zinc-binding group were systematically varied and preliminary conclusions regarding structure–activity relationships were discussed. Among the synthesized hybrid compounds, compound **9a** showed the most potent HDAC inhibitory activity at a low nanomolar level(IC_{50} for HDAC-1: ~ 11 nM and for HDAC-6: ~ 6 nM) and exhibited antiproliferative activity towards HCT116 colon carcinoma cells at micromolar level (IC_{50} ~ 3 μM). Further exploration of this series led to the discovery of the potent dual inhibitor **9b**, which exhibited the strongest *in vitro* cytotoxic activity, comparable to podophyllotoxin alone (etoposide). The zinc-binding group was found to play a significant role in the cytotoxicity. In fact, the introduction of anilides as ZBGs resulted in increased antiproliferative activity against HCT116 and A549 tumor cells.

A series of novel evodiamine/SAHA hybrids were identified as triple inhibitors of Topo I/Topo II/HDAC [28]. In particular, compound **10** was proven to be a potent inhibitor of of HDAC (IC_{50} for HDAC1, HDAC6, HDAC8: 24 nM, 13 nM and 9 μM, respectively) and of topoisomerase I/II, comparable to standard inhibitors (camptothecin and etoposide). . The hybrids of these series may be of potential interest taking into account the multiple actions of evodiamines [29].

Colchicine-SAHA hybrids (**11**) have been described as dual inhibitors of HDAC and microtubule function [30]. The tested compounds exhibited a moderate HDAC inhibitory activity (lower than SAHA), but a substantial reduction of antiproliferative activity as compared with colchicine. A similar study was performed using a benzamide-based HDAC inhibitor [31]. Compounds of this series retained some HDAC inhibitory activity. The most potent cytotoxic agent (**12**) exhibited a marked inhibition on tubulin polymerization but a moderate HDAC inhibitory activity. This

observation could suggest that the biological activity reflects the predominant effect of one of the two components rather than dual action.

3.2 Hybrids incorporating HDAC inhibitors and target-specific agents

The identification of a large number of molecularly targeted agents, which have been designed to disrupt the function of targets within specific oncogenic signaling processes, offers the opportunity to explore the therapeutic benefit of concomitant inhibition of multiple pathways [4]. The clinical efficacy of single-agent therapy with target–specific drugs is still limited to a small fraction of treated patients as a consequence of molecular heterogeneity among tumors and selection of resistant cells [3]. To overcome the drawbacks of low responsiveness and drug resistance, several efforts have been devoted to optimize the use of novel target-specific agents and to elucidate the determinants of treatment response [7]. The compelling evidence that protein kinase inhibitors play an important role in the treatment of diverse tumors supports the potential interest for their combination with cytotoxic drugs or other target-specific agents [4,5]. In particular, based on the role of epidermal growth factor receptor (EGFR) and related kinase receptors in regulation of the malignant behaviour of several solid tumors, including breast and lung cancer, promising hybrid compounds incorporating HDAC inhibitors and tyrosine kinase inhibitors have been investigated. Specifically, in this approach the use of multi-kinase inhibitors may provide the additional advantage of targeting diverse oncogene-associated signalling pathways [6]. Indeed, some multi-kinase inhibitors have been reported to be effective for receptor tyrosine kinases (RTK) of the EGFR family, c-Met/VEGFR or protein kinases PI3K/mTOR.

Initial efforts to combine the pharmacological properties of multitarget inhibitors have been reported in two independent studies in which chimeric molecules were designed as inhibitors of EGFR, HER2 and HDAC activity [32, 33]. CUDC-101 (**13**), [33] displayed a potent antiproliferative and antitumor activity against a number of tumor models, including lapatinib- and erlotinib- resistant tumor cell lines. This hybrid inhibited EGFR- and HER2-dependent signalling

and other survival signalling pathways, involving AKT and MET. *In vivo*, it promoted tumor regression or inhibition in various cancer xenograft models including nonsmall cell lung cancer (NSCLC), liver, breast, head and neck, colon, and pancreatic cancers. CUDC-101 is characterized by a favourable safety profile and, based on these promising features, has entered phase I clinical trials. Recently, the same group [34] has described CUDC-907 (**14**), a hybrid integrating the structural elements required to inhibit both HDAC and PI3K. The Authors found that CUDC-907 effectively inhibited PI3K-AKT-mTOR pathway and was capable to modulate multiple signalling factors, an effect consistent with HDAC inhibition. The hybrid compound exhibited a potent antiproliferative activity, higher than single agent, PI3K or HDAC inhibitors, or their combination. A pharmacological feature relevant for therapeutic implications is the antitumor efficacy following oral administration. The higher oral bioavalability of CUDC-907 as compared to other hydroxamate-based HDAC inhibitors and the increased tumor accumulation may account for the potential therapeutic benefit of the compound.

HDAC inhibitor-based compounds were designed to incorporate the VEGFR2 inhibitor Semaxanib (SU5416) [35]. The study provided preliminary data concerning antiproliferative activity, biochemical inhibition and SAR of the hybrids. The lead molecule (Z)-N1-(3-((1H-pyrrol-2-yl)methylene)-2-oxoindolin-5-yl)-N8-hydroxyoctanediamide **15** was found to be an effective dual-action hybrid on the basis of biochemical assays and a potent antiproliferative agent in selected tumor cell lines. This approach is of potential interest, given the modulation of angiogenesis by HDAC inhibitors and their favourable interaction with antiangiogenic agents [19, 21].

A novel series of N-aryl salicylamides were synthesized as EGFR inhibitors with a hydroxamic acid moiety at 5-position (**16**) [36]. The compounds exhibited distinct inhibitory activity against EGFR and HDACs and antiproliferative activities *in vitro* in the micromolar range.

On the basis of a similar multitargeting concept, a series of dual HDAC-Src inhibitors containing varied hydrophobic linkers were synthesized [37]. The optimized inhibitor **17** was found to be a HDAC1 inhibitor (Ki : 260 pM) and also a c-Src inhibitor (Ki : 138 nM). Profiling of compound **17**

against a panel of 11 HDAC isoforms revealed that it was a non-selective HDAC inhibitor. Moreover, it was selective for c-Src over homologous kinases. The compound showed an improvement in therapeutic index significantly higher than when targeting c-Src and HDACs with the combination of two separate compounds, suggesting that the synergistic activity against cancer cells may be achieved without increasing the toxicity of each agent.

In a recent work macrocyclic compounds simultaneously inhibiting HDAC, Fms-like tyrosine kinase 3 (FLT3) and Janus kinase 2 (JAK2) have been reported (**18**) [38]. Most of these macrocyles exhibited HDAC inhibition as well as FLT3 and JAK2 inhibition either under cell-free conditions or in cell systems. *In vitro* antiproliferative assays indicated that these compounds were more cytotoxic to MV4-11 cells bearing the FLT3-ITD mutation and HEL cells bearing the JAK2V617F mutation.

Dual-action agents have been designed by structure-based approaches to target both HDAC and hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) [39]. Again, this study was based on the knowledge that a combination of HDACi and statins (known as HMGR inhibitors) exhibit a synergistic induction of apoptosis. These compounds showed potent inhibitory activities against HDACs and HMGR with IC_{50} values in the nanomolar range. Compound **19** showed inhibitory activity against HDAC1 (IC_{50} ~ 65 nM) and inhibitory activity against HMGR (IC_{50} ~ 17 nM). An interesting feature is some selectivity for human cancer cells as compared to normal cells. However, *in vivo* studies of these dual-action hybrids are yet not available.

On the basis of the evidence that HDAC inhibitors could increase the antitumor effect of RXR agonists, Chen et al. [40] designed and synthesized a bifunctional compound (**20**, DW22) which targeted both RXR and HADC. This hybrid agent was derived from bexarotene, a prototypical RXR agonist, and SAHA. Molecular docking studies demonstrated that this agent had a relatively strong affinity for RXR and HADC. It presented the potential of activation of RXR and inhibition of HDAC (IC_{50} ~ 7 μM), and displayed anti-proliferative effects on representative cancer cell lines (IC_{50} : 9-24 μM), including drug-resistant cell lines.

Recently, the Ras inhibitor farnesylthiosalicylic acid (FTS, salirasib) was conjugated to hydroxamic acid with various linkers to obtain dual Ras-related signalling and HDAC inhibitory effects [41]. The hybrid molecule **21** demonstrated the highest potency against cancer cell lines with IC_{50} values in the range of 5-8 μm. Compound **21** also exhibited the most potent inhibitory activity against HDAC1 and HDAC8 and blocked the Ras-related signaling pathways in a dose-dependent manner.

4. Bcl-xL/Mcl-1 hybrid inhibitors

The cellular response to drug treatment critically influences the therapeutic outcome. Defects or alterations in cell death pathways may contribute to drug resistance of tumor cells. In particular, targeting the antiapoptotic factors has emerged as a potentially useful strategy to modulate drug resistance. Giving the interplay of antiapoptotic proteins of Bcl-2 family, a promising approach could be the concomitant inhibition of Bcl-2/Bcl-xL and McL-1.

Bcl-2 family proteins are overexpressed in many types of cancer or are involved in the resistance to chemotherapy. Inhibition of Bcl-xL and/or Bcl-2 results in upregulation of Mcl-1 that elicits resistance to cancer cells [42]. Therefore, effective treatment may require simultaneous inhibition of Bcl-xL or Bcl-2 and Mcl-1.

Some dual inhibitors of these factors have been recently designed starting from the structure of natural compounds [43, 44]. Tanaka and co-workers [45] designed a small library of hybrid compounds using structure-guided analyses of selective Mcl-1 and Bcl-xL inhibitors. Following further optimization, the most active compound showed potent Mcl-1/Bcl-xL dual inhibitory activity (Mcl-1, IC_{50}: ~ 88 nM; and Bcl-xL, IC_{50} : ~ 4nM). In these studies, the identification of inhibitors was based on structural analysis performed to exploit optimal interactions. Unfortunately, no pharmacological studies are available to support their therapeutic potential.

5. Bifunctional agents with cleavable linkers

In the approaches described above, the conjugation of the two molecular entities was generally performed using a non-cleavable linker, on the basis of the hypothesis that both pharmacophores retain their biological activity and their specific affinity for biological targets.

An alternative strategy is the connection of two entities through cleavable linkages (e.g., esters, disulfides or carbamates). The approach is based on the release of two parental molecular structures under the physiological or enzymatic conditions that prevail at the site of activity. The main purpose of using cleavable linkers is to modulate the release of individual drugs *in vivo* or to improve the selectivity of the drugs. In this design strategy the cooperation between the two agents may be more predictable, if the released molecules retain activity and specificity for their intracellular targets.

Figure 4

Pan-HDAC inhibitors have been reported to potentiate the cytotoxic/antitumor activity of antimicrotubule agents [2]. The efficacy of the combination has been ascribed to hyperacetylation of tubulin resulting in microtubule stabilization. However, the mechanism of interaction appears to be complex and likely involves modulation of protective factors [46]. Based on the evidence of synergistic interaction, antimicrotubule agents have been conjugated with a HDAC inhibitor through a disulfide linker to allow thiol-mediated release of the two moieties inside the cell [47]. In spite of the rational design, these compounds were characterized by a reduction of the cytotoxic potency. A plausible explanation for this disappointing result is an inefficient release of the active components. In the case of the paclitaxel conjugate (substituted at 2'-position) (**22**, Figure 4) the low potency, as compared with the natural taxane, is consistent with an impairment of drug-microtubule interaction following substitution in this critical position. If this interpretation is correct, the activity of the conjugate more closely reflects the inhibition of HDAC rather than the antimitotic activity of the taxane. This behaviour is reminiscent of the SAHA-CPT hybrid [26] where the CPT moiety

likely plays the role of the hydrophobic cap group with a (partial) loss of the contribution of the primary target inhibition.

Novel conjugates have been reported (**23**) [48] based on the hypothesis that vitamin receptors overexpressed by tumor cells may be exploited for tumor-targeting drug delivery. They incorporate a taxoid and a CPT as potent cytotoxic agents and biotin for enhancing selectivity through receptor-mediated endocytosis. These conjugates were designed to allow drug release via cleavage of the self-immmolative disulfide linker by intracellular glutathione. Studies of internalization revealed an increased selectivity for tumor cells. In spite of the complex synthesis, given the clinical relevance of the agents used, this type of conjugates may have therapeutic implications in an effort to improve tumor selectivity and to overcome the disadvantages of co-administration of the two drugs.

A number of platinum complexes have been reported as prodrugs by conjugation with other agents of potential therapeutic interest to enhance platinum cytotoxicity. Specifically, the conjugation with cyclooxygenase inhibitors was reported to facilitate the cellular accumulation of Pt(IV) complexes and to overcome cisplatin resistance [49]. In the reported conjugates (**24**) the COX-2 inhibitor served as axial ligand, allowing the release of platinum complex and of two molecules of the COX-2 inhibitor following intracellular thio-mediated reduction.

Platinum(IV) complexes [50] were designed as dual-targeting prodrugs following the introduction of the vitamin E analog, α-tocopherol succinate (a-TOS), as the axial ligand(s) of platinum(IV) (**25**). In this conjugate the platinum is expected to cause DNA damage, while a-TOS disrupts Bcl-xL-Bax interactions leading to mitochondrial dysfunction, thus enhancing the intrinsic mitochondria-mediated pathway of apotosis activated by DNA damage. Indeed, the conjugate displays potent in vitro cytotoxicity, higher than cisplatin, in a variety of tumor cell types.

With the same purpose of improving the efficacy by enhancing the apoptotic response, a dual-targeting platinum(IV) prodrug (**26**) was designed by the conjugation of the platinum moiety to a small-molecule inhibitor (chalcone) of the p53–MDM2 interaction [51]. Chalcoplatin displayed significantly increased cytotoxicity in p53 wild-type but not in p53 null. Cellular pharmacokinetic

studies revealed that chalcoplatin effectively entered cells. It is conceivable that the enhanced cellular uptake of platinum, related to the hydrophobic features of the chalcone moiety, may contribute to p53 induction and increase cytotoxicity in p53 wild-type tumor cells.

Various ester prodrugs were also prepared by coniugation of lantadene (a pentacyclic triterpenoid inhibitor of NF-kB) with non steroidal anti-inflammatory agents (**27**, Schema 8). The synthesis of these hybrid compounds was based on the evidence that the transcription factor NF-kappa B and cyclooxygenase-2 are implicated in influencing inflammation and promoting tumor growth and survival. [52]. The compounds showed dual inhibition in the micromolar range and cytotoxic activity in the sub-micromolar range.

The PI3K/AKT/mTOR signaling pathway is often hyperactivated in tumor cells, and PI3K and mTOR act synergistically in promoting tumor growth, survival, and resistance to chemotherapy. Inhibition of this pathway therefore is an exploitable strategy for cancer chemotherapy. Kaloustian et al. conjugated 17-hydroxywortmannin (PI3K inhibitor) analogues to rapamycin (mTOR inhibitor) analogues via a diester linker [53]. Conjugate **28** showed activity in U87MG tumor xenografts, following weekly intravenous dosing. At 15 mg/kg, **28** completely inhibited the growth of HT29 tumors, whereas an equivalent mixture of the inhibitors was poorly tolerated.

Future Directions

The design of hybrid drugs capable of amplifying the effects of individual entities is rapidly evolving, indicating that this area of research excites great interest for the scientific community.

In contrast to multi-kinase inhibitors which are characterized by a single pharmacophore structure, the hybrid bifunctional agents are designed to achieve multiple actions mediated by functionally different components potentially able to interact with specific targets.

The expected advantages of hybrid compounds as antitumor agents could be a) improvement of the efficacy by exploiting synergistic interactions; b) enhancement of the selectivity resulting in a better tolerability; c) modulation of drug resistance. In addition, the use of a hybrid molecule may provide

a pharmacokinetic advantage over the separate administration of the two drug components. The hybrid is expected to produce sufficient simultaneous concentrations of the pharmacologically active entities to exploit favourable interactions, avoiding the disadvantages of co-administration of multiple agents. However, a large number of the reported hybrid molecules have not progressed beyond the biochemical/cellular characterization. With few exceptions (e.g., ref. 34, 35), the approaches describing the synthesis of novel hybrid molecules are substantially proof-of-concept or feasibility studies without pharmacological details. Thus, the actual advantages of compounds of this class remain to be defined. Only preliminary information concerning the potential toxicity of hybrid agents are available. Relevant to this point is the observation that hybrid molecules incorporating target-specific agents are characterized by a good profile of tolerability [34, 35].

The success of hybrid drug approaches will depend on several challenges related to drug design, most of which still need further implementation. A large number of the recently reported hybrid agents are characterized by non-cleavable linkages. The design of dual inhibitors is based on the premise that each agent can be structurally modified without compromising its inhibitory activity against the specific target(s). However, the presence of a stable linkage does not allow a clear interpretation of the molecular/cellular basis of activity of the bifunctional agent, and it may be difficult to predict whether the *in vivo* antitumor effects reflect a single or multiple mechanisms. This is a critical aspect for the design of hybrid molecules when the two components are characterized by substantially different potency. For example, the combination of a cytotoxic agent, effective in the nanomolar range of concentrations, with a target-specific drug, effective in the micromolar range, does not ensure sufficient concentration of the less potent component to achieve the expected synergy. In this case, the biological activity of the hybrid molecule could reflect the predominant mechanism of the most potent moiety.

The most promising and rational design of novel molecules should be the chemical combination of agents that exhibit biological/biochemical activity in a comparable range of concentrations, as it happens for several target-specific compounds, including HDAC inhibitors and protein kinase

inhibitors. Anyway, a rational design should take into account specific structural features, including the length of the linker and the linker position to allow the chemical flexibility required to retain activity against each putative target.

HDAC inhibitors have been widely employed in efforts to combine various target-specific agents. The interest for HDAC inhibitors is related to their pleiotropic effects including modulation of expression of proteins involved in signaling pathways and regulation of critical processes such as proliferation and apoptosis. Some HDAC inhibitors may have also efficacy in modulation of tumor-induced angiogenesis [19,21]. This effect, related to down-regulation of HIF-1 alpha and proangiogenic factors, offers the opportunity to design hybrid molecules contaning HDAC inhibitors and other agents effective in the angiogenesis process. Some promising approaches aimed at exploiting the combination of HDAC inhibitors with tyrosine kinases implicated in angiogenesis (e.g., VEGFR) have been described [35].

In addition to a proper selection of targets for drug combination, the potential "druggability" of the hybrid agents plays a key role for the success of multitarget approaches. Several compounds reported above have high molecular size and high lipophilicity. The poor aqueous solubility makes it difficult to develop i.v. formulations. Thus, one of the future directions for the construction of hybrid anticancer molecules should be the identification of leads with an appropriate molecular size and physicochemical properties to optimize their drug-like profile. In addition, it should be considered that for many of these agents the complexity of the chemical structure and the high number of synthetic steps may reduce the potential of large scale production. Ligand-based and/or structure-based computational approaches can be extremely helpful for generation and optimization of hybrid drugs [45, 54].

In conclusion, the administration of a bifunctional molecule is expected to exploit the concomitant effects of the two active components at the target tissue, thus providing pharmacokinetic and pharmacodynamic advantages over combined administration of drugs. Among the various hybrid

molecules, the compounds incorporating two cytotoxic moieties could be successful in overcoming some mechanisms of resistance, but it is unlikely that the strategy may result in improvement of selectivity. In this context, the introduction of a tumor-targeting moiety to obtain "vectorized" hybrids could have promising applications, as suggested by recent efforts in this field [9]. On the other hand, the conjugation of a cytotoxic agent with a less toxic target-specific compound may not ensure the expected synergistic interaction which could be observed at cellular level, when the separate agents are combined under favourable exposure conditions. The most promising approach appears to be the conjugation of two target-specific agents which, due to a comparable potency at cellular/ biochemical level, may act in a context more favourable to exploit synergistic interactions and biochemical specificity.

The above considerations highlight that hybridization of bioactive agents into multifunctional drugs could be a very challenging and promising approach, but still needs further implementation and methodological development. To address this issue, dedicated efforts should be made to integrate complementary fields of study, including cellular/biochemical pharmacology, medicinal and computational chemistry, for a rational design of hybrid drugs.

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Figure Legends

Figure 1. General structure of hybrid drugs. a) molecules containing the pharmacophores of different drugs; b) molecules resulting by combination of entire drugs connected by a linking arm.

Figure 2. Hybrid compounds incorporating cytotoxic agents.

Figure 3. HDACi-containing hybrid agents.

Figure 4. Hybrid drugs with disulfide or ester cleavable linkages.

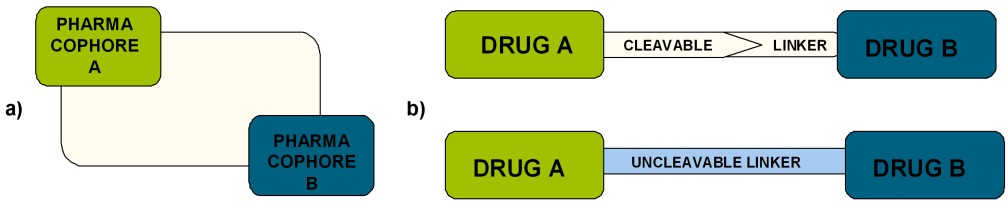
a)
PHARMA COPHORE A
PHARMA COPHORE B
b)
DRUG A
CLEAVABLE
LINKER
DRUG B
DRUG A
UNCLEAVABLE LINKER
DRUG B

Fig.1

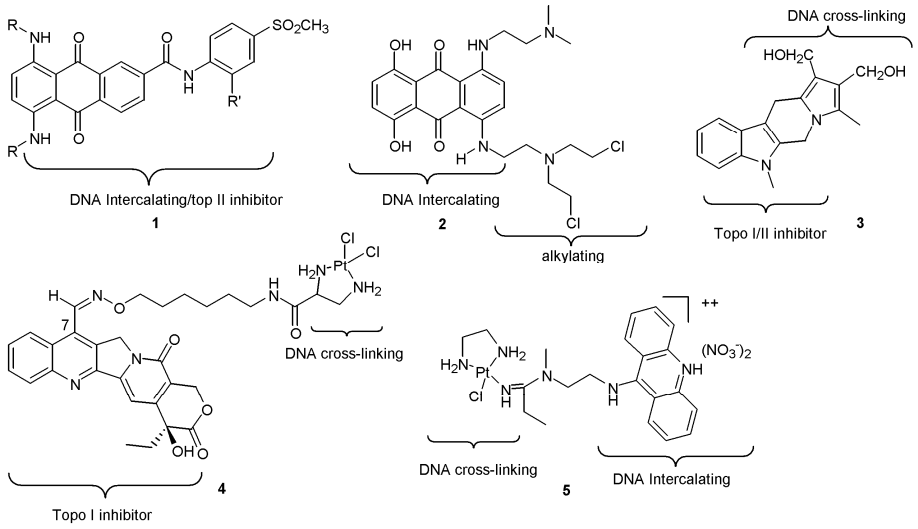
DNA Intercalating/top II inhibitor
1
DNA Intercalating
2
alkylating
DNA cross-linking
Topo I/II inhibitor
3
DNA cross-linking
4
Topo I inhibitor
DNA cross-linking
DNA Intercalating
5

Fig. 2

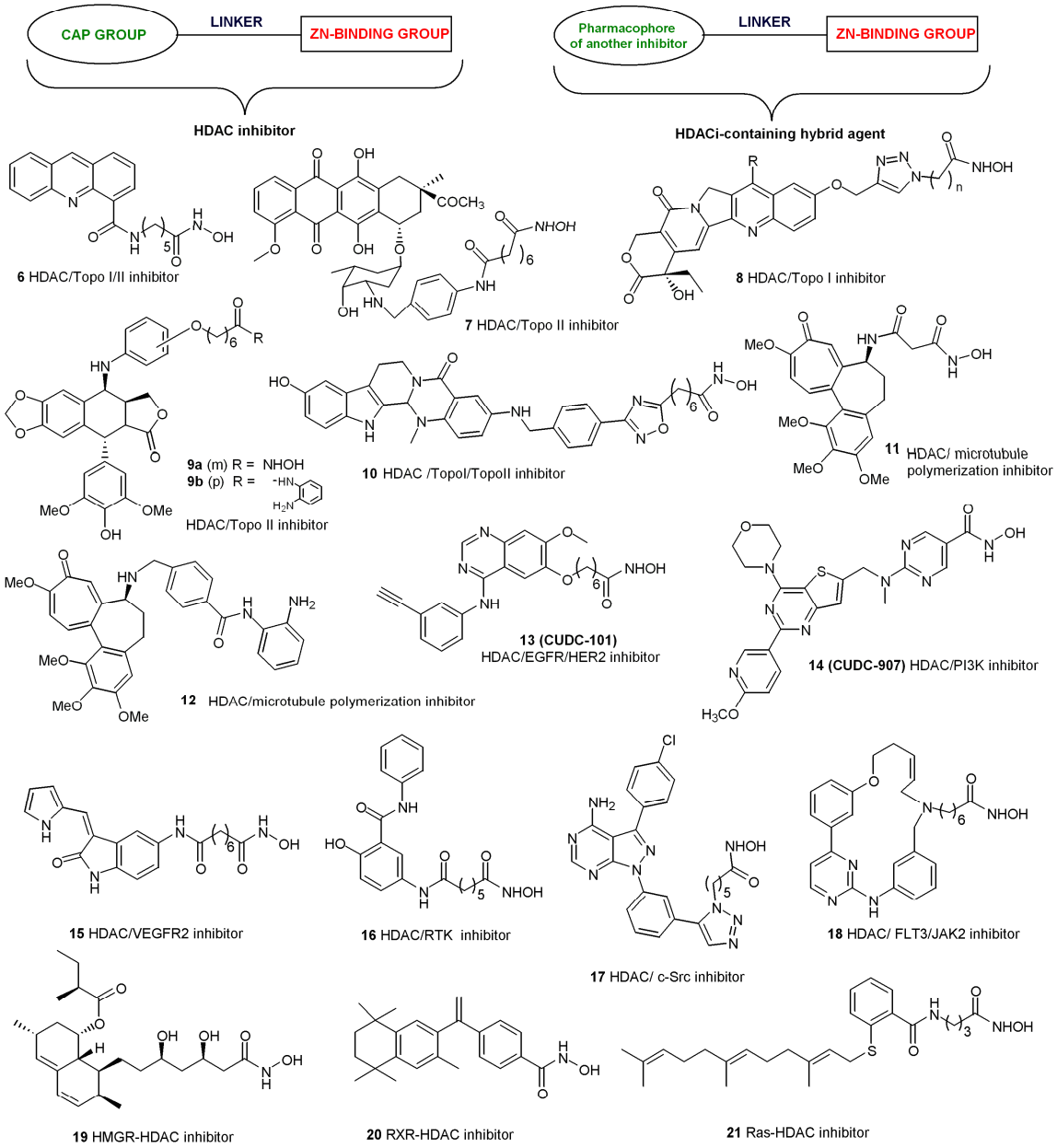
CAP GROUP
LINKER
ZN-BINDING GROUP
HDAC inhibitor
Pharmacophore of another inhibitor
LINKER
ZN-BINDING GROUP
HDACi-containing hybrid agent
6 HDAC/Topo I/II inhibitor
7 HDAC/Topo II inhibitor
8 HDAC/Topo I inhibitor
9a (m) R = NHOH
9b (p) R =
HDAC/Topo II inhibitor
10 HDAC /TopoI/TopoII inhibitor
11 HDAC/ microtubule polymerization inhibitor
12 HDAC/microtubule polymerization inhibitor
13 (CUDC-101) HDAC/EGFR/HER2 inhibitor
14 (CUDC-907) HDAC/PI3K inhibitor
15 HDAC/VEGFR2 inhibitor
16 HDAC/RTK inhibitor
17 HDAC/ c-Src inhibitor
18 HDAC/ FLT3/JAK2 inhibitor
19 HMGR-HDAC inhibitor
20 RXR-HDAC inhibitor
21 Ras-HDAC inhibitor

Fig. 3.

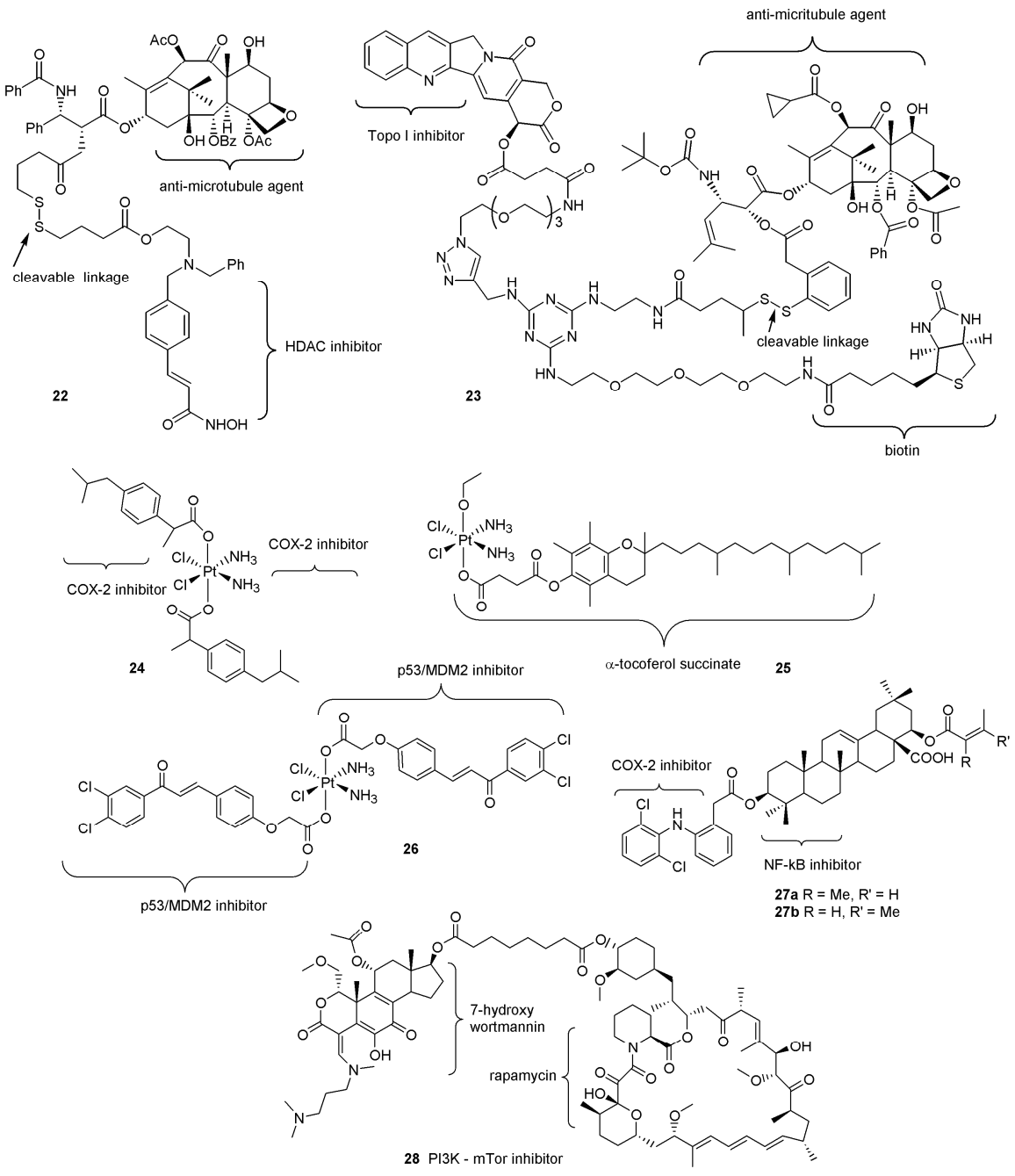
anti-micritubule agent
Topo I inhibitor
anti-microtubule agent
cleavable linkage
HDAC inhibitor
22
23
cleavable linkage
biotin
COX-2 inhibitor
COX-2 inhibitor
24
α-tocoferol succinate
25
p53/MDM2 inhibitor
26
p53/MDM2 inhibitor
COX-2 inhibitor
NF-kB inhibitor
27a R = Me, R' = H
27b R = H, R' = Me
7-hydroxy
wortmannin
rapamycin
28 PI3K - mTor inhibitor

Fig. 4.