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PERSPECTIVE

Hybrids of amino acids and acetylenic DNA-photocleavers: optimising efficiency and selectivity for cancer phototherapy

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Hybrid agents which combine potent DNA-photocleavers with tunable amino acids or small peptides were designed to improve selectivity of Nature's most potent class of antibiotics towards cancer cells. The ability of these compounds to photocleave DNA is controlled by their incorporation into hybrid architectures with functional elements derived from natural amino acids. These conjugates are highly effective at inducing double-strand DNA cleavage and, in some cases, rival or even surpass both naturally occurring DNA cleavers and anticancer agents that are currently in clinical use. The possibility of triggering their activity in a photochemical and pH-sensitive fashion allows for a high degree of selectivity over activation. The conjugates were shown to penetrate cell membranes and induce efficient intracellular DNA cleavage. Initial *in vitro* tests against a variety of cancer cell lines confirm the potential of these compounds as anticancer agents at low nanomolar concentrations.

Naturally occurring enediynes: a modular architecture to accomplish multiple purposes

The record-setting cytotoxicity of enediyne antibiotics, often hailed as the most potent family of anticancer agents known to date,[1] is based on their ability to cause efficient double-strand (ds) DNA cleavage and subsequent apoptosis (self-programmed cell death). The naturally occurring enediynes were developed by microorganisms as a self-defence tool, targeting the DNA of competing species with astounding efficiency. Compounds of this class typically have elaborate modular structures where different parts serve different purposes.

The key structural element responsible for the biological activity is the six-carbon motif ("the enediyne warhead") highlighted in red (Fig. 1). This subunit is responsible for the DNA-damage due to its ability to transform into a reactive *p*-benzyne diradical *via* the Bergman cyclisation[2] (Fig. 2). This highly reactive species abstracts a hydrogen atom from either strand of double stranded DNA – a process that leads to ds DNA cleavage and causes cells to undergo apoptosis.

Although the parent version of the Bergman cyclisation shown in Fig. 2 proceeds only at elevated temperatures, which are incompatible with biological systems, incorporation of the enediyne moiety into a nine- or ten-membered cycle allows the cyclisation to proceed at much lower temperatures. Unfortunately, even though the increased reactivity of cyclic enediynes makes them suitable for biological purposes, it also introduces

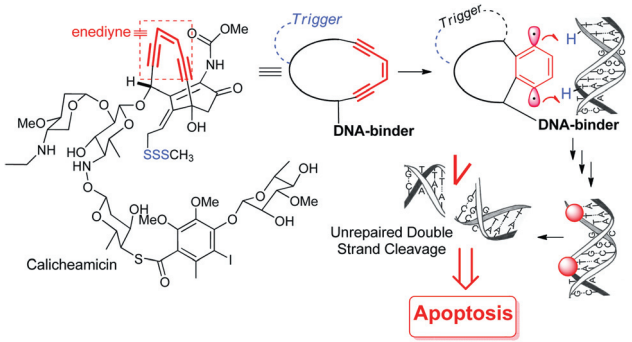

Fig. 1 Structure of calicheamicin, highlighting the modular nature of the naturally occurring enediynes and mechanism for inducing double-strand (ds) DNA cleavage. The enediyne warhead is shown in red, the trigger is shown in blue.

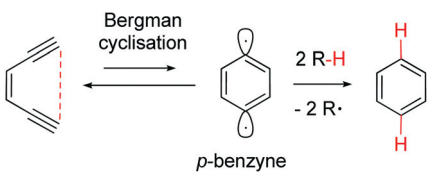

Fig. 2 Simplest version of the Bergman cyclisation.

an additional challenge because thermal activation provides a narrow time window for separating the production of these compounds from their activation and transformation into the highly reactive diradical. In calicheamicin, an additional level of control is accomplished by the trisulfide trigger (shown in blue in

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Fig. 1) which is responsible for activating the warhead *via* reductive S–S bond cleavage followed by intramolecular Michael cyclisation. The remaining functionalities (shown in black, Fig. 1) have multiple roles in addition to contributing to the solubility and DNA binding. For example, the sugar residues of esperamicins have been recently implicated in preventing premature cycloaromatisation and providing a mechanism for the auto-resistance to natural enediyne antibiotics by the enediyne-producing microorganisms.[3]

In general, efficient ds DNA cleavage is hard to achieve as illustrated by the fact that even calicheamicin, the best ds DNA cleaver among anticancer drugs, induces only about 25% of ds cleavage (a ~3 : 1 ss : ds ratio).[4] Remarkably, not only does such a seemingly low ds : ss ratio far surpass other popular DNA-cleavers, such as bleomycins (1 : 6–1 : 20),[5,6] but is still sufficient to account for their record-breaking biological activity because of the connection between ds-DNA cleavage and self-programmed cell death (apoptosis). ds DNA cleavage is much more difficult to repair and more important therapeutically than single strand (ss) DNA cleavage.[7] These data provide a compelling rationale for the development of DNA-damaging species as a strategy towards new cancer therapies.

Despite the complexity of naturally occurring enediynes, natural selection did not optimize these molecules for their pharmaceutical use as anticancer agents. Most importantly, natural enediynes are not able to distinguish between healthy and cancer cells and, as a result, are highly toxic. Because one can take full advantage of the remarkable enediyne reactivity only if the cancer cells are targeted *selectively*, so far only one of the enediynes, calicheamicin, has been approved by the FDA as a conjugate with Gemtuzumab ozogamicin, a monoclonal antibody (marketed as "Mylotarg") for treating acute myelogenous leukemia. Not only is this approach very expensive but, recently, Pfizer voluntary withdrew Mylotarg from the US market after results from a recent clinical trial raised new concerns about the product's safety as the drug failed to clear the risk–benefit threshold.[8] The low selectivity towards cancer cells remains a very important factor in preventing these molecules from realising their full potential in cancer chemotherapy. Finding the right balance between reactivity and selectivity has been the focus of concentrated research efforts aimed at either fine-tuning the reactivity of the enediyne warhead through strain,[9] chelation[10] and/or electronic effects,[11,12] or/and at increasing the selectivity of enediynes *via* a variety of approaches including conjugation to monoclonal antibodies,[13] use of reductive environment of hypoxic tumours, designing pH-sensitive warheads for acidified solid tumours and triggering the enediyne warhead photochemically.[14,15]

The appeal of photochemical activation

Photocatalytic processes *vs.* photo-Bergman cyclisation

The use of tissue-penetrating light allows for spatial and temporal control over prodrug activation, as light can be delivered directly to the tumour with a high concentration of the prodrug. This advantage is illustrated by the emergence of photodynamic therapy (PDT) as a powerful and convenient alternative to many traditional cancer therapy methods.[16] In addition to skin cancer,[17] PDT can also be used to treat tumours on the lining of internal organs or cavities such lung, throat, esophagus and colon cancers.[18] Potentially, other tumours can be targeted with low-energy tissue-penetrating photons.

Conventional PDT is based on a three-component system which includes photosensitiser (*e.g.*, Photofrin), light and oxygen. Absorption of light transforms the photosensitiser (usually a porphyrin or a related compound) into its triplet excited state which can transfer excitation energy to molecular oxygen present in the tissues. This process generates highly reactive singlet oxygen which gives rise to a number of other oxygen species capable of damaging numerous targets such as cancer cells themselves or the blood vessels supplying them with nutrients. Other reactive species often used to damage DNA targets are hydroxyl-radicals, which are typically produced by Fe(II) or Cu(I)-containing complexes.[19]

In contrast to the reactivity of enediyne warheads, the above processes are catalytic in nature. This design accounts for many advantages of conventional PDT but also creates several problems. The most important of them is lasting sensitivity to light. Because the photosensitising drug used for PDT stays in the human body for up to several weeks, its presence leads to continuous generation of singlet oxygen when the patient is exposed to sunlight. As a result, damage to healthy tissues may continue even after the target cancer cells have been already destroyed. Additionally, since the conventional PDT approach requires oxygen, it is less effective in hypoxic tumours.

Not surprisingly, the idea of developing a photochemically triggered version of the Bergman cyclisation in the context of cancer therapy gained significant popularity. The alternative strategies involved either a photochemically triggered but thermal Bergman cyclisation[20] or a truly photochemical ring closure due to the direct excitation of the enediyne moiety (Fig. 3).

Although the photo-Bergman cyclisation has been known since the 1968 report of Campbell and Eglington (as long as the thermal ring closure!),[21] more targeted efforts were undertaken only after the importance of the enediyne warhead had been discovered. Acylic enediynes undergo *cis–trans* isomerisation of the central double bond.[22] Several groups avoided this reaction pathway by incorporating the central part of the enediyne moiety into an aromatic[23,24] or aliphatic[25] cycle, obtaining low to moderate yields of the photocyclised product in presence of suitable hydrogen donors (Fig. 3).

A more efficient photo-Bergman cyclisation is observed when both alkynes are incorporated in a strained cycle[26] (Fig. 3e). However, even for these activated substrates, other processes can successfully compete with the Bergman cycloaromatisation (Fig. 4). For example, even though the conversion of cyclodec-3-ene-1,5-diyne to the respective *p*-benzyne intermediate apparently proceeds efficiently,[27] this intermediate is not trapped by hydrogen abstraction sufficiently fast to prevent the retro-Bergman opening leading to the formation of 1,2-ethynylcyclohexene. The overall transformation can be considered as a strain-driven photo-Cope rearrangement. In another example, Branda and coworkers reported the elegant design of a cyclic photochromic enediyne which undergoes hexatriene electrocyclisation when activated with 365 nm light.[28] The cyclised compound can be reopened with >525 nm light. Interestingly, photochemical

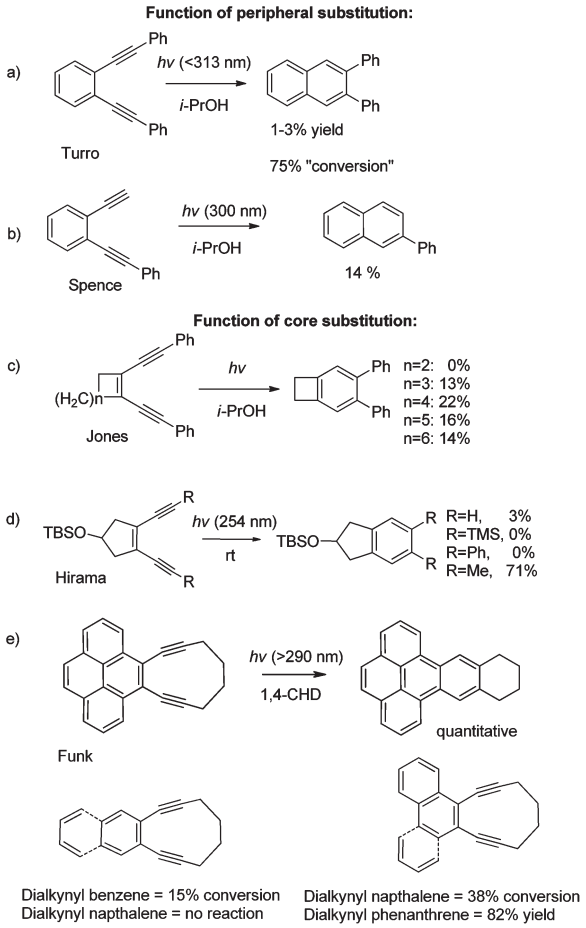

Fig. 3 Sensitivity of photo-Bergman cyclisation to peripheral and core substitution.

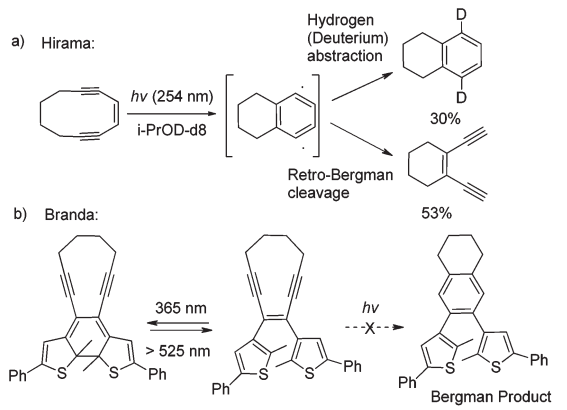

Fig. 4 Photochemical activation of cyclic enediynes leading to transformations other than the Bergman cyclisations.

Bergman cyclisation does not occur, suggesting that this process is much slower than the electrocyclic ring closure.

DNA photocleavage using enediyne warheads

Considering the multiple possibilities for the photochemical transformations, light-activated reactions of enediynes with DNA can potentially proceed *via* a number of pathways, which are not limited to the photochemical Bergman cyclisation (and/or C1–C5-cyclisation discussed in the next section), but may also include nucleobase alkylation, photoinduced electron transfer (oxidative damage), direct H-transfer to the enediyne excited state, sensitisation of singlet oxygen and other reactive oxygen species (ROS) formation as well as the photothermal Bergman cyclisation, where irradiation with light provides the energy needed for a ground-state reaction.[29]

To further complicate the comparison, the efficiency of DNA-cleavage depends not only on the efficiency of the radical-generating step but also on such factors as solubility, binding mode (*e.g.*, intercalation *vs.* groove binding) and overall affinity to DNA, involvement of electron transfer in quenching of excited states and in the propagation of damage by hole- or exciton-hopping, the intermediacy of diffusing oxygen-species *etc.* As a result, some of the literature data reflect a complex combination of multiple factors.

Interestingly, the hybrid antitumour antibiotic dynemicine A (20 μM) induces considerable DNA cleavage when activated by visible (<580 nm) light.[30] This process is suggested to proceed through photoreduction of the antibiotic quinone core, followed by a thermal Bergman cyclisation. The natural enediynes esperamicin and neocarzinostatin were also reported to cause DNA cleavage upon photochemical activation.[31] About 13% of ds DNA cleavage by 1 μM esperamicin A_1 has been observed after 15 min of 254 nm irradiation. The exact chemical mechanism of these photochemical processes is still unknown although spin-trapping experiments support the formation of radical species. Esperamicin shows the same base selectivity for the photochemical DNA cleavage as with the usual thiol-activated thermal pathway. In contrast, light-activated neocazinostatin attacks G-bases which are rarely damaged by the thiol-activated neocarzinostatin, suggesting that the involved chemistry is different for the photochemical DNA cleavage caused by the natural enediyne.

De novo designed enediynes can induce photochemical DNA cleavage as well. Whereas single-strand (ss) cleavage is rather common, efficient double-strand (ds) cleavage remains a difficult goal to accomplish. Representative examples are summarized in Fig. 5. A water-soluble dialkynylpyrene reported by Funk *et al.*[26] provided some ds DNA cleavage at 20 μM enediyne concentration after only 15 min of irradiation. Russell's pyrimidine alcohol yields significant DNA ss-cleavage at 40 μM and signs

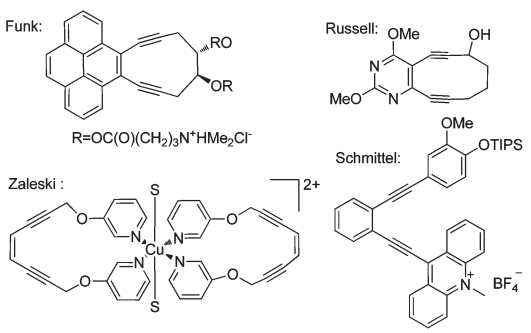

Fig. 5 Selected photochemically activated enediynes capable of causing ds DNA cleavage.

of ds-cleavage at 4000 μM under irradiation with 313 nm light. A report of Zaleski *et al.* showed that copper complexes of bis (pyridine) enediynes lead to a mixture of ds- and ss-cleavage with complete consumption of the supercoiled plasmid DNA after 8 h irradiation at concentrations of enediynes in the range of 50–500 μM.[29] At the lower concentrations, only ss-cleavage is observed. Schmittel *et al.*[32] reported that a mixture of ds- and ss-DNA cleavage was observed at a rather high (1 mM) concentration of an enediyne activated *via* intramolecular electron transfer between a donor and an acceptor attached at the opposite enediyne termini.

In the above examples, it is generally not clear whether the observed formation of double strand breaks corresponds to true ds cleavage events or simply results from the accumulation of ss nicks. These results do not suggest the relative inefficiency of the photo-Bergman cyclisation in comparison to the thermal Bergman cyclisation because most literature examples of DNA cleavage promoted by thermal activation of enediynes are single strand cleavage as well. The general scarcity of efficient ds DNA cleavers is not surprising considering that even the most efficient of natural enediynes, calicheamicin γ1, only leads to 1 : 2–1 : 3 ds : ss cleavage ratios. For a *p*-benzyne diradical to cause ds cleavage, the two radical centers should target opposite DNA strands with 100% efficiency. In practice, one of the radical centers is often quenched by reaction with the environment or by abstraction of hydrogen atoms from an already damaged strand ("silent damage"). Clearly, there is room for improvement.

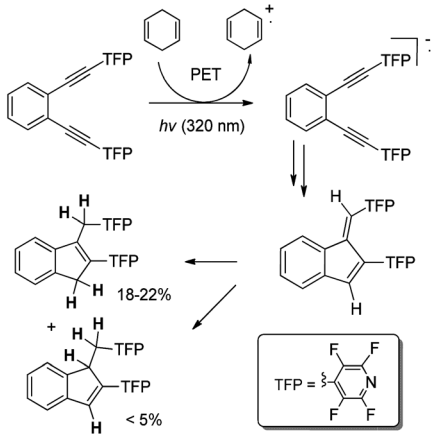

Fig. 6 Photochemical C1–C5 cyclisation of enediynes. Hydrogen atoms abstracted from the environment are shown in bold.

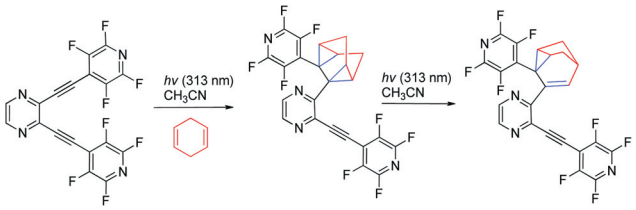

Fig. 7 Switch from a C1–C5 cyclisation to photochemical cycloaddition promoted by variations at the core of the enediyne chromophore.

New warheads, new chemistry. More efficient DNA cleavage?

Considering the above, we set out to design a more powerful DNA-damaging warhead capable of a larger number of hydrogen abstractions, which would provide a promising approach towards increasing the ds cleavage efficiency. The first breakthrough occurred when we found that enediynes with highly electron-withdrawing tetrafluoropyridyl substituents at the alkyne termini undergo a photochemical cyclisation to yield substituted indenes.[33] We were encouraged by this finding because this reaction (C1–C5 cyclisation) leads to the formal abstraction of four H-atoms (compared to only two H-abstractions in the Bergman cyclisation). The transformation is also different from the Bergman cyclisation mechanistically because it is initiated by Photoinduced Electron Transfer (PET) (the thermal version of this cycloaromatisation is impossible under the mild conditions employed[34]) and sensitive to the effects of remote substituents. From a synthetic perspective, it provides a five- rather than a six-membered ring (Fig. 6). Our mechanistic studies found that the four H-atoms abstracted by the enediyne warhead from the environment (*e.g.*, 1,4-cyclohexadiene (CHD) as a DNA surrogate) are delivered through a combination of H-atom transfers, electron transfers and proton transfers.[33,35]

The increase in the net number of DNA damaging events suggests that these compounds can damage both the ribose backbone and nucleobases through a synergistic combination of hydrogen atom abstraction, oxidative damage (initiated *via* photoinduced electron transfer from DNA) and nucleobase alkylation.

The photochemistry and photophysics of TFP-enediynes is sensitive to substitution. A subtle modification of the core part of the chromophore (change of benzene to pyrazine) led to a dramatic change in the observed reaction course. Instead of a cyclisation (C1–C5 or Bergman), the enediyne underwent a cycloaddition step (Fig. 7). A detailed mechanistic study revealed that this reaction proceeds *via* an electrophilic triplet π,π* state. The Intersystem Crossing (ISC) step which provides the reactive triplet is facilitated by the presence of a higher energy n,π* "phantom" triplet state which serves as an internal sensitizer. Fast ISC allows the molecule to bypass the PET step needed for the C1–C5 closure and directs reactivity in the new manifold.[36]

The latter reaction may provide a key to the understanding of a surprising finding of unexpectedly efficient DNA damage by TFP-*mono*acetylenes incapable of either the Bergman or C1–C5 cyclisation (*vide infra*) because it illustrates the potential of electrophilic alkyne triplet states to alkylate electron-rich π-systems.

Finding the right partner for the enediyne warheads in Nature's amino acid toolbox

Despite the high DNA-damage potential, the TFP-enediyne warheads represent only one part in the design of a multifunctional DNA photocleaver for cancer therapy. In order to utilize these relatively hydrophobic molecules in a biological setting, they have to be attached to a functional group which can provide water-solubility and biocompatibility. An attractive choice is

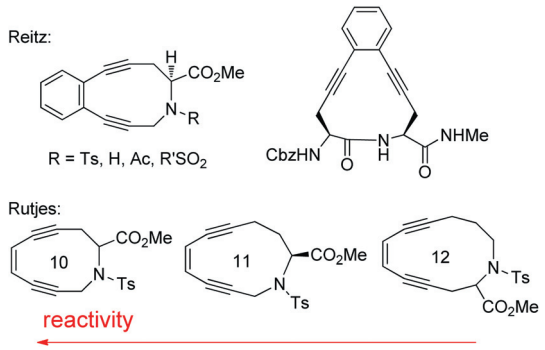

Fig. 8 Use of the peptide bond for the formation of covalent tether between the terminal carbons of enediyne moiety.

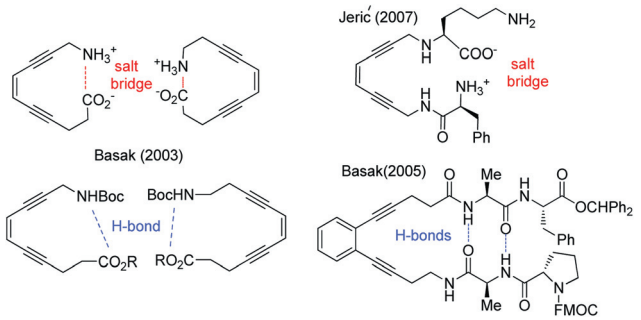

Fig. 9 Use of supramolecular interactions for controlling the distance between the terminal carbons of enediyne moiety.

provided by amino acids, one of the most diverse set of building blocks at Nature's disposal, which can deliver many combinations of properties with broad variations in charge, hydrogen bonding ability, lipophilicity, steric bulk, *etc*.

Several roles of amino acid residues in enediyne conjugates have been reported in the literature. Reitz and coworkers[37] as well as Rutjes and coworkers[38] used the peptide bond formation for the preparation of cyclic enediynes activated by strain (Fig. 8). In these cases, the amino acid part serves as a covalent linker bringing the reacting termini of the enediyne system closer. As expected, the Bergman cyclisation rate is directly proportional to the ring strain and inversely proportional to the linker length.

Basak *et al.*[39] and later Jerić and Chen[40] also explored the possibility of supramolecular activation in enediyne-amino acid conjugates where the ring closure was assisted by the formation of a salt bridge between ammonium and carboxylate groups (Fig. 9). Such assistance is more efficient than H-bonding in the protected neutral analogues. In 2005, Basak *et al.* expanded this concept to a pentapeptide amino acid enediyne conjugate with several H-bonds between the terminal peptides.[41]

Basak *et al*. also used amino acid linkers to connect two enediynes to a photochemically switchable azo-benzene unit[42] (Fig. 10).

Jones and coworkers found that the acidic moieties in enediyne amino acid conjugates (*e.g.*, the tri-(Asp) conjugate in Fig. 11) help to target basic proteins such as the H1 histone protein.[43]

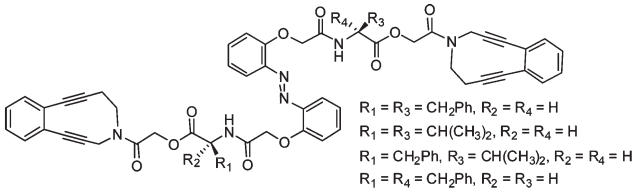

Fig. 10 Photochemically switchable bis-enediyne amino acid hybrids

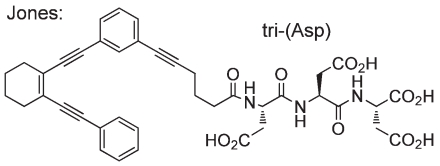

Fig. 11 Conjugates with acidic side-chains for cationic targets.

Lysine-conjugates

For an anionic target such as DNA, attachment of basic amino groups to the "warhead" appears to be a logical choice. Protonation of these groups renders the conjugate cationic, adding affinity to the negatively charged phosphate backbone of DNA.[44] Attachment of the TFP-substituted enediyne to lysine would mimic the well-known interaction between cellular DNA and lysine-rich histone proteins. Several other considerations make this choice even more intriguing: (a) lysine residues of histone proteins have been implicated in conversion of oxidised DNA into spiro-adducts and DNA cross-links (b) the lysine moiety in the catalytic center of DNA repair enzymes is known to assist in removal of oxidised DNA bases with transient DNA strand scission. We discuss these possibilities below.

Lysine-mediated DNA-cross links

A large body of research documents the formation of cross-links *via* a reaction of lysine residues with nucleobases.[45] Several mechanistic scenarios operate for different types of oxidative DNA-damage (*e.g.* 8-oxo-guanine (OG), guanine radical cation or oxidised 8-oxo-guanine (OG_{ox})). Although the selectivity of nucleophilic attack of the lysine amino group at the oxidised DNA depends on the type of DNA damage, both C5 and C8 attacks can lead, after a rearrangement, to the formation of cross-linked spiro-adducts.[46] Alternatively, formation of lysine-DNA adducts can also occur through initial oxidation of lysine and reaction of an N-centered radical with DNA[47] (Fig. 12). This DNA-protein cross linking can block DNA replication and, if not repaired, induce cell death.

Mimicking 8-oxoguanine-DNA glycosylases (OGG1): creating abasic sites and converting them into strand cleavage

A very interesting model for the possible role of lysine in converting OG sites into strand scission is suggested by the mechanism of action of DNA glycosylase/β-lyase OGG1 in which lysine acts both to displace the oxoguanine base (with the

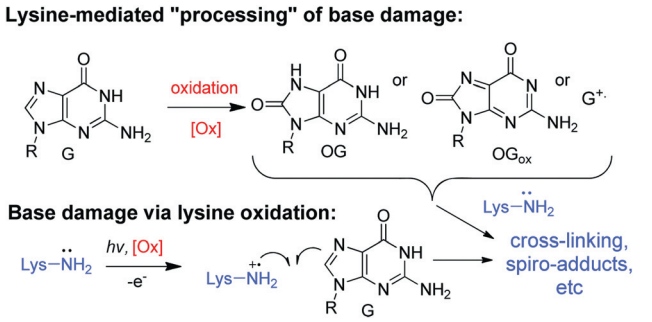

Fig. 12 Formation of deoxyguanosine-lysine adducts *via* a two-step mechanism which involves chemical or photochemical DNA oxidation followed by reaction of with lysine or oxidation of lysine followed by electron transfer from DNA.

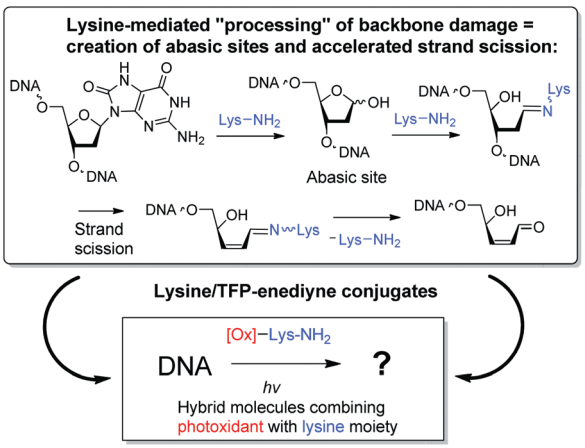

Fig. 13 Acceleration of strand scission *via* Schiff base formation with lysine (top) and the potential ability of lysine conjugates to emulate both steps in the DNA oxidation/cleavage mechanism (bottom).

formation of an abasic site) and to promote subsequent elimination *via* a transient formation of a Schiff base (Fig. 13).[48]

Note that hybrid ED/AC-Lys conjugates can perform all the steps involved in the OGG1-mediated DNA cleavage: (a) oxidative damage by the excited chromophore (a step that an enzyme cannot perform!), (b) formation of the abasic site *via* 8-OG displacement by one of the amino groups of the conjugate, (c) conversion of the abasic site into cleaved DNA (again by one of the amino groups).

C- vs. N-conjugates: introducing the pH-gating ability

In order to take full advantage of lysine's potential as a DNA binding group, we have chosen to use its carboxyl group for the attachment to the DNA-cleaving moieties (C-lysine conjugates in Fig. 14 top).[49,50]

This mode of attachment is different from the more common formation of a classic peptide bond *via* the α-amino lysine group moieties (*N*-lysine conjugates in Fig. 14 bottom).[51] Importantly, both amino groups of the lysine residue of C-conjugates are free for facilitation of DNA-damage. The α-amine is particularly

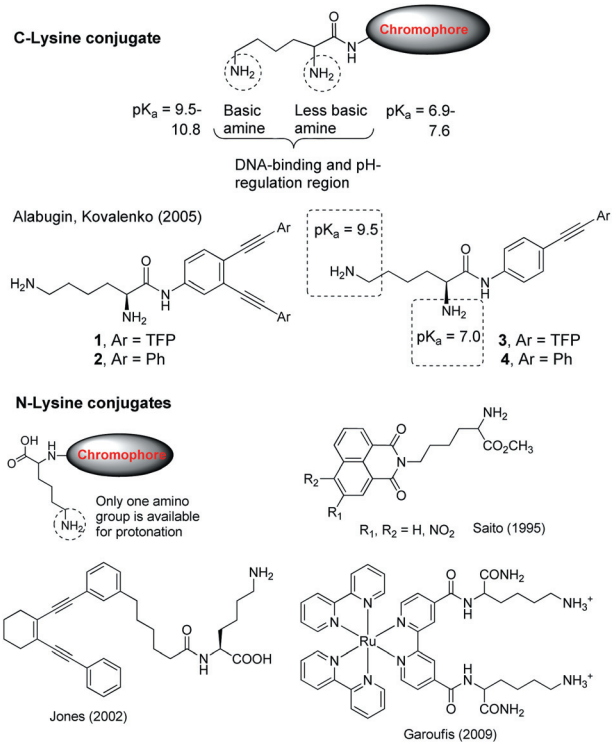

Fig. 14 Two approaches to amino acid/DNA cleaver hybrids and structures of lysine conjugates **1**–**4**.

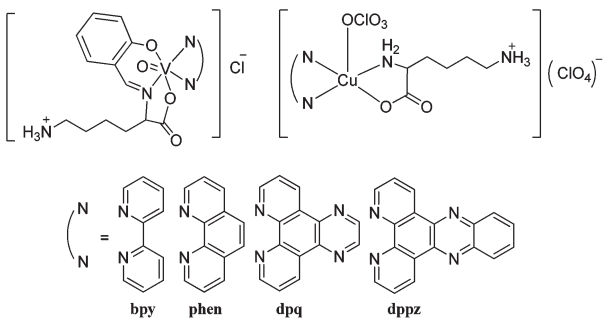

Fig. 15 The structures of Lysine–metal complexes with different ligands.

important for the design of pH-controlled DNA-cleavers (*vide infra*).

Yet another use of the α-amino group of lysine was reported by Chakravarty and coworkers who took advantage of the amine nucleophilicity to form either a covalent or a dative bond with either a carbonyl or a metal center from the DNA-cleaving part. Lysine-copper[52] and lysine-oxovanadium[53] complexes in Fig. 15 show DNA ss cleavage upon photo excitation.

Cleavage of DNA by TFP-hybrids

Unusual efficiency of ds DNA cleavage

Encouragingly, these simple lysine–enediyne conjugates, such as compound **1** (Fig. 14), showed the ability to cleave plasmid DNA at relatively low concentrations (high nanomolar for ss

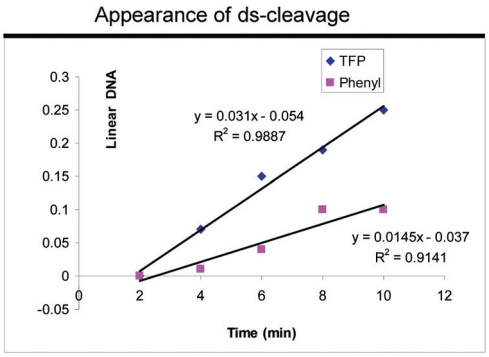

Fig. 16 Relative efficiencies of pBR322 plasmid DNA cleavage (20 μM/bp) by 10 μM lysine conjugates **1** and **2** at pH 8 as a function of irradiation time.

cleavage and low micromolar for ds cleavage). The TFP-enediyne **1** was significantly more efficient in causing ds DNA photocleavage than the analogous diphenyl substituted enediyne (Fig. 16). Statistical analysis revealed a high ratio of ds–single strand (ss) breaks with 100–1000 times more ds breaks than could be accounted for by random ss breaks on opposing strands.[49]

These findings compared very favourably with the results for other photoactivated enediyne warheads shown in Fig. 5. Not only were the required concentrations two orders of magnitude lower but the very favourable (~3 : 1) ss–ds cleavage ratio (Fig. 16) approached that of calicheamicin!

Selectivity of DNA cleavage: combining orthogonal factors

A common approach to understanding the mechanism of DNA photocleavage by new chemical agents is to analyse the cleavage pattern in labelled DNA oligomers. Radical damage, for example, is typically localised near the binding site of the DNA cleaver, while oxidative cleavage is funnelled away to adjacent, easily oxidisable G_n sites.

Intriguingly, when this standard test, using a DNA oligomer with a ^{32}P-phosphate label (incorporated as a terminal phosphate group), was performed with lysine conjugates of TFP-enediynes and related fulvenes and acetylenes, it failed repeatedly, as no cleavage pattern could be observed despite rapid disappearance of the DNA label.[54] These observations were traced back to the photochemical removal of the terminal phosphate label (see next section for additional discussion of this unexpected behaviour). Only when the label was no longer part of a terminal phosphate group but rather "hidden" in the backbone of the DNA oligomer, could a cleavage pattern be observed.

The results were surprising. While TFP-substituted enediyne **1** produced the expected cleavage pattern that was consistent with oxidative DNA damage, the same pattern was observed for phenyl-substituted enediyne **2** – a compound that had always been assumed to damage DNA by a radical mechanism, as expected from the Bergman cyclisation. An even bigger surprise was that lysine-conjugates of acetylenes **3** and **4**, originally intended as mere controls for photophysical measurements, could also serve as DNA cleavers. As they are incapable of cyclisation, they represent a new class of oxidative DNA-damaging warheads.

Interestingly, for all lysine conjugates, the selectivity of damage was found to be a compromise between G selectivity for activation *via* PET and affinity of protonated amines for narrow AT-rich regions of DNA (AT-tracts). Consequently, most of the damage was observed at guanines flanking the AT-tracts. Interestingly, some cleavage was also observed at single guanine in the AT-tract suggesting guanine alkylation as an additional path for the interaction of photochemically excited alkynes and DNA.

New strategy to generate double strand breaks: photochemical ss → ds break conversion. towards photochemical RNA interference

The failure of the terminal phosphate group to serve as a suitable DNA label in the presence of C-lysine conjugates indicated a remarkable ability of these hybrid agents to locate a single phosphate monoester in the presence of multiple phosphate diesters of the DNA backbone. Because DNA-cleavage leads to the transformation of an internal phosphate (a diester) to a terminal phosphate (a monoester), we exploited whether this preference can be used to direct DNA photocleavage and accomplish an unprecedented photochemical conversion of a single strand break into ds cleavage (Fig. 17).[55]

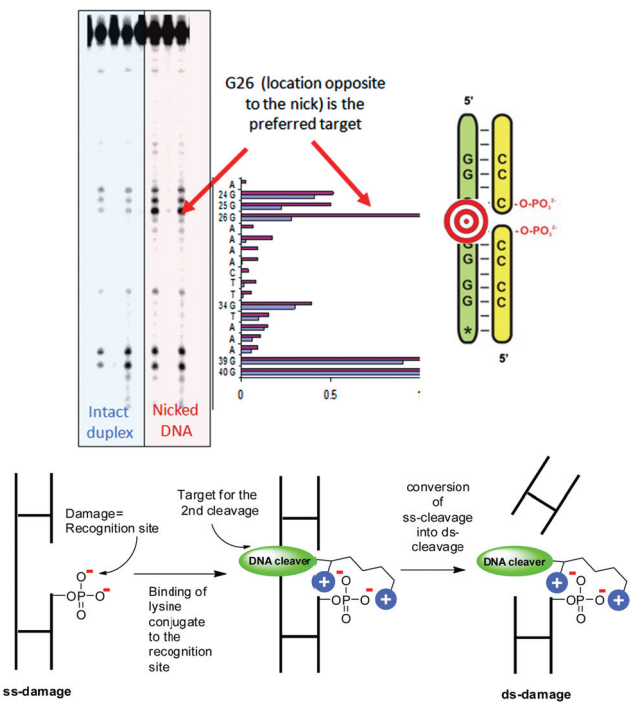

Fig. 17 Photochemical conversion of ss DNA damage to ds DNA damage based on recognition of DNA ss cleavage by a lysine conjugate. (Top) Comparison of photocleavage efficiency and selectivity in an intact DNA 54-mer and DNA with a single break introduced across a minor damage site (G26). The data are shown for a gapped DNA with two phosphates flanking the gap. Similar increase in selectivity is also observed for nicked and gapped DNA with a varying number of phosphate monoester moieties at the damage site.[55] (Bottom) Possible mechanism for the lysine-mediated transformation of ss damage into ds-damage.

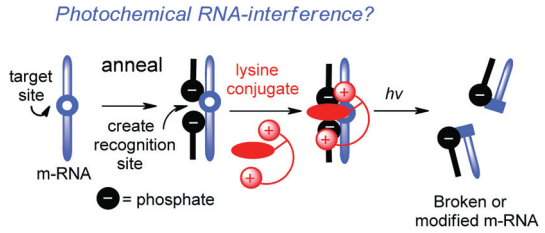

Fig. 18 Potential design for photo RNA interference experiments. By annealing a single strand nucleotide with complementary oligonucleotides bearing terminal phosphate groups, a target site for lysine recognition is created on the targeted nucleotide backbone. Lysine conjugates are then photochemically excited to perform photocleavage at that site, akin to the DNA experiments shown earlier.

In particular, we have synthesised a variety of possible DNA ss damage sites and tested whether they can serve as recognition sites for the lysine conjugates and direct cleavage at the intact strand opposite to the damaged site. Success of these experiments provided the first example of photochemical transformation of an easily repairable ss damage site to a therapeutically more important ds damage site. A variety of damage sites was tested (both nicked and gapped DNA with phosphates at 3′ and/or 5′ locations) at different locations of the target within the sequence.

At this points, it is not clear whether the highly efficient ds DNA cleavage in Fig. 16 is due to scission of both strands by the same conjugate molecule during a single binding event, or if a second molecule can bind to the nicked site and cleave the opposite strand at that same location. Each of the two scenarios will provide more ds damage than statistical nicking.

Because a terminal phosphate group could be used to direct the lysine-conjugates to specific locations within DNA, these findings constitute a significant advance in the field of small-molecule–DNA interaction. Recognition of DNA damage sites had been thought to be the exclusive domain of large (repair) enzymes and some rather elaborate natural products, but with this study, it was expanded to small molecules (MV ≤ 500). It is also a suitable explanation of the high ds–ss cleavage ratio observed in the reactions of lysine conjugates with DNA. Not only does the conversion of ss breaks to ds breaks also point to a new strategy in the development of chemotherapy agents, but it also provides a chemical analogy to siRNA with a potential for photochemical triggering (Fig. 18).

pH-Gated DNA cleavage: targeting hypoxic tumours

The relatively acidic extracellular environment of solid tumours[56,57] lends itself for the design of tumour-specific pH-activated chemical agents.[58] Hyperglycemia and/or such drugs as amiloride, nigericin, and hydralyzine, are able to lower the intracellular pH of cancer cells as well. At dosages that do not affect the normal cells, amiloride and nigericin have been reported to drop the intracellular pH in a number of tumour cell types from 7.2 to 6.2–6.6.[59–62] When combined with hyperglycemia and/or hypoxia, further acidification to a pH as low as 5.5 is possible.[63,64]

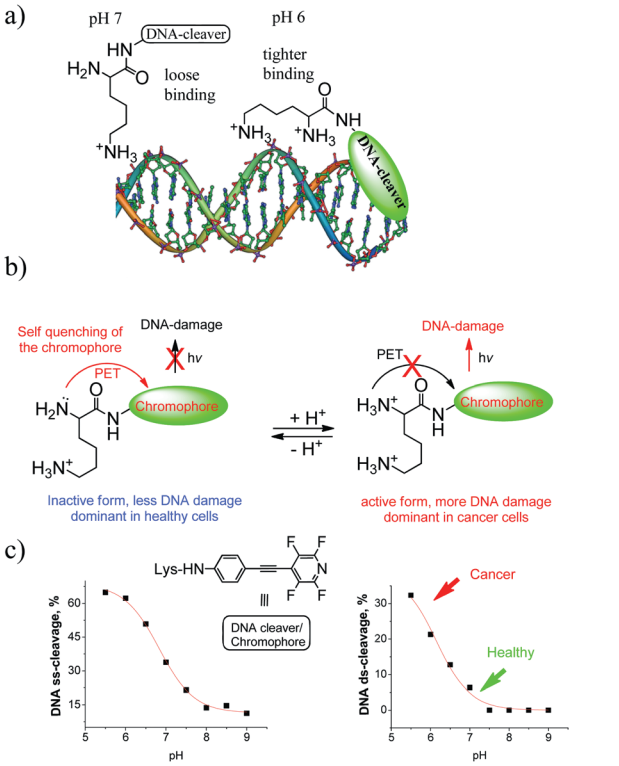

Fig. 19 pH-Dependent changes in the activity of lysine conjugates is based on two effects. (a) tighter binding of diprotonated species, (b) change in photophysics (deactivation of intramolecular photoinduced electron transfer): (c) ss- and ds-DNA cleavage (excitation at >300 nm) with lysine TFP-alkyne conjugate **3** as a function of pH.

Several approaches have been developed to make phototherapy agents, and enediyne warheads in particular, more active at lower pH. Strategies have usually focused on switching from an inactive form of the enediyne warhead to an active one upon the lowering of pH, or by taking advantage of the change in electron density in the in-plane orbitals of the enediyne warhead upon protonation, which results in a lowering of the activation barrier for the Bergman cyclisation.[65]

The modular structure of lysine conjugates enabled a conceptually different approach based on pH-gated photoinduced electron transfer (Fig. 19). Although both amino groups in C-lysine conjugates are available for protonation, their basicities are vastly different: While the ε-ammonium group has a pK_a of ~10–11, remaining protonated at all physiologically relevant pH, the α-ammonium group has a pK_a of ~6.5–7. This lower basicity of the α-amino group allows for a change in protonation at a pH threshold separating healthy and cancer cells, which is highly relevant to the intended goal of maximizing the activity toward cancer cells.

Protonation of the less basic α-amino group leads to an increase in the warheads' activity as DNA cleavers *via* a combination of synergistic effects. First, the added positive charge modifies binding of the lysine conjugates to DNA. Not only does this result in a higher binding constant, but there is also a likely change in the binding mode. By having the α-amino acid protonated and bound to DNA, the warhead is positioned closer to the intended damage site, increasing cleavage activity.

Secondly, the difference in protonation results in marked photophysical changes. The non-protonated amino groups have a lone pair of electrons available for either intra- or intermolecular electron transfer quenching of the warhead's excited state. In other words, the photoinduced electron transfer responsible for the DNA-damaging action of the warhead will not happen from DNA to the warhead's excited state, but instead from the lone pair of the α-amino group, rendering the warhead harmless to DNA. In contrast, when the α-amine is protonated, the DNA-damaging photochemistry is possible.

When tested against plasmid DNA at a range of pH values, it was found that both the overall amount of cleavage and the relative amount of ds DNA breaks followed a classical pH-titration curve with the same pK_a as the α-amino group of the C-lysine conjugate. As ss damage is easier to repair for living cells than ds damage, this is yet an additional bonus in the quest to differentiate between healthy cells and cancer cells. For example, at the conditions shown in Fig. 19, hardly any ds cleavage is observed at the pH of healthy tissues whereas >25% ds cleavage (on par with calicheamicin[4]) is observed at pH 5.5 (the minimum pH value reported for hypoxic tumours).[64]

Optimizing structural design

Simple lysine–enediyne conjugates provide a remarkable set of interesting properties. However, because the DNA-cleaving and biological activity depends on a number of other factors, such as the compounds' solubility in biological medium, their affinity to DNA, and whether the caused damage is localised (*e.g.*, radical damage, alkylation) or has the ability to spread (*e.g.*, hole hopping in case of oxidative damage, diffusion of reactive oxygen species), we have investigated the effect of further structural modifications.

Modification of the DNA cleaving core

Lysine conjugates with several TFP-substituted chromophores displayed DNA-cleaving ability. Although enediyne conjugates were the most reactive, they tend to aggregate due to limited solubility in aqueous media, which complicates the mechanistic studies. The putative fulvene intermediates in the enediyne → indene transformation were also prepared and found to cause DNA cleavage at guanines, lending support to the proposed mechanism for the C1–C5 cyclisation of TFP-substituted enediynes. The fulvene–lysine conjugates also possessed moderate DNA-cleaving reactivity but offered no obvious advantages over enediynes and were not pursued further.

On the other hand, acetylene–lysine conjugates possessed a promising combination of sufficient DNA cleaving ability with good water solubility and relatively low toxicity. Because mono alkynes represented a new type of DNA photocleaver, we have investigated them in more detail. In particular, in order to test for a connection between the alkylating ability and DNA-damaging properties of these compounds, we investigated the photoreactivity of three isomeric aryl-TFP (tetrafluoropyridinyl) alkynes with different positions (*o*-, *m*- and *p*-) of amide substituents towards a model π-system (Fig. 20).[66] Reactions with 1,4-cyclohexadiene (1,4-CHD) were used to probe the alkylating properties of the

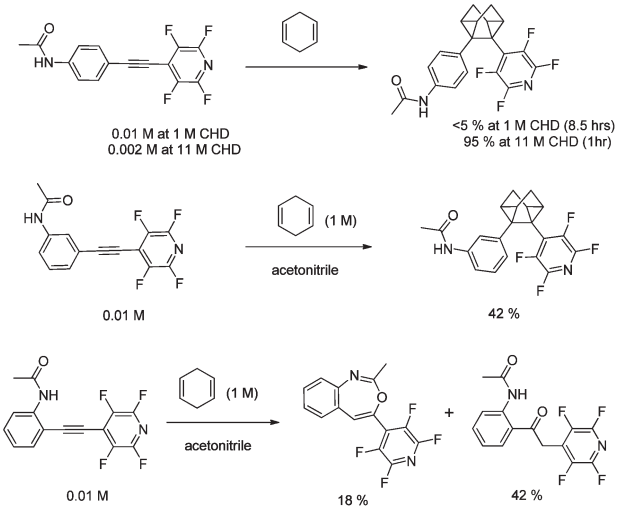

Fig. 20 Effect of structural modifications in the alkyne part on photoreactivity towards 1,4-CHD. UVB (313 nm) was used for the photoreactions.

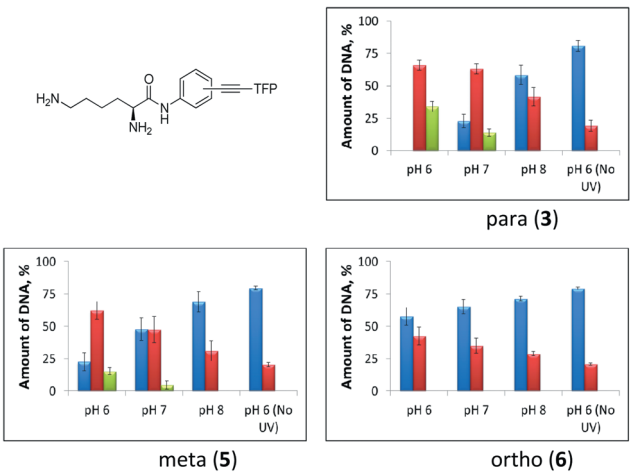

Fig. 21 Effect of structural modifications in the alkyne part on DNA photocleavage (10 min of >300 nm irradiation). No damage – blue; ss cleavage – red; ds cleavage – green.

triplet excited states in these three isomers whereas Stern–Volmer quenching experiments were used to investigate the kinetics of PET. The three analogous isomeric lysine conjugates cleaved DNA with differences in efficiency (34%, 15% and 0% of ds DNA cleavage for *p*-, *m*- and *o*-substituted lysine conjugates, respectively) consistent with the alkylating ability of the respective acetamides (Fig. 21). A significant protecting effect of hydroxyl radical and singlet oxygen scavengers to DNA cleavage was shown only with the *m*-lysine conjugate.

Modification of DNA binding part

The idea to use two amino groups that differ in basicity for achieving pH-gated drug activation was expanded further. By attaching warheads to two lysine groups, dipeptide conjugates of enediynes and acetylenes were created, either with two α- and

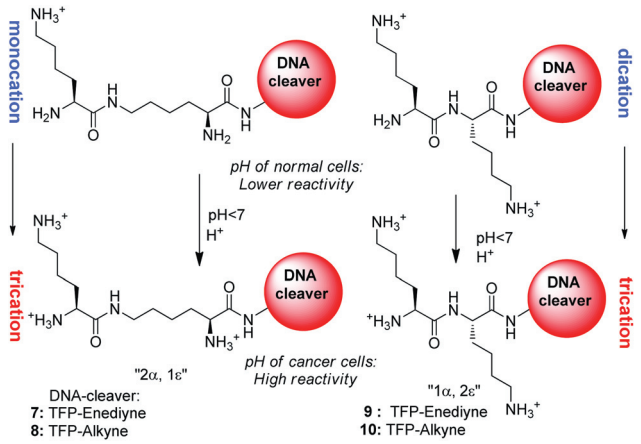

Fig. 22 Second generation of lysine-conjugates for pH-dependent DNA cleavage.

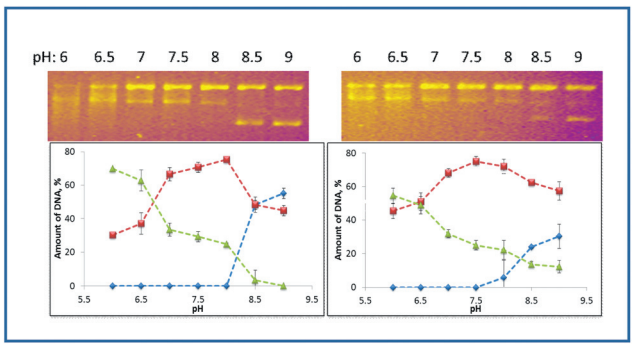

Fig. 23 Quantified cleavage data for plasmid relaxation assay for DNA photocleavage with 15 μM of acetylenic conjugates **8** (left) and **10** (right) and 38 μM (b.p.) of pBR322 plasmid DNA at pH range of 6–9 after 10 min. of UV (>300 nm) irradiation. Reported values represent the average of three experiments. Code: intact DNA, blue diamond; ss-cleavage, red square; ds-cleavage, green triangle.

one ε-amino group, or with one α- and two ε-amino groups (Fig. 22).[67]

These hybrids feature a shift at physiologically relevant pH either *from dication to trication* or *from monocation to trication*. Although the chemical mechanism for the DNA cleavage and the possibility of DNA alkylation and DNA-lysine cross linking are still under investigation, the bis-lysine conjugates unambiguously show a significant increase in the efficiency of DNA cleavage (Fig. 23) due to the change from a mono-lysine to a bis-lysine moiety. The observed ds/ss ratios of up to 2 : 1 by far exceed those of the naturally occurring calicheamicin and bleomycin, indicating that not only is the cleavage activity increased at low pH, but also the recognition of initial ss break sites by these conjugates, creating subsequent damage that leads to an overall ds break, may be strongly enhanced.

The higher reactivity of bis-lysine conjugates results in significant ds DNA cleavage even at pH 8, conditions where the same concentrations of mono-lysine conjugates do not cause this damage (Fig. 24). The correlation between the efficiency of ds DNA cleavage and the relative abundance of the tricationic form of the dipeptide moieties suggests that this protonation state

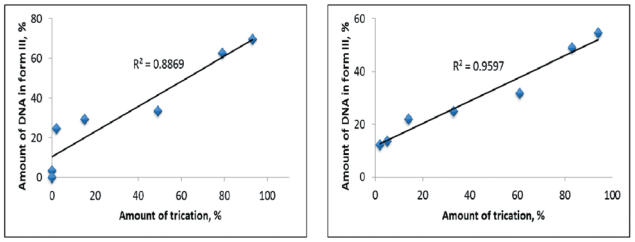

Fig. 24 Correlation of DNA ds-cleavage with the trication mole fraction (bottom) for conjugates **8** (left) and **10** (right).

plays a particularly significant role in ds-cleavage.[67] Further research aimed at finding even more efficient combinations of pH-gated selectivity with reactivity is in progress.

Statistical tests[68] unambiguously show that these compounds are true ds DNA cleavers.[69] To the best of our knowledge, the above results correspond to the most efficient ds DNA photocleavage by a small molecule known to date.

Cellular uptake, intracellular DNA-damage and cytotoxicity toward cancer cell lines

Testing the DNA-cleaving ability of compounds against isolated DNA serves as a useful tool for quantifying cleavage activity, or for comparing various compounds and optimising the chemistry responsible for DNA cleavage. However, in order to determine whether a compound could become a viable pharmaceutical tool, tests against live targets are indispensable.

In particular, damaging intracellular DNA is more challenging than cleaving isolated DNA. Not only does the DNA-cleaving agent have to penetrate through the cell and nucleus membranes, it also needs to be able to attack the more compactly organized intracellular DNA. By using single cell gel electrophoresis (SCGE, or "Comet") assays (Fig. 25), we confirmed that intracellular DNA damage indeed occurs when the cells are exposed to DNA-cleaving agent (both lysine and bis-lysine conjugates) and light.[65,70] In the absence of the photoactivated conjugates, irradiation for 10 min does not produce efficient DNA damage. These results confirm that compound **3** and **10** can penetrate into the cancer cell nucleus and damage highly compacted DNA upon photoactivation.

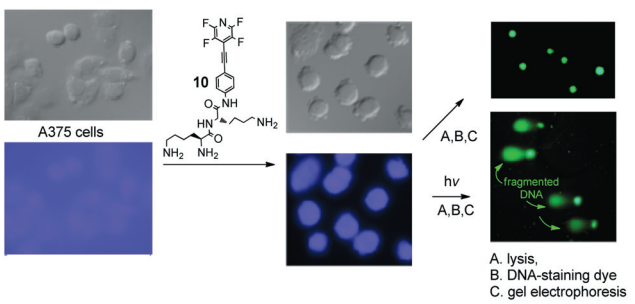

Fig. 25 Left: the low auto-fluorescence of A375 cells. Center: increased fluorescence due to the penetration of acetylene-bis-lysine conjugate into A375 cells. Right: SCGE (Comet) assays for A375 cells + UV (365 nm) + compound **10**. The characteristic "comet" shape confirms DNA fragmentation due to the presence of the conjugate. All photochemical irradiations were carried out for 10 min.

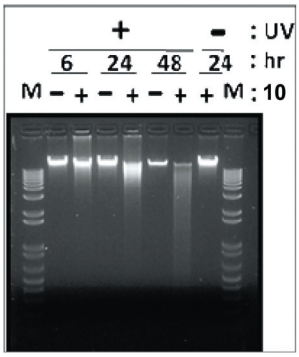

Fig. 26 UV activated bis-lysine conjugate **10** causes DNA damage in A375 cells. 30 μM of **10** was added to cells and incubated for 3 hours. Treated and untreated cells were exposed to UV radiation (365 nm) for 20 min and harvested at 6, 24 and 48 hours post UV radiation. Smaller − and + symbols indicates absence or presence of conjugate **10**. Time points are indicated by numbers in hours; M stands for DNA marker. Bigger − and + indicates whether cells were UV irradiated.

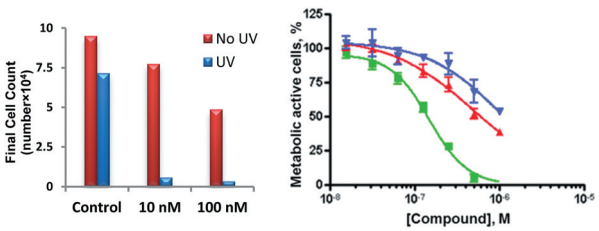

Fig. 27 Left: Cell proliferation assay using LNCaP cells and acetylene-lysine conjugate **3** after 10 min. of UVC (254 nm) irradiation. Right: cell proliferation assay using A375 cells (human melanoma) and *p*-(green square), *m*-(red up-pointing triangle) and *o*-(blue down-pointing triangle) alkyne lysine conjugates **3**, **5** and **6** after 10 min. of photoactivation (365 nm) at concentration where toxicity in the dark is very low.

In order to further elucidate the cellular effects of these compounds, in particular their ability to cause apoptosis, we studied time evolution of DNA damage in A375 human melanoma cells. Fig. 26 shows that the DNA degradation was observed at all-time points but it progressively increased with time, even hours after irradiation has been stopped. The continuous increase in DNA degradation is considered as one of signs of apoptosis due to the release of endogenous endonucleases.[71] The appearance of fragmented DNA on the gel was similar to that reported earlier by Wyllie and coworkers for the fraction of apoptotic cell lysates which has been cross-linked to the nucleus with the involvement of histone proteins.[72]

Lysine-conjugates of enediynes and acetylenes were tested in cell proliferation assays against several cancer lines both in the dark and under UV irradiation (Fig. 27). While all compounds tested showed strong photocytotoxicity, some compounds were toxic in the dark as well. The mechanism of the dark cytotoxicity remains unknown but it is unlikely to be associated with a ground state cycloaromatization process. Most encouragingly, the lysine–TFP–acetylene conjugate **3** showed a high level of activity in presence of light, while exhibiting little effect on cell growth in the dark. Remarkably, even at a 10 nM concentration of compound, a strong effect could be observed against LNCaP human prostate adenocarcinoma cells.[50] While with UV activation, more than 90% of LNCaP cells are destroyed after a single 10 min treatment, almost no effect was observed in absence of light. In a similar way, all three isomeric lysine conjugates inhibited human melanoma cell growth under photoactivation but the *p*-conjugate has the lowest CC_{50} (50% cell cytotoxicity) value of 1.49×10^{-7} M.

This is the same level of activity that is typically observed in cell assays with Photofrin,[73] a commonly used photodynamic therapy agent. This is noteworthy because Photofrin acts as a *catalyst* for the production of singlet oxygen, while the conjugates described here act *stoichiometrically* and are inactivated once they performed their cleavage reaction. The comparison with other, more conventional chemotherapy agents is also favourable. Cisplatin – a DNA crosslinker – requires concentrations that are typically two orders of magnitude higher[74] to achieve LD_{90}. And while Taxol, perhaps the greatest success story in cancer therapy, is effective at approximately equal concentration as the lysine–acetylene conjugate, its actions require treatment times that are longer by 2–3 orders of magnitude.[75]

Beyond DNA-cleavage

Other interesting features of enediyne aminoacids conjugates go beyond the scope of this review but deserve to be mentioned. For example, recent work of Jerić and coworkers uncovered thermally induced cyclisation–elimination pathways of enediynes with terminal amino acid residues at both propargylic carbons. In these systems, Bergman cyclisation led to loss of one of the amino acid residues with a concomitant formation of a five-membered cycle[76] (Fig. 28).

Another interesting observation was reported by Bertrand and co-workers who found a remarkable memory of chirality in cascade transformation of enediynes with appropriately positioned chiral amino acid residues[77] (Fig. 29). The cascade is initiated by base-catalyzed enediyne–enyne allene rearrangement

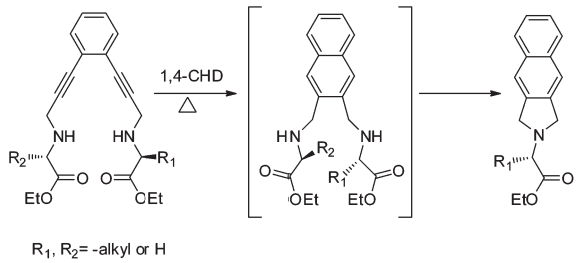

Fig. 28 Bergman cyclisation of acyclic amino acid derived enediynes.

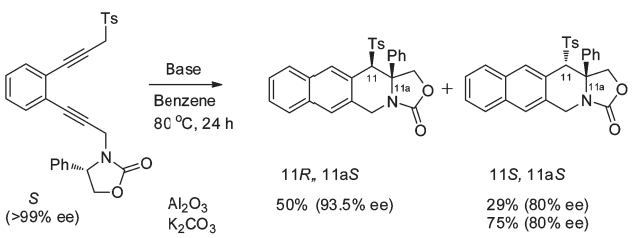

Fig. 29 The cascade transformation of enediyne-amino acid hybrids through base-catalysed isomerisation and Saito–Myers cyclisation.

followed by Saito–Myers cyclisation that gives rise to a biradical. An intramolecular-hydrogen atom abstraction leads to another biradical which undergoes intramolecular coupling without significant loss of chiral integrity.

Conclusions

Hybrid agents which combine potent DNA-photocleavers with tunable amino acids or small peptides display selectivity and efficiency which compares favourably with Nature's most potent class of anticancer antibiotics. These small molecules can be assembled *via* efficient modular approaches and show biological activity and intricate combination of synergistic effects that was thought to be the sole domain of compounds that are at least two orders of magnitude larger. The record-breaking DNA-cleaving ability of these compounds stems from new chemistry, which is enhanced by the ability of lysine residues to detect the sites of initial (ss) DNA damage sites and convert them to the therapeutically important ds breaks. These compounds show high photo-induced cytotoxicity against several cancer cell lines. Encouragingly, the DNA-cleaving ability is dramatically amplified at the slightly acidic pH of hypoxic cancer tissues.

For future practical applications, it is necessary to consider the limits of photoactivation in living patients. Tissue is not uniformly transparent, but strongly absorbs in the UV-Vis range, mostly due to hemoglobin absorbance, and in the NIR range, due to water. There is a range between ~650 and 900 nm, the "therapeutic window of tissue", where light can penetrate deeply into tissues, and consequently, viable drugs that go beyond activation on the surface of tissues need to be activated by light that matches this range of wavelengths. A particularly attractive option here is two-photon activation, as this method not only uses long-wavelength photons, but is also highly intensity-dependent, aiding in spatial control over drug activation. Although simple enediyne chromophores require activation with light of <360 nm, it was shown that these molecules can be also activated in a two-photon fashion with lower energy ~600 nm photons.[78]

Lysine–acetylene and lysine–enediyne conjugates have successfully passed the initial tests in the development of anticancer drugs ranging from mechanistic studies on DNA cleavage to *in vitro* tests against cell lines. So far, these compounds have exceeded expectations. *In vivo* tests need to follow for these compounds to move to clinical trials.

Acknowledgements

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