Antitumor Antibiotics: Bleomycin, Enediynes, and Mitomycin

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Contents

1. Introduction

Natural-product-derived cytotoxics remain a mainstay in current chemotherapy.[1] This review focuses on the current level of understanding and emerging trends relevant to the DNA-damaging metabolite families of the bleomycins, 9- and 10-membered enediynes, and mitomycins. Within the context of their clinical utilities and shortcomings, a comparison of resistance mechanisms within producing organisms to those predominant among tumor cells reveals remarkable potential for continued development of these essential anticancer agents.

2. Bleomycin

2.1. Discovery and Biological Activities

The bleomycins (BLMs), such as bleomycinic acid (**1**), BLM A2 (**2**), or BLM B2 (**3**), are a family of glycopeptide-derived antibiotics originally isolated from several *Streptomyces* species.[2,3] Several structure variations of the naturally occurring BLMs have been identified from fermentation broths, primarily differing at the C-terminus of the glycopeptide. The BLM structure was revised in 1978[4] and confirmed by total synthesis in 1982.[5,6] Structurally and biosynthetically related to the BLMs are the phleomycins (PLMs), such as PLM 12 (**4**) or PLM D1 (**5**),[7−10] and tallysomycins (TLMs), such as TLM S_2B (**6**) and TLM S_{10}B (**7**)[11,12] (Figure 1).

BLMs are thought to exert their biological effects through a sequence-selective, metal-dependent oxidative cleavage of DNA and RNA in the presence of oxygen.[13−16] The BLMs can be dissected into four functional domains: (i) the pyrimidoblamic acid (PBA) subunit along with the adjacent β-hydroxyl histidine constitutes the metal-binding domain that provides the coordination sites required for Fe(II) complexation and molecular oxygen activation responsible for DNA cleavage; (ii) the bithiazole and C-terminal amine provide the majority of the BLM−DNA affinity and may contribute to polynucleotide recognition and the DNA cleavage selectivity; (iii) the (2*S*,3*S*,4*R*)-4-amino-3-hydroxy-2-methylpentanoic acid (AHM) subunit not only provides the connectivity between the metal-binding and DNA-binding sites but also plays an important role in the efficiency of DNA cleavage by BLMs; (iv) the sugar moiety is likely to participate in cell recognition by BLMs and possibly in cellular uptake and metal-ion coordination. Consequently, there have been continuing attempts to develop new BLM congeners to define the fundamental functional roles of the individual domains and search for anticancer drugs with better clinical efficacy and lower toxicity. However, the structural complexity of BLMs has limited most of the modifications at either the C-terminal amine or the N-terminal β-aminoalaninamide moiety by either directed biosynthesis or semisynthesis. Total chemi-

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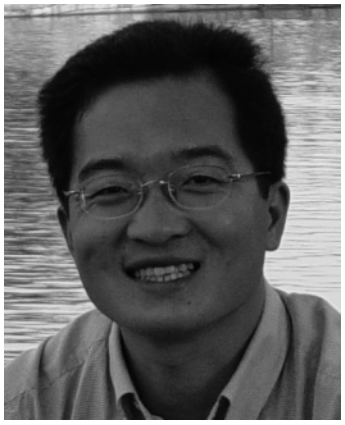
Ute Galm studied pharmacy at the University of Tuebingen, Germany. In 2003 she finished her doctoral research on aminocoumarin antibiotics under Professor Lutz Heide and received her Ph.D. degree from the University of Tuebingen. She was appointed as a postdoctoral associate at the University of Wisconsin—Madison in 2004, where she works with Professor Ben Shen. Her current research interests are focused on biosynthetic studies of the bleomycin family of compounds in different *Streptomyces* strains.

Martin Hager, born in 1972 in Muenster, Germany, began his studies in Biology in 1993 at the University of Tuebingen. He received his diploma in 1998 and his Ph.D. degree in 2002 with Professor Ralph Bock at the University of Freiburg for his work on the functional analyses of open reading frames by reverse genetics. He then joined the laboratory of Professor Albrecht E. Sippel to investigate EGF-receptor inhibitors and new approaches to tumor targeting by exploiting antibody avidity. In 2003 he joined the laboratory of Professor Jon S. Thorson. His current research objective is the directed evolution of glycosyltransferases and structural analysis of resistance proteins. Martin Hager is a postdoctoral fellow of the German Academy of Scientists Leopoldina. He also received grants from the German Research Foundation and the German Ministry of Education and Research.

Steven G. Van Lanen received his B.S. (1998) degree in Molecular Biology from the University of Wisconsin—Madison. In 2003 he received his Ph.D. degree in Chemistry at Portland State University under the guidance of Professor Dirk Iwata-Reuyl. He currently is a postdoctoral fellow in the laboratory of Professor Ben Shen, studying the biosynthesis of C-1027 and other enediyne natural products.



Jianhua Ju was born in 1972 in Shandong, China. He received his B.S. degree in Pharmacy from Shandong University in 1995 and obtained his Ph.D. degree in Medicinal and Natural Product Chemistry at Peking Union Medical College in 2000. Then he worked as a faculty member at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences in Beijing, where he was engaged in new natural products discovery research. He received the Servier Young Investigator Award in Medicinal Chemistry in 2002 issued by the Chinese Pharmaceutical Association & Institut de Recherches Internatinales Servier. In 2003 he joined the research group of Professor Ben Shen as a postdoctoral research associate at the School of Pharmacy, University of Wisconsin—Madison working in the field of natural product biosynthesis.

cal synthesis of BLMs is expensive, thus limiting the practicality in pharmaceutical applications. While numerous BLM analogues have been synthesized in the past two decades, none has improved properties.[13,14,17] Therefore, the development of methods to manufacture novel BLMs, particularly those unavailable or extremely difficult to prepare by chemical synthesis, remains an important research goal.

BLMs exhibit strong antitumor activity and are currently used clinically in combination with a number of other agents for the treatment of several types of tumors, notably squamous cell carcinomas and malignant lymphomas.[13,14,18] The commercial product, Blenoxane, contains **2** and **3** as the principal constituents. Unique to most anticancer drugs, BLMs do not cause myelosuppression, promoting its wide application in combination chemotherapy. Early development of drug resistance and cumulative pulmonary toxicity are the major limitations of BLMs in chemotherapy.[18]

2.2. Clinical Resistance

Thus far there is no proof of a mechanism that explains the development of BLM resistance in some tumor cells, although alteration of drug uptake and efflux, enhanced repair of BLM-induced DNA lesions, and increased inactivation of BLM might be possible mechanisms.[19−24] Several pathogenic microorganisms have been found to exhibit BLM resistance, seemingly caused by the existence of a BLM-binding protein in the respective organisms.[25−28]

Jon Thorson received his B.A. degree in Chemistry (1986) from Augsburg College and his Ph.D. degree in Organic Chemistry (1993) from the University of Minnesota with Professor Hung-wen (Ben) Liu. He held a postdoctoral appointment as a Merck Postdoctoral Fellow of the Helen Hay Whitney Foundation (1993–1996) at the University of California, Berkeley, with Professor Peter Schultz. From 1996 to 2001 Jon held appointments as an assistant member of the Memorial Sloan-Kettering Cancer Center and assistant professor of Sloan-Kettering Division, Joan and Sanford I. Weill Graduate School of Medical Sciences, Cornell University, during which he was named a Rita Allen Foundation Scholar (1998–2002) and Alfred P. Sloan Fellow (2000–2002). Professor Thorson joined the School of Pharmacy in the summer of 2001, and since moving to UW he has been awarded the American Society of Pharmacognosy Matt Suffness Award (2004) and selected as a H. I. Romnes Fellow (2004). His research interests include understanding and exploiting biosynthetic pathways in various microorganisms, microbial pathway genomics, mechanistic enzymology, mechanisms of resistance to highly reactive metabolites, enzyme engineering and evolution to generate novel catalysts, and development of chemoenzymatic and chemoselective ligation strategies for natural product glycorandomization.

Ben Shen received his B.S. degree from Hangzhou University (1982), M.S. degree from the Chinese Academy of Sciences (1984), and Ph.D. degree from Oregon State University (1991, with Professor Steven J. Gould) and carried out postdoctoral research in Professor C. Richard Hutchinson's laboratory at the University of Wisconsin—Madison (1991–1995). He started his independent career at the Department of Chemistry, University of California, Davis, in 1995 and moved to the University of Wisconsin—Madison in 2001, where he currently holds the Charles M. Johnson Chair in Pharmacy and Professor of Pharmaceutical Sciences in the School of Pharmacy and Professor of Chemistry in the Department of Chemistry. He was a Searle Scholar (1997) and recipient of a CAREER award (1998–2003) from the National Science Foundation, the Matt Suffness award (2000) and Jack L. Beal Award (2001) from the American Society of Pharmacognosy, and an Independent Scientist Award (2001–2006) from the National Institutes of health. His current research interests are the chemistry, biochemistry, and genetics of secondary metabolite biosynthesis in *Streptomyces* and drug discovery and development by combinatorial biosynthesis methods. Natural products currently under investigation include aromatic polyketides, modular polyketides, the enediynes, nonribosomal peptides, and hybrid peptide–polyketides.

2.2.1. Bleomycin Hydrolase

BLM can be metabolically inactivated in normal and tumor tissues by an enzyme called BLM hydrolase as demonstrated by several earlier studies. BLM hydrolase is a thiol protease that hydrolyzes the C-terminus of BLM to generate the inactive deamido metabolite.[19,22,29] As evidence that this mechanism may contribute to clinical BLM resistance,[30−32] BLM-resistant mammalian cells that were exposed to E64, a specific thiol protease inhibitor that blocks BLM hydrolase activity, became more sensitive to BLM.[33] These experiments led to identification of the corresponding genes that encode BLM hydrolase from yeast and mammalian cells.[34−37]

X-ray crystallographic studies of yeast[38] and human[37] BLM hydrolase revealed that both enzymes share the same hexameric ring barrel structure with the active sites embedded in a central cavity. The central channel, which displays a very prominent net positive charge in the yeast homologue and a slightly negative net charge in the human enzyme, may explain the differences in DNA binding among the human and yeast BLM hydrolases.[37] The three-dimensional structures of these proteins have inspired mechanistic speculations regarding BLM hydrolysis. Notably, the primary amino group of the metal-binding β-aminoalanine moiety of BLM has been proposed to serve as the "amino terminus" anchoring to the C-terminal carboxylate of hydrolase with the bulk of the BLM molecule protruding into the large cavity in the center of the protein hexamer.[39] In this orientation the first BLM peptide bond is cleaved via an aminopeptidase reaction. Such mechanistic insights may ultimately be useful in designing novel BLM analogues that are resistant to BLM hydrolase.

Expression of the yeast BLM hydrolase gene *blh1* in mammalian cells conferred a nearly 8-fold increase in resistance to BLM, which could be antagonized by the E64 inhibitor.[40] This suggested that inactivation of *blh1* from yeast should result in a BLM-hypersensitive phenotype. However, independent studies revealed conflicting data regarding the role of Blh1 in the detoxification of BLM.[35,36] While two studies suggested Δ*blh1* mutants were mildly sensitive to BLM, two alternative studies indicated that these mutants lacked BLM sensitivity.[35,36,41,42] Moreover, overexpression of *blh1* in either parental or BLM-sensitive yeast cells confers no additional resistance to BLM,[42,43] while the overproduced Blh1 clearly inactivates BLM in vitro.[42] Thus, the role of BLM hydrolase in producing tumor resistance remains controversial.

Furthermore, Blh1 binds specifically to the Gal4 transcription factor DNA-binding site and acts as a repressor to negatively control the galactose metabolism pathway.[38,44,45] Therefore, Blh1 could also play a more general role in the cells to degrade proteins or perhaps to regulate gene expression by degrading certain transcription factors.[45−47] If this is the case, the BLM resistance observed by overexpression of yeast *blh1* in mammalian cells may be explained, for

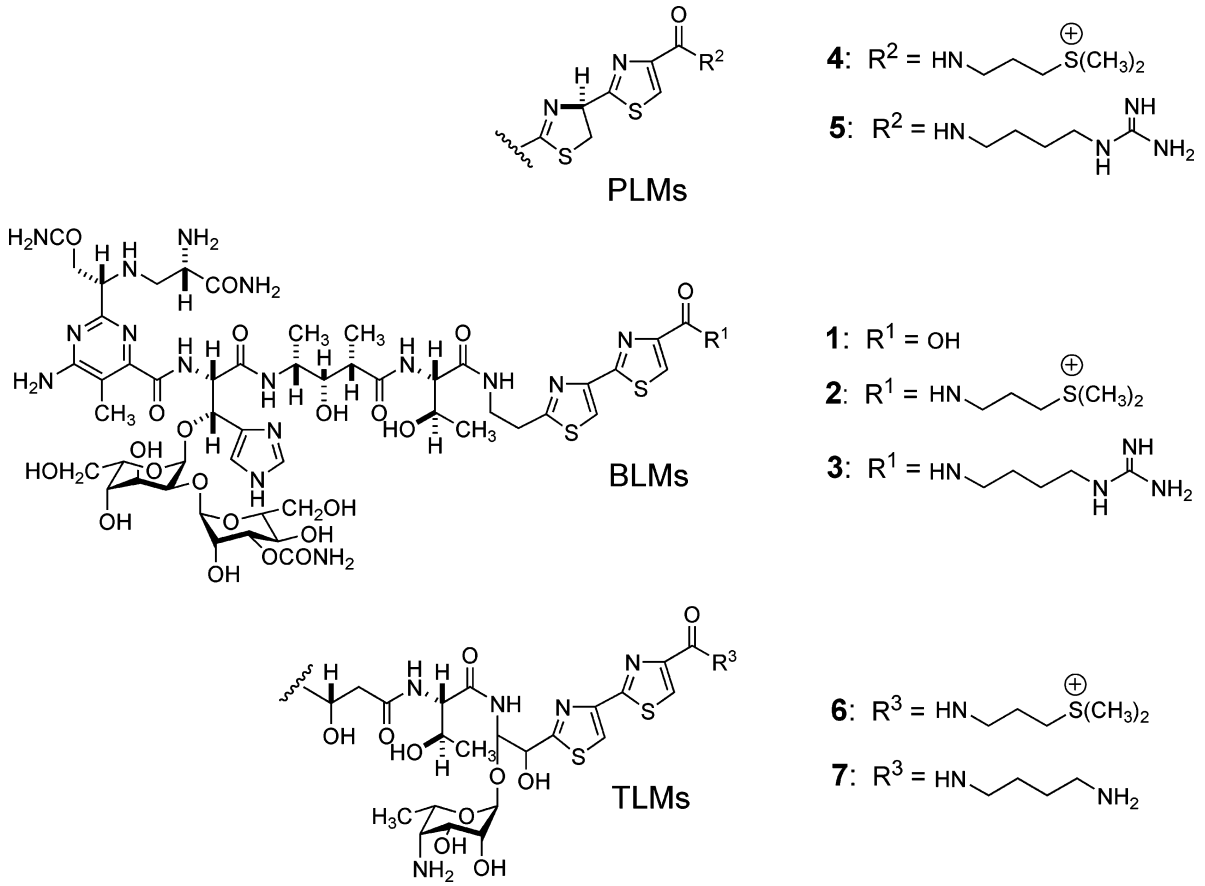


Figure 1. Structures of bleomycinic acid (**1**), bleomycin A2 (**2**), bleomycin B2 (**3**), phleomycin 12 (**4**), phleomycin D1 (**5**), and tallysomycin S_2B (**6**), and tallysomycin $S_{10}B$ (**7**).

example, by degrading pro-apoptotic factors, thus preventing cell death.

2.2.2. Enhanced DNA Repair

To overcome BLM-induced genotoxicity, DNA repair may be the most important mechanism used by cells. Thus, organisms exposed to BLM must recruit a variety of enzymes and/or proteins to repair the diverse types of BLM-induced DNA lesions. Apn1 was the first yeast enzyme discovered to process BLM-induced DNA lesions in vitro.[48,49] Apn1 is equipped with an apurinic/apyrimidinic (AP) endonuclease activity that cleaves the DNA backbone at AP sites as well as a 3′-diesterase activity that removes 3′-blocking groups (such as 3′-phosphoglycolate) at strand breaks. These enzymatic activities regenerate 3′-hydroxyl groups that subsequently allow DNA repair synthesis by DNA polymerase and ligase.[50−52] Two additional yeast 3′-diesterases, Apn2 and Tpp1, also repair BLM-induced DNA lesions. Notably, only the inactivation of all three 3′-diesterase genes, *apn1*, *apn2,* and *tpp1*, resulted in remarkable BLM sensitivity.[53,54] In human cells only the Tpp1 and Apn2 homologues , hPNKP and Hap1, respectively, have been identified to date.[53,54] There is some evidence that overproduction of Hap1 in mammalian cells can lead to enhanced resistance to BLM,[55] although the contribution of hPNKP and Hap1 to the repair of BLM-induced DNA lesions in human cells has not been investigated.

Rad52 and Rad6 proteins, which are part of the recombination and postreplication DNA repair pathways in yeast, respectively, are also involved in the repair of BLM-induced DNA lesions. These findings suggest that the repair of such lesions may not be restricted to enzymes with the ability to cleave AP sites or remove 3′-blocking groups.[56,57] The Rad52 and Rad6 pathways also repair a wide spectrum of other DNA lesions, including those generated by the alkylating agents, such as methyl methane sulfonate that produces AP sites and 4-nitroquinoline-1-oxide that forms bulky DNA adducts, and γ-rays.[58−62] While the exact contributions of these two proteins to DNA repair of BLM-induced lesions has not been established, some evidence suggests that their contribution depends on the extent of damage to the DNA.[57]

In Yeast expression of the DNA repair proteins Apn1 and Rad6 resulted in restoration of wild-type BLM resistance in the respective deletion mutants but did not exceed the resistance level of the parental cells.[49,58] However, in mammalian cells one study revealed the overproduction of the DNA repair enzyme Hap1 to enhance BLM resistance in normal or tumor cells.[55] This latter finding is in agreement with one of the earlier predictions that tumor resistance to BLM may be attributed to elevated DNA repair activities.[23] Therefore, attempts to promote the antitumor potential of BLM must take into consideration the importance of locally diminishing the DNA repair capacity within the respective tumor cells.

2.2.3. Bleomycin Binding Protein

Although BLM has never been used as an antibacterial agent, many clinically isolated methicillin-

resistant *Staphylococcus aureus* (MRSA) strains were found to be resistant to this drug at high levels.[28] The respective resistance gene *blmS* from one of these strains was investigated in detail. Sequence analysis revealed that it was identical with a gene located on the staphylococcal plasmid pUB110,[25] the gene product of which (BlmS) was determined to be a BLM-binding protein.[28] Another gene, *ble*, located on the transposon Tn5 (originally isolated from *Klebsiella pneumoniae*),[26,27] confers BLM resistance upon *Escherichia coli* with the gene product (BlmT) found to also be a BLM-binding protein.[62] While it could be argued that pathogenic bacteria like *K. pneumoniae* and *S. aureus* recruited their BLM-resistance genes from BLM-producing organisms, BlmS and BlmT share only ~20% identity/~40% similarity between each other and to the BLM-binding proteins from BLM producers. In contrast, the latter are highly homologous with ~60%identity/~70% similarity among each other (see section 2.3). Cumulatively, the most common bacterial resistance mechanism against BLM and its analogues appears to be development and utilization of binding proteins via drug sequestering.

2.2.4. Other Mechanisms

Proper cell wall maintenance appears to play an important role in protecting yeast against the lethal effects of BLM. Certain mutants that were sensitive to BLM were also identified to be defective in genes encoding proteins that maintain proper cell wall structure such as Fks1, a subunit of β-1,3-glucan synthetase.[63] Defects in the cell wall integrity signaling pathway are therefore also able to cause sensitivity to BLM, likely due to improper cell wall structure and enhanced permeability. Slg1, a plasma membrane sensor, detects cell wall perturbations and signals activation of protein kinase C (Pkc1), and varying sensitivities to BLM correlate to mutants lacking either Slg1 or Pkc1.[63]

The Slg1 protein has been shown to be required to attenuate the toxicity of BLM to yeast cells.[63] Although mammalian cells do not have a cell wall, their extracellular matrix is related to the yeast cell wall and contains a class of protein receptors known as integrins.[64,65] The structural organization of integrins is similar to yeast Slg1 protein.[65,66] It is therefore conceivable that a member of the integrin family could function to sense BLM, in a manner comparable to yeast Slg1 protein, and activate a signal transduction pathway leading to a defense mechanism against BLM.

Furthermore, a transcriptional activator, Imp2, was found to be involved in detoxification of BLM. Imp2 is a small protein that can activate transcription of a reporter gene by virtue of an acidic domain,[43] and at least two genes, *malT* and *malS*, have been identified to be positively regulated by Imp2.[67] Deletion of *imp2* resulted in mutants that displayed hypersensitivity to BLM.[43] Since Imp2 is a transcriptional activator, this protein may positively regulate the expression of at least one gene encoding a protein that repairs BLM-induced DNA lesions. The exact mechanism of gene activation by Imp2, however, remains to be elucidated.[24]

Interestingly, no drug transporters belonging to the ATP-binding cassette (ABC) and major facilitator superfamilies, such as Snq2, Yor1, Atr1, and Flr1, have been found to be involved in BLM resistance, as deficient mutants are not sensitive to the drug.[68−74]

2.3. Resistance by the Producing Organisms

The antibiotic-producing microorganisms must protect themselves from the lethal effects of their own products, and multiple mechanisms of drug resistance are common for many antibiotic-producing organisms. In most cases antibiotic production genes have been found to be clustered in one region of the bacterial chromosome, consisting of structural, resistance, and regulatory genes. Two resistance genes, *blmA* and *blmB*, have been characterized whose deduced products confer BLM resistance to the producing organism by drug sequestering (BlmA) or modification (BlmB), respectively.[75−80]

2.3.1. Bleomycin N-Acetyltranferase (BlmB)

The *blmB* gene from *Streptomyces verticillus* encodes an *N*-acetyltransferase that acetylates the α-amine of the BLM β-aminoalanine moiety in the presence of CoA. The latter moiety is critical for metal binding, and the acetylated BLM is no longer able to chelate metal and, thus, lacks activity.[75,79] BlmB has been overproduced in *E. coli,* and the recombinant protein has been purified and biochemically characterized.[80] Surprisingly, neither the carboxylic acid congener of BLM such as **1** nor PLMs such as **4** could serve as substrates for the BlmB ***N***-acetyltransferase.[75,80] On the basis of this remarkable substrate specificity it would now be of interest to determine the three-dimensional structure of BlmB and thereby shed light on the molecular interactions between BLM and the BlmB ***N***-acetyltransferase.

Within the TLM biosynthetic gene cluster from *Streptoalloteichus hindustanus* a *blmB* homolog, *tlmB,* was identified, the deduced product of which showed 57% identity/64% similarity to BlmB (George, N. P.; Wendt-Pienkowski, E.; Shen, B. Unpublished data). On the basis of these results TlmB is presumed to be the *N*-acetyltransferase responsible for inactivation of PLMs in *S. hindustanus*. In contrast, no *blmB* homologue could be identified within the sequenced PLM biosynthetic gene cluster from *Streptomyces flavoviridis* (Oh, T.-J.; George, N. P.; Wendt-Pienkowski, E.; Yi, F.; Shen, B. Unpublished data). From these findings two important questions arise: (i) could the putative PLM *N*-acetyltransferase reside outside the sequenced cluster or does it not exist within the PLM producer and (ii) what is the relevance of the *N*-acetyltransferase for the BLM- and TLM-producing organisms in order to maintain resistance against their own products while the PLM producer does not seem to depend on this self-resistance mechanism? Both questions remain unresolved.

2.3.2. Bleomycin Binding Protein (BlmA)

In addition to the aforementioned BlmB *N*-acetyltransferase, *S. verticillus* has the *blm*A gene encoding

for a binding protein (BlmA) that displays a strong affinity for BLM.[28,76] The expression of both *blmA* and *blmB* has been shown to increase simultaneously with BLM production in the late exponential growth phase of *S. verticillus*.[80] This finding was not surprising since both gene products are likely to be responsible for self-resistance of the producer strain and therefore need to be available in sufficient amounts as BLM production increases. BlmA, an acidic protein consisting of 122 amino acids with a calculated molecular size of 13.2 kD, has been extensively characterized biochemically.[28] Determination of the X-ray crystal structure revealed that BlmA forms a dimer through N-terminal arm exchange.[78] The resulting concavity and groove may contribute to the binding of two BLM molecules, and the formation of a dimer may be necessary to retain an affinity for BLM. The recent X-ray crystal structure of the BlmA−BLM complex confirmed that two BLM molecules are indeed bound by the BlmA dimer.[81] Additionally, these studies revealed the binding of the first BLM molecule to support cooperative binding of the second BLM.[81] The interaction of BlmA and BLM is assumed to result from an electrostatic interaction between the basic antibiotic and acidic protein, the affinity of which is enhanced upon Fe^{2+}-chelation. Moreover, the N-terminal Pro-9 of BlmA may play a role as a hinge to support the dimer structure. Replacement of Pro-9 in BlmA by Leu abolished the binding affinity for BLM due to disruption of the quaternary structure.[77]

A BLM-binding protein was also isolated from *S. hindustanus*, the producer of TLMs such as **6** and **7**. This protein was named Sh Ble and is able to form 1:1 protein−drug complex with high affinity to BLM.[82] We recently completed the cloning and sequencing of the TLM biosynthetic gene cluster from *S. hindustanus* and indeed localized the gene, *tlmA*, within the sequenced TLM gene cluster that encodes the Sh Ble protein (George, N. P.; Wendt-Pienkowski, E.; Shen, B. Unpublished data). Finally, the PLM biosynthetic gene cluster from *S. flavoviridis* ATCC21892 has also been sequenced recently (Oh, T.-J.; George, N. P.; Wendt-Pienkowski, E.; Yi, F.; Shen, B. Unpublished data). The sequenced PLM cluster also contains a gene, *plmA*, whose deduced gene product is highly homologous to BlmA and TlmA, supporting its role as PLM-binding protein. The three binding proteins, BlmA, PlmA, and TlmA, show high sequence homology (53−61% identities/63−73% similarities). Identification of these binding proteins in all three producers implicates drug sequestering as a main mechanism of self-resistance in these organisms.

Streptomyces lavendulae produces mitomycin (MTM) C (**19**), a potent anticancer agent, and a resistance protein, Mrd, was identified to act as a MTM C binding protein[83] (see section 4.3). Mrd belongs to a larger family of proteins containing tandem $\beta\alpha\beta\beta\beta$ motifs as representative structural elements. This family also includes the BLM-resistance proteins of *S. hindustanus* (Sh Ble), *S. verticillus* (BlmA), and *E. coli* (BlmT on transposon Tn5). Although the BLM- and Mrd-binding proteins show low sequence similarity, the X-ray crystal structure of Mrd is very similar to that of the BLM-binding proteins.[84] Both structures share overall tertiary and quaternary structural features and display a pair of symmetric cavities that serve as binding sites. However, BLM is a far larger molecule than MTM C, and these compounds are chemically distinct. From a crystallographic study performed with the BlmT−BLM complex[85] it could be shown that the bithiazole moiety of BLM is sequestered between two tryptophans of the binding protein, a feature that has also been observed for Mrd−MTM C complex. Indeed, Mrd was recently confirmed to bind BLM and confer BLM resistance.[86] Thus, even though the nature of the ligand is very different, the mode of drug binding by Mrd and the BLM-binding proteins is very similar. Taking these findings into consideration, it will be interesting to explore whether the converse is true and, more importantly, whether this drug-binding motif serves as a general cross-resistance mechanism for drug sequestering.

Since BLM and its homologues are excellent DNA cleavage agents, the *Sh ble* gene also serves as an invaluable resistance marker in commercial cloning plasmids, particularly for prokaryotic−eukaryotic shuttle plasmids. Although no naturally occurring BLM-binding protein has been identified in eukaryotic cells so far, these proteins should also be considered as a possible emerging mechanism of resistance in eukaryotes in the future.

2.3.3. Transport Proteins

In the BLM-producer *S. verticillus* Blm−Orf7 has been proposed to be a member of the ABC-transporter family of proteins.[76] The latter proteins confer resistance by transporting the drug out of the cells,[87] although this function has not been confirmed for Blm−Orf7.[76] The gene product of Blm−Orf29 is closely related to a family of transmembrane transporters and could also be involved in BLM resistance by drug efflux.[88] Further investigation of both transporters regarding their contribution to self-resistance of the producer strain would be informative, although BLM is not a substrate for the corresponding P-glycoprotein transporters (Pgp) involved in multidrug resistance (MDR) in yeast or mammals.[89]

3. Enediynes—Nine-Membered Enediyne Core Subfamily

The enediynes represent a steadily growing family of natural products with unprecedented molecular architecture. They have garnered much interest because of their unique structure and mode of action that confer clinically desirable attributes such as antibiotic and antitumor activity.[90,91] The enediynes are structurally characterized by an unsaturated core with two acetylenic groups conjugated to a double bond or incipient double bond and have been categorized into two subfamilies: 9-membered ring chromophore cores or 10-membered rings (see section 4). This portion of the review focuses on recent advances in our understanding of the mechanism of the nine-membered ring enediynes and the potential applica-

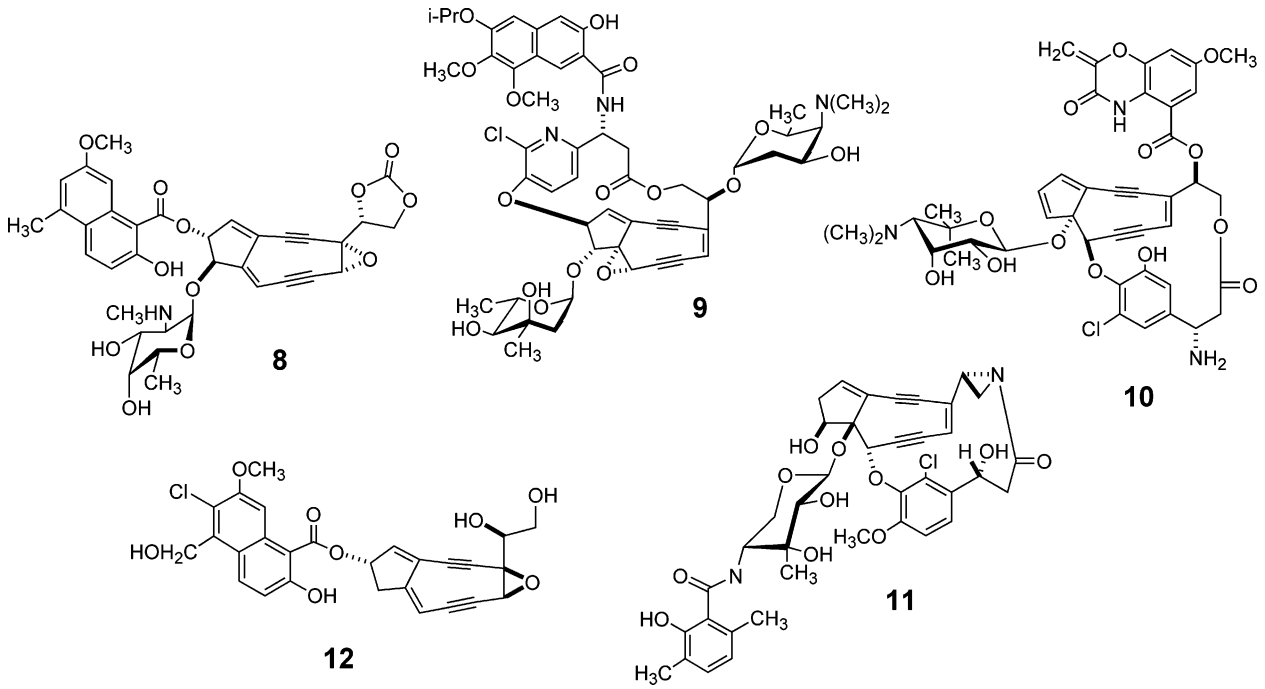


Figure 2. Nine-membered enediyne chromophores whose structures have been elucidated: neocarzinostatin (**8**), kedarcidin (**9**), C-1027 (**10**), maduropeptin (**11**), and N1999A2 (**12**).

tions of these natural products as therapeutic agents. The discussion includes the general characteristics of the family followed by specific information regarding individual enediynes with an emphasis on C-1027 and neocarzinostatin (NCS) as models for the nine-membered enediynes.

3.1. Discovery and Biological Activities

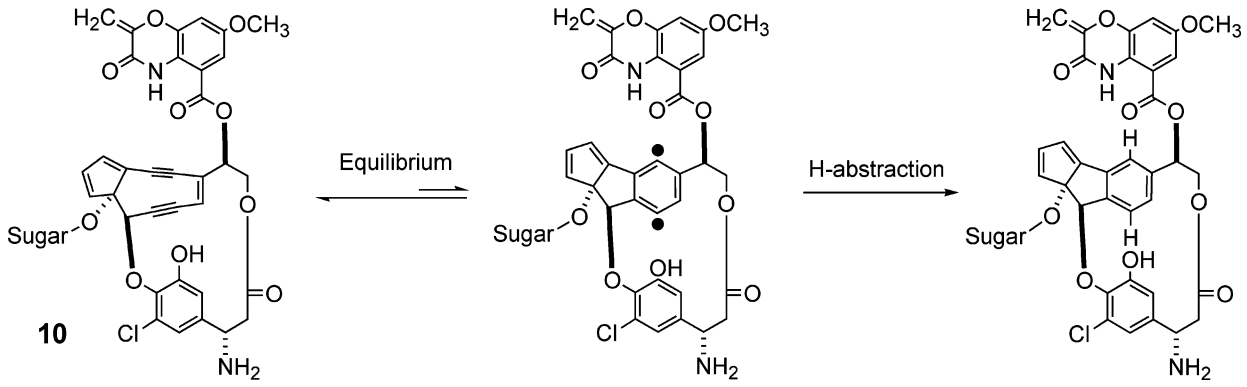
With the elucidation of the NCS chromophore (**8**) in 1985[92] the nine-membered chromoprotein family of enediyne has steadily grown to currently consist of nine natural products: NCS from *Streptomyces carzinostaticus*,[93−95] kedarcidin (**9**) from *Actinomycete* L585-6,[96,97] C-1027 (**10**) from *Streptomyces globisporus*,[98] maduropeptin (**11**) from *Actinomadura madurea*,[99] N1999A2 (**12**) from *Streptomyces* sp. AJ9493,[100] actinoxanthin from *Actinomyces globisporus*,[101,102] largomycin from *Streptomyces pluricolorescens*,[103] auromomycin from *Streptomyces macromomyceticus*,[104−106] and sporamycin from *Streptosporangium pseudovulgare*.[107,108] Although all of the known nine-membered enediynes contain a common bicylo[7.3.0]dodecdadiynene chromophore, only five of the complete structures, **8**−**12**, have been established (Figure 2). Recently, numerous cryptic gene clusters encoding enediyne biosynthesis in a variety of actinomycetes have been unveiled,[109] suggesting that these organisms have the potential to produce uncharacterized enediynes. The latter finding may significantly increase the pool of nine-membered enediynes in the years to come.

As a group the nine-membered enediynes are the most potent family of anticancer agents discovered, some members of which are 5−8000 times more potent than adriamycin, an antitumor antibiotic that has been very effective in clinical use.[110] For example, C-1027 shows extremely potent cytotoxicity against KB carcinoma cells (IC_{50} 0.1 ng/mL) in vitro[111] and powerful antitumor activity toward tumor-bearing mice in vivo.[110] However, the enediynes have shown delayed toxicity, limiting their use in clinical applications.[110,112] To overcome this shortcoming a few modified enediynes have been prepared for clinical purposes and have shown great promise. Conjugation of NCS with poly(styrene-*co*-maleic acid) (SMA) or its various alkyl esters has dramatically improved the uptake and overall toxicological profile, and as a consequence, the polymer conjugated derivative of NCS, SMANCS, has been used to treat hepatoma in Japan since 1994.[113] SMANCS in conjunction with Lipiodol, a lipid contrast agent, has also shown great promise for treatment of tumors in the lung, stomach, pancreas, and gall bladder as well as lymphoma and melanoma.[113]

An equally exciting development has been the creation of monoclonal antibody (mAB)-enediyne conjugates. The best example is the 10-membered enediyne calicheamicin (CAL), which has been prepared as a mAB−CAL conjugate and is currently approved by the FDA under the trade name of Mylotarg to treat acute myeloid leukemia (AML) (see section 4).[114] Several mAB−C-1027 conjugates have also been prepared and are characterized by increased tumor specificity and strong inhibition on the growth of tumor xenografts.[115−117] These conjugates are currently being evaluated for clinical significance as anticancer drugs[118] and highlight the potential for enediynes in therapeutic treatments.

The mode of action of nine-membered enediynes, which is generally accepted for all enediynes, is the ability to produce single-stranded or double-stranded DNA lesions (and, in some cases, RNA lesions)[119] by a common mechanism shown for **10** in Scheme 1.[120,121] The chromophore binds the minor groove of DNA and undergoes an electronic rearrangement to form a benzenoid diradical species (via a Bergman

Scheme 1



or Myers rearrangement), which in turn abstracts hydrogen atoms from the deoxyribose of DNA. Molecular oxygen can react with the newly formed carbon-centered radicals, leading to site-specific DNA breaks. The DNA damage in turn causes a significant decrease in DNA replication competency and ultimately leads to cell death.

Similar to 10-membered enediynes, the 9-membered ring enediyne chromophores require activation for biological activity, although they are remarkably less stable overall. The most biologically relevant mode of diradical initiation is thiol activation to trigger radical formation, although other activators such as acidic or basic pH and light have been shown to initiate the electronic rearrangement.[91] It is notable that **10** is the most labile enediyne studied among the family as the free chromophore. Unlike the others, DNA cleavage by C-1027 proceeds even in the absence of thiol groups or nucleophiles.[122,123] It has also been reported that maduropeptin, like C-1027, is also capable of diradical formation without activation.[124]

3.2. Resistance by the Producing Organisms

Unlike the 10-membered enediynes, the 9-membered enediyne cores are relatively unstable, and therefore, it is of utmost importance for the producing organism to control the production, transportation, and export of the enediyne product. Similar to several *Streptomyces* antibiotic-producing organisms, multiple mechanisms of self-resistance have been evolved including drug sequestering, efflux transport, and DNA repair.

3.2.1. Apo-Protein

The primary mechanism utilized by nine-membered enediyne-producing organisms is drug sequestering by the production of an apo-protein that tightly, but noncovalently, binds and stabilizes the chromophore.[90,91] The observations that apo-proteins for C-1027 and macromomycin are constitutively produced and independent of the chromophore production suggest that the apo-protein function is necessary for self-resistance.[125,126] Furthermore, it has been proposed that **10** is in equilibrium with its *p*-benzyne form and is stabilized kinetically by the CagA apo-protein.[127] This hypothesis was tested and supported by electron paramagnetic resonance analyses of the C-1027 chromoprotein complex and a spin-trapping study of DNA cleavage induced by C-1027.[128,129]

The apo-protein sequences for **8** (NcsA),[130,131] **9** (Ked),[124,132] **10** (CagA),[133] actinoxanthin (AxnA),[102] and auromomycin (McmA)[134] have been reported and show ~40% identity among them. The apo-protein sequence for **11** is presumably known, but the authors reported size ambiguities ranging from 13 kD by mass spectroscopy to 29 kD or 31 kD by SDS−PAGE.[124,135] We recently sequenced the maduropeptin biosynthetic gene cluster from *A. madurea*. While sequence analysis failed to identify an apo-protein candidate that is homologous to known apo-proteins, a single ORF, encoding a hypothetical protein with unknown function that is small (16 kD) and acidic (pI 3.5), was identified. The latter could potentially function as the chromophore-binding protein, although our current study falls short of excluding the possibility that the apo-protein may reside outside of the sequenced cluster (Liu, W.; Wendt-Pienkowski, E.; Oh, T.-J.; Van Lanen, S. G.; Shen, B. Unpublished data).

The structures for both apo-proteins (CagA and NcsA) and chromoprotein complexes (C-1027 and NCS) have been established by NMR spectroscopy.[136−140] Preliminary X-ray diffraction data for actinoxanthin, macromycin, and C-1027 apo-proteins or chromoproteins have also been reported,[141−143] although detailed descriptions of the crystal structures are lacking. The apo-proteins share several characteristics as expected from high sequence homology, including being small, acidic peptides that are rich in β-sheet secondary structure.

The apo-protein for C-1027, CagA, was revealed to consist of three antiparallel β-sheets: a four-stranded β-sheet, a three-stranded β-sheet, and a two-stranded β-sheet. A hydrophobic pocket is formed by the four-stranded β-sheet and three loops. Modeling of the C-1027 chromoprotein complex demonstrated that the chromophore is packaged compactly by folding its benzoxazolinate and aminosugar moieties in a manner to interact with several hydrophobic side chains of CagA, which include Tyr-32, Ala-34, Pro-47, Ala-50, and Pro-76. Electrostatic interactions are also plausible within the holo-protein, which include a salt bridge and two hydrogen bonds.

In the case of the aromatized C-1027 chromophore−CagA complex, the benzodihydropentalene core is located in the center of the pocket and its molecular

plane is nearly perpendicular to the bottom of the pocket. The benzene ring of the core, where the C3 and C6 of the carbon-centered diradicals are formed, faces toward the bottom of the pocket and is masked by the β-tyrosine, benzoxazolinate, and aminosugar moieties. A mechanism for diradical stabilization was proposed in which the H-α of Gly-96 serves as a candidate for hydrogen abstraction by the C6 radical while H-β1 and H-β2 of Pro-76 present to the C3 radical.[128,129] The former interaction has recently been tested by observing kinetic isotope effects which revealed that the [U-^{2}H]Gly-96 CagA exhibited a better chromophore-stabilizing ability.[144]

The structure for apo-protein of NCS, NcsA, has been solved by NMR spectroscopy.[136,137,145] Similar to CagA, NcsA is comprised primarily of β-sheets. In addition to structural analysis of NcsA, binding data was obtained using fluorescence and NMR spectroscopy. Binding of **8** to NcsA, analogous to **10** to CagA, was attributed mainly to hydrophobic interactions between the naphthoic acid moiety and several hydrophobic side chains of NcsA including Gly-35, Leu-45, Phe-78, Val-95, and Trp-39. NcsA binds the naphthoic acid moiety deep in the pocket, and the enediyne core is located above a Cys-47-Cys-37 disulfide bond and surrounded by the aromatic rings of Phe-52 and Phe-78, the former of which, in conjunction with Asp-33, Ser-98, and the protonated methylamino group, infringe upon nucleophilic attack and thereby inhibit diradical formation. The carbonate carbonyl and aminosugar group provide some binding energy but to a much lesser extent.

Although the primary function of the apo-protein is to sequester and stabilize the enediyne for proper delivery, the apo-proteins have been proposed to exhibit protease activity. As demonstrated for kedarcidin and NCS, Ked or NcsA isolated from the native producer had specific endopeptidase activity, preferentially cleaving histone H1 compared to other histones and various proteins.[124,135] This putative activity was very attractive as it enabled the specific delivery of the chromoprotein complex to the DNA target. However, the protease activity has recently been reexamined for NCS by production and isolation of a recombinant NcsA from *E. coli*, and results from two separate groups confirmed that protease activity can be separated from NcsA, suggesting this activity is due to minor contaminating proteases.[146,147] The recombinant NcsA maintained its structural integrity based on far-UV, CD, and NMR spectroscopy and functioned in binding **8,** although with a much greater K_d (>200-fold). It is unclear whether the differences in **8** binding were due to experimental variations or if the recombinant NcsA protein had lost some functionality during production in a heterologous host. In general, the native apo-proteins have >1000-fold lower specific activities and no primary sequence or tertiary structure similarities to known proteases,[135,148−151] supporting the conclusion that the protease activity is indeed an artifact of the purification and assay conditions. The possibility still remains that the apo-protein could require a cofactor for protease activity that is lost upon recombinant expression and purification (perhaps the chromophore itself) or could recruit a specific protease by noncovalent interactions. The availability of numerous genes and the strategies to heterologously express them for apo-protein production should facilitate further experiments to ascertain the nature of the protease activity, such as site-directed mutagenesis and chemical trapping of potential substrate−enzyme intermediates.

Regardless of the protease function, the binding of the apo-protein to the enediyne chromophore affords protection for the producer but also provides a blueprint as a natural drug delivery system. Having the wealth of structural data will now allow opportunities to genetically engineer and chemically manipulate the chromophore complex for specific targeting and optimized drug delivery during future clinical trials.

3.2.2. DNA Repair

A pivotal discovery in our understanding the resistance mechanisms for the enediyne family was the identification and cloning of the gene clusters for C-1027 and CAL.[152,153] Primary sequence analysis of the gene products revealed several possible candidates responsible for self-resistance in conjunction with drug binding by the apo-protein and the putative involvement of encoded DNA repair enzymes.

The upstream boundary of the C-1027 gene cluster contains an ORF, *sgcB2*, whose deduced product showed high sequence similarity to *E. coli* UvrA, a protein involved in excision repair,[154] and *Streptomyces peucetius* DrrC, a UvrA-like drug-resistance protein.[156,157] UvrA is part of a three-component protein assembly that recognizes, unwinds, and excises damaged DNA, and UvrA has been shown to strongly bind DNA and release DNA-bound anthramycin.[155] Similar to UvrA, DrrC was shown to have DNA-binding activity that was mediated by ATP and enhanced in the presence of the antibiotic daunorubicin.[156,157] Therefore, SgcB2, which has 27% identity/37% similar to DrrC, is speculated to bind to DNA regions as a general mechanism to inhibit C-1027 binding or could hinder the activation of the enediyne core while inside the producer.

3.2.3. Transport

Sequencing of the gene clusters for NCS and C-1027 and BLAST searches of the gene products revealed homologues to efflux pumps and other candidates for transport, one of which is conserved between the two clusters: SgcB for C-1027 and NcsA1 for NCS. These proteins are putative efflux transporters that have several homologues within *Streptomyces* including Pur8 in the puromycin gene cluster from *Streptomyces alboniger*.[158] Pur8, when expressed in *S. lividans*, induced specific antibiotic resistance and was implicated in the excretion of the last intermediate in the puromycin biosynthetic pathway, *N*-acetylpuromycin.[159] Sequence analysis revealed Pur8 to contain 14 transmembrane-spanning regions, and as a result, Pur8 is believed to be necessary for puromycin efflux energized by a proton-dependent electrochemical gradient. From the high sequence homology (SgcB/Pur8, 36% identity/56%

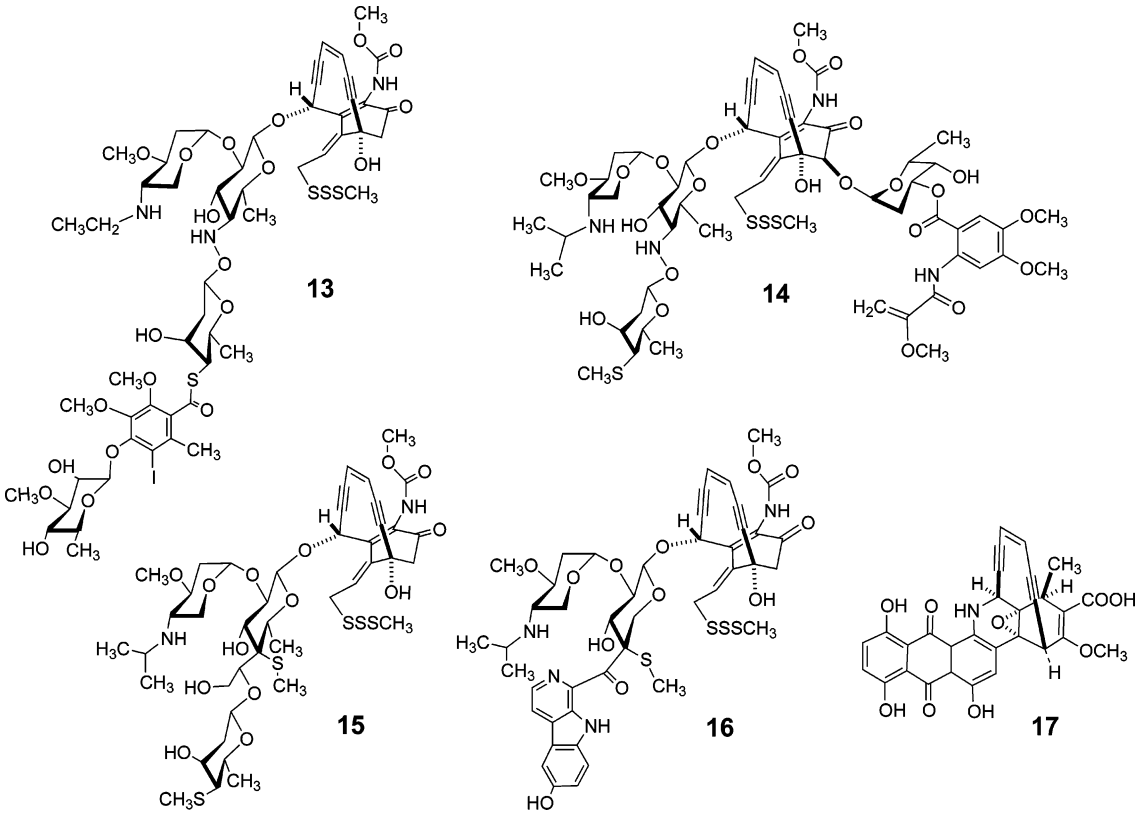


Figure 3. Structures of 10-membered enediynes: calicheamicin (**13**), esperamicin (**14**), namenamicin (**15**), shishijimicin (**16**), and dynemicin (**17**).

similarity and NcsA1/Pur8, 34% identity/53% similarity), it is reasonable to assume that SgcB and NcsA1 have similar activity and provide the means for enediyne efflux transport.

Interestingly, the C-1027 gene cluster also contains an unshared antibiotic transporter homolog, SgcB4, which consists of conserved domains from a family of predicted drug exporters of the resistance-nodulation-cell division permease superfamily,[160] and AcrB, a cation/multidrug efflux pump utilized as a defense mechanism. The latter family consists of proteins that have been biochemically confirmed to be involved in multidrug efflux with wide substrate specificity as demonstrated in *E. coli*,[161] the stress-induced efflux system of *E. coli*,[162] and the secretion of the siderophore pyoverdine in *Pseudomonas aeruginosa*.[163,164] SgcB4, therefore, represents an additional candidate for C-1027 efflux that is not shared in the nine-membered enediyne core subfamily and could be a general mechanism for C-1027 resistance.

4. Enediynes—Ten-Membered Enediyne Core Subfamily

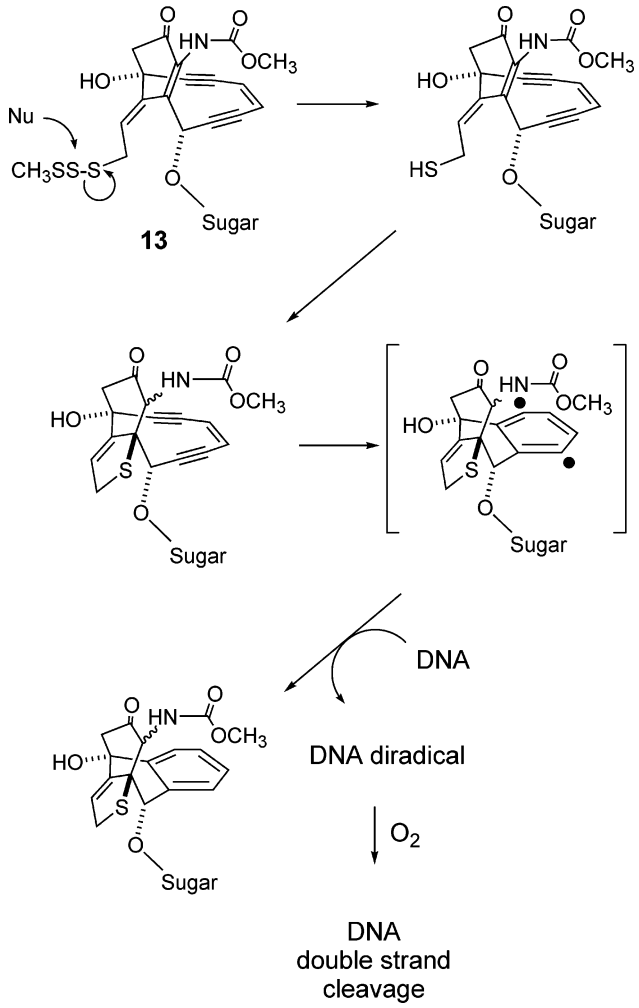
The second set of enediynes is very similar to those previously mentioned in that they also contain three critical functional domains—the enediyne moiety (or 'warhead'), a recognition unit which delivers the enediyne moiety to its DNA target, and a trigger device that initiates the generation of the reactive chemical species. The notable difference, however, is that the enediyne is found within a 10-membered ring system, presenting metabolites that do not require sequestration by an apo-protein. To date, this subfamily encompasses the natural products CAL (**13**) from *Micromonospora echinospora* ssp. calichensis,[165,166] esperamicin (**14**) from *Actinomadura verrucosospora*,[167] namenamicin (**15**) from *Polysyncraton lithostrotum*,[168] shishijimicin (**16**) from *Didemnum proliferum,*[169] and dynemicin (**17**) from *Micromonospora chersina* (Figure 3).[170,171] Like the other members of the enediyne antibiotic family, the 10-membered enediynes also function in vitro and in vivo as DNA-damaging agents.[172,173]

4.1. Discovery and Biological Activities

Discovery of the 10-membered enediynes began with **14** in 1985 and sparked a great interest in this new class of antitumor antibiotics.[167] In the following years the structures of **13**, **15,** and **17** were reported with the discovery of **16** in 2003 representing the most recent family member.[169] In a manner similar to that described for the 9-membered enediynes, the 10-membered enediynes bind DNA with high affinity, and some reported sequence specificity, culminating in sequence-selective oxidative strand cleavage. DNA cleavage results from quenching of the benzenoid diradical formed upon reductive activation via DNA backbone hydrogen abstraction (Scheme 2).[174−176]

The staggering cytotoxicity of the enediynes represents their greatest strength. However, the relatively unspecific mode of action poses the problem of general toxicity and subsequent systemic side effects. Therefore, current enediyne research is focused upon the development of enediyne analogues with enhanced selectivity toward cancerous cells. One promising approach to compensate for this limitation has

Scheme 2



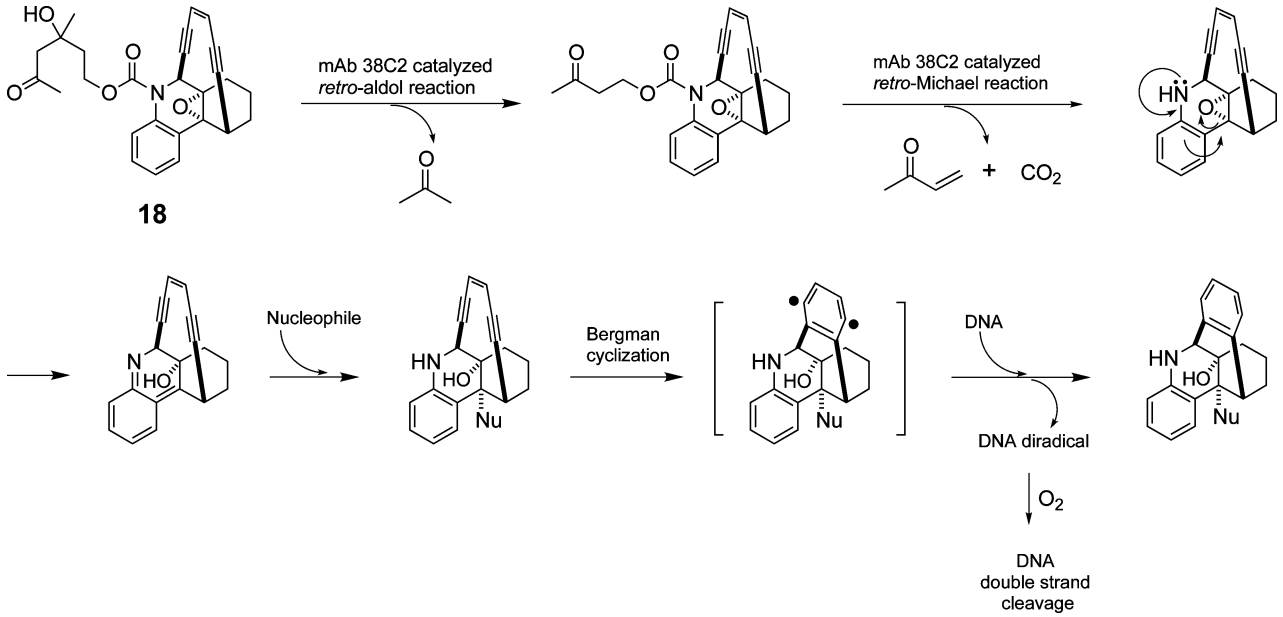
been to conjugate 10-membered enediynes to tumor-directed mAbs.[177] Such antibody-targeted chemotherapy is heavily dependent upon the specific delivery of the enediyne to tumor cells via the tumor-associated antigen-mAb recognition to provide a localized exposure to the cytotoxic agent.[178] The high toxicity of the 10-membered enediynes (they are capable of triggering cell cycle arrest and apoptosis in the picomolar range) favors this approach as only a small number of immunoconjugates bind to the cell surface and are internalized.[179] Two different mAB-directed strategies have proven successful in the application of 10-membered enediynes. An alternative strategy employs the specific tissue-localized enzymatic activation of enediynes. Both strategies ultimately limit overall general toxicity and are described in more detail below.[180,181]

As the pioneering example of localized delivery, the first mAB−CAL conjugate strategically focused upon the treatment of AML,[112,182] a disease for which **13** had already displayed notable antileukemic potency. To reduce general **13** cytotoxicity a semisynthetic derivative of the drug was covalently coupled to a humanized mAb (HuM195) specific for the antigen CD33.[183] The combination of mAb and immunoconjugate linker turned out to be the key to the success of this new approach. The antibody HuM195 binds the antigen CD33, a glycosylated transmembrane protein with an expression pattern that is basically confined to the hematopoietic system. In 90% of patients with AML this 67 kD glycoprotein is absent from healthy hematopoietic stem cells and nonhematopoietic tissues.[184] Furthermore, binding of CD33 results in endocytosis of the antibody−drug−complex, further enhancing specific drug delivery. The linker was designed as a metabolically stable but acid-labile hydrazone conjugate such that lysosomal acid hydrolysis of the mAb-CAL conjugate efficiently releases the 10-membered enediyne.[185] This anti-CD33 antibody-CAL conjugate was named gemtuzumab ozogamicin (CMA-676) and later given FDA approval under the name Mylotarg for the treatment of first-relapse AML in patients >60 years of age. Due to its reduced toxicity, antibody-targeted chemotherapy with Mylotarg is today a clinically validated therapeutic option for CD33-positive AML.[186]

Mylotarg represents the first and is presently the only approved antibody-targeted cytotoxic small molecule agent for clinical use in the United States. Several attempts have been made to expand this strategy. CD22 emerged in these studies as an attractive candidate for immunotoxin-based therapeutic strategies involving the treatment of B-lymphoid malignancies.[187] As a sialic-acid-binding lectin, CD22 combines two desirable qualities—its expression is restricted to B-lymphocytes[188,189] and CD22 is rapidly internalized when bound by ligand or antibody.[190,191] In an approach paralleling the treatment of AML with Mylotarg, a CD22-targeted immunoconjugate of CAL (CMC-544 or inotuzumab ozogamicin) bound CD22 with subnanomolar affinity and exhibited a potent cytotoxicity against $CD22^+$ B-lymphoma cells. These results bode well for the future use of CMC-544 in the treatment of patients with non-Hodgkin B-cell lymphoma.[192]

The versatility of CAL-immunoconjugates has recently been demonstrated by two more applications that have been advanced to the stage of clinical evaluation. The compound CMB-401 employs a mAb that is directed against polymorphic epithelial mucin (PEM), a glycoprotein that shows aberrant expression levels in malignancies of epithelial origin and is implicated in the increased metastatic potential of ovarian cancers. This new derivative of a CAL-immunoconjugate showed potent selective cytotoxicity toward PEM-positive cell lines in tissue culture. Therefore, CMB-401 is now being evaluated as a monotherapy for the treatment of epithelial ovarian carcinoma.[193] The oligosaccharide $Lewis^y$ was also considered as a promising antigen for antibody-targeted chemotherapy. Following the concept established for Mylotarg, CMC-544 and CMB-401, CAL has also been conjugated to the humanized IgG1 antibody Hu3S193 which recognizes the $Lewis^y$ antigen. This specific antigen is highly expressed on carcinomas of the colon, breast, lung, ovary, and prostate, while expression of $Lewis^y$ in normal tissues is restricted to the gastric mucosa, small intestine, and pancreas. This conjugate may therefore prove useful for selectively targeting tumors that express $Lewis^y$.[194]

Scheme 3



In a related strategy, a simplified analogue of another naturally occurring 10-membered enediyne, **17**, has been employed in mAb-directed enzyme prodrug therapy (ADEPT). This approach relies upon directing an enzyme specifically to tumor cells via a tumor-specific mAb. Subsequently, the enediyne prodrug is administered and locally activated at the tumor site by the tumor-associated mAb-enzyme conjugate, thereby greatly reducing toxicity to normal tissue.[195] Among the 10-membered enediynes **17** is unique in that it combines structures that are characteristic of both anthracycline and enediyne antibiotics.[180,196] As a DNA-damaging agent, **17** induces single- and double-strand breaks after reductive activation. Interaction with the DNA is established by intercalation of the anthraquinone into DNA presenting the enediyne in the minor groove.[197] Reductive activation of the anthraquinone, mediated by physiological reductants, initiates the cascade of events toward Bergman−cycloaromatization and ultimately DNA cleavage.[196,198,199]

The enediyne−ADEPT strategy employed a simplified analogue (**18**; Scheme 3) of **17** and the catalytic mAb 38C2. Antibody 38C2 is a monoclonal aldolase antibody with dual function, providing both tumor specificity through an integrin-targeting RGD peptidomimetic and catalytic activity through aldolase catalysis.[200] As illustrated in Scheme 3, **18** serves as a substrate for 38C2 and was specifically equipped with a consecutive aldol and oxa-Michael-dependent linker for enediyne activation.[201]

In this elegant design only the carbon−carbon bond-cleaving retro-aldol reaction catalyzed by 38C2 was able to reveal the hidden retro-Michael substrate. Once exposed the oxa-Michael motif underwent retro-aldol and β-elimination to result in the activated, cycloaromatized drug (Scheme 3).[195] While 38C2 was able to trigger prodrug activation by a reaction cascade and the free drug inhibited tumor cell growth in vitro,[201] the immunogenicity of 38C2 remains a major limitation for the transition of this strategy from preclinical to clinical evaluation. Yet, the demonstration of enediyne−ADEPT technology is highly versatile and opens new therapeutic perspectives.

4.2. Clinical Resistance

The demonstrated potency of enediynes coupled with their unique architecture and mechanism attracted great attention, which ultimately led to their expanding clinical utility. However, clinical enediyne use has also been accompanied by growing reports of clinical drug resistance in enediyne-treated patients.[202] Notably, the Mylotarg treatment of elderly relapsed and secondary AML patients revealed clinical resistance to correlate with both disease resurgence and the expression of functional MDR.[203] Although there has been great progress in the treatment of AML and most adult de novo AML patients achieve complete remission (CR) with conventional chemotherapies, the majority of responding patients relapse and ultimately die with treatment-refractory disease. In these cases, MDR was the leading cause of this therapeutic failure and accounts for a poor prognosis in AML.[204,205]

MDR characterizes a series of events by which leukemia cells become resistant to various chemotherapeutic drugs that are structurally and functionally unrelated. It can result from the overexpression of ATP-dependent efflux pumps that are members of the ABC transporter superfamily.[206] The Pgp is the most prominent member of this family and responsible for the efflux of several chemotherapeutic drugs currently employed for AML, including anthracyclines and etoposide. Pgp is expressed in 70% of older patients with de novo AML, whereas only 40% of younger subjects are typically affected. Pgp was also shown to have a high frequency of expression in patients with secondary and relapsed adult AML.[207,208] Since the drugs that are used for chemotherapy in AML patients are substrates of Pgp, it is assumed that treatment with various chemotherapy regimens

induces and upregulates Pgp expression. This overexpression of Pgp in leukemia cell lines was shown to result from drug-induced changes in mRNA stability and transcriptional activation.[206] Therefore, Pgp expression accounts for increased efflux of CAL and is linked to lower CR, higher rates of refractory disease, and shorter overall survival after treatment with these chemotherapeutics. Multidrug resistance and its impact on the treatment of AML with CAL−immunoconjugates has prompted the development of MDR modifiers that inhibit efflux of antileukemia agents and restore the effect of chemotherapeutic agents in resistant cell lines.[208,213,214] Cyclosporine and PSC 833 are Pgp antagonists that are used as chemosensitizing agents in AML treatment trials. These MDR modifiers are able to restore the cytocidal effect of Mylotarg in Pgp-expressing cell lines.

In the context of this review it is interesting to note that MTM C (**19**, vide infra) is able to suppress the activity of Pgp. The underlying mechanism by which this occurs, however, remains elusive.[209] Taken together, these observations indicate that avoiding the induction of Pgp expression is of paramount importance when it comes to choosing an appropriate and successful therapeutic regimen. It also points to reconsidering the role of mAB−CAL conjugates in the treatment of relapsed and refractory AML since the higher specificity and lower side effects cannot compensate for developed resistance mechanisms. Given the lower expected frequency of MDR expression in de novo AML, Mylotarg may show greater efficacy in this group of patients.

Recent observations suggest that non-Pgp transporters and mechanisms other than drug efflux may also contribute to clinical **13** resistance. These resistance mechanisms involve the multidrug-resistance-associated proteins (MRPs) that also belong to the ABC-transporter family and the major vault protein LRP, a ribonucleoprotein found to be overexpressed in many chemoresistant cancer cell lines and implicated in the sequestration of drugs.[206,210] Vaults are large-sized complexes that have an estimated molecular mass of 13 MDa. Their barrel-like structures indicate a function in intracellular drug transport, and they have been linked to drug resistance. However, they share no similarity with the apo-proteins of nine-membered enediynes, the MTM C-resistance protein Mrd (see section 5.3) or BlmA (see section 2.3).[206,210] Recently, the breast-cancer-resistant protein (BCRP), the equivalent of mitoxantrone-resistant protein or placental ABC transporter, was described in AML and shown to play an important role in the development of MDR.[211] This unique transporter belongs to the family of ABC transporters yet is evolutionarily distinct from Pgp or MRPs as it requires dimerization. The AML chemotherapeutics mitoxantrone, daunorubicin, and etoposide are all substrates for this transporter. Notably, BCRP mRNA levels in patients resistant to Mylotarg that did not achieve remission after the first chemotherapy were found to be 10-times higher as compared to patients who did achieve remission.[212]

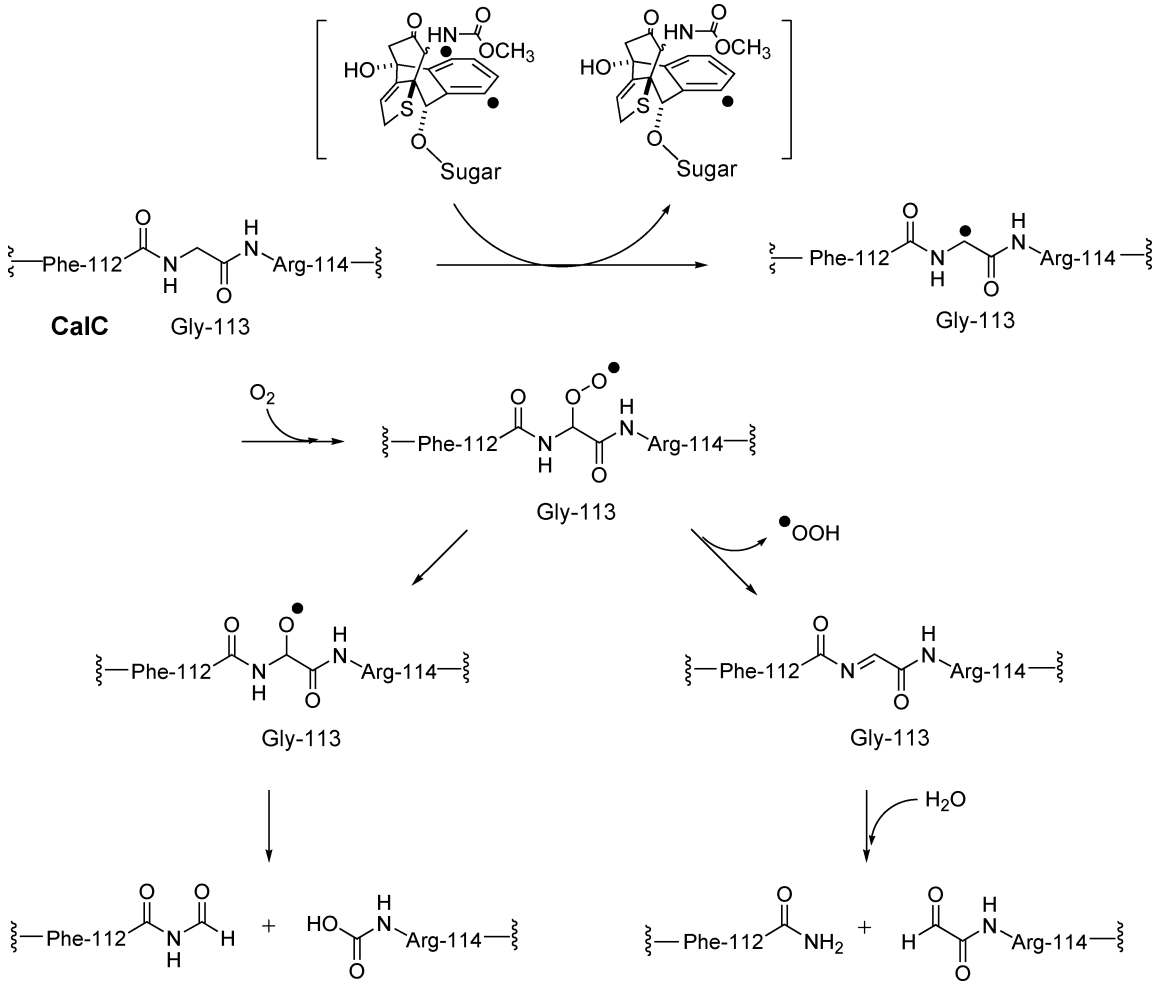
4.3. Resistance by the Producing Organisms

Similar to the nine-membered enediynes, the members of this group also require activation to become biologically active, yet unlike most nine-membered enediynes, the 10-membered analogues do not spontaneously cycloaromatize.[176] Thus, all reported 10-membered enediynes lack an apo-protein, and until recently, the predominant mechanism of self-resistance among 10-membered enediyne-producing organisms was lacking. This issue was recently addressed by using one of the best characterized 10-membered enediynes, **13**, in a screen as a resistance marker. From a genomic cosmid library of the CAL-producing *M. echinospora* expressed in *E. coli*, six colonies showed growth on CAL-impregnated media and eventually led to the discovery of a single gene (*calC*) that was able to confer resistance to **13**.[152,215] Expression of this gene in *E. coli* rendered the organism resistant to CAL at concentrations that were 3 orders of magnitude higher than the lethal dose for wild-type *E. coli*.[216] This finding demonstrated the protective potential of the CalC protein and prompted studies to explore the underlying mechanism. The early in vivo studies also revealed the CalC−CAL interaction to be specific since the *calC*-expressing *E. coli* failed to grow in the presence of either **14** or **17** yet could thrive in the presence of **15** and **16**.

The key in vitro evidence pointing to an unprecedented mechanism came from gel electrophoresis experiments that revealed cleavage of CalC by reductively activated **13** with proteolytic-like precision to give two specific peptide fragments. Further analyses suggested a CAL-catalyzed proteolysis through hydrogen abstraction from Gly-113 with direct α-hydrogen transfer from Gly-113 to the cycloaromatized **13** (CLMϵ) observed via isotopic labeling.[216] These key experiments further corroborated the hypothesis that CalC was acting as a quencher for the enediyne diradical in a mechanism parallel to hydrogen abstraction from the deoxyribose of DNA (Scheme 4). Amino acid point mutagenesis of CalC at position 113 reduced resistance, thereby providing additional support for the proposed CalC mechanism and the importance of Gly-113.[216]

The elucidation of the CalC-mediated resistance to **13** represents a truly unprecedented and unusually extravagant paradigm in antibiotic self-resistance. Although it seems counterintuitive that *M. echinospora* would destroy its own biosynthetic product while sacrificing the resistance protein, the catastrophic reactivity of the enediynes may dictate such an excessive response. CalC has, to date, no significant homologues, and it is also not known if the other producers of 10-membered enediynes employ a similar self-sacrifice mechanism. However, given CalC was able to confer resistance to the 10-membered enediynes **15** and **16**, it is reasonable to assume the gene clusters from *Polysyncraton lithostrotum* and *Didemnum proliferum* could harbor *calC* homologues. It also remains to be determined whether CalC expression correlates directly with the production of **13** in *M. echinospora*. Finally, it is unclear if CalC (or a CalC variant) can, at some stage, act as a

Scheme 4



binding protein for **13** prior to hydrogen abstraction, i.e., whether CalC is in fact derived from an "apoprotein"-like progenitor. Intriguingly, the secondary structure prediction for CalC shows a symmetrical pattern with a $\beta\alpha\beta\beta\beta-\beta\beta\beta\alpha\alpha$ fold for the putative monomeric protein. In light of the recently unraveled $\beta\alpha\beta\beta\beta-\beta\alpha\beta\beta\beta$ monomeric signatures of the MTM C-resistance protein Mrd and the BLM-resistance protein BlmA (see section 2.3), the secondary structure prediction of CalC might indicate a possible functional conservation of the CalC structure. This is further supported by recent CalC NMR experiments that reveal CalC contains predominantly a β-sheet secondary structure (Hager, M. H.; Hallenga, K.; Thorson, J. S. Unpublished data).

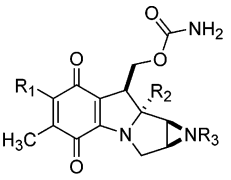
In view of the recently established resistance mechanism of *M. echinospora*, it will be interesting to see if the producers of 10-membered enediynes all rely solely on the strategy of providing a surrogate substrate for detoxification or if there are other parameters that play a role in self-resistance.[152] In the CAL producer *M. echinospora* the biosynthetic gene cluster harbors several genes of putative efflux proteins that belong to the family of ABC transporters. The gene *calT5*, for example, encodes a protein with high homology to DrrA, an ATP-binding protein which confers resistance to daunorubicin. A counterpart to *calT5*, *orf 42*, is found in the **15**-producer *M. chersina* (Gao, Q.; Thorson, J. S. Unpublished data), which points to a possible role of this gene in the efflux of **13** and **17**. This is consistent with the role of CalC homologues as a last-ditch "emergency brake" and the predominant mechanism of self-resistance in 10-membered enediyne producers via efflux transporters.

5. Mitomycin

The mitomycins (MTMs) are potent antibiotics that belong to the family of antitumor quinones. In contrast to BLM and the enediyne antibiotics, MTMs do not cause DNA-backbone cleavage but rather form covalent linkages with DNA and function as alkylating agents.[217] A unique hallmark of the MTMs is their conversion to the active drug through an enzymatic reduction process that preferentially proceeds in the absence of oxygen.[218]

5.1. Discovery and Biological Activities

In 1956 mitomycin A (**20**) and B (**21**) were isolated from *Streptomyces caespitosus,*[219−221] and shortly after mitomycin C (**19**) was found from the same strain (Figure 4).[222] The *N*-methyl derivative of **19**, porfiromycin, was isolated in 1960 from *Streptomyces ardus*, followed by the discovery of mitiromycin from *Streptomyces verticillatus*.[223,224] Among all these different



19: $R_1 = NH_2$, $R_2 = OCH_3$, $R_3 = H$
20: $R_1 = OCH_3$, $R_2 = OCH_3$, $R_3 = H$
21: $R_1 = OCH_3$, $R_2 = OH$, $R_3 = CH_3$

Figure 4. Structures of mitomycin C (**19**) and analogues mitomycin A (**20**) and mitomycin B (**21**).

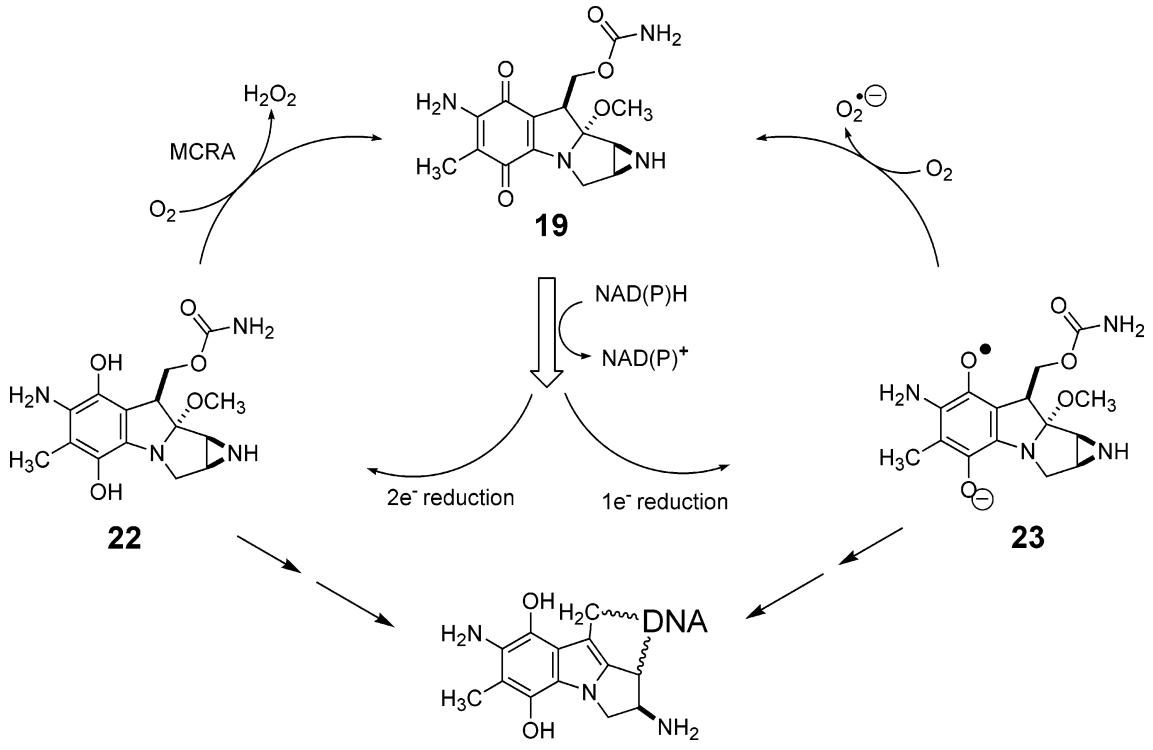
MTMs, **19** led to early widespread clinical use given its uniquely superior activity against solid tumors and reduced toxicity as compared to the natural counterparts **20** and **21**.[225,226]

The MTMs are comprised of aziridine, quinone, and carbamate moieties arranged in a compact pyrrolo-[1,2-a] indole structure,[227,228] which presents the extraordinary ability to cross-link DNA with high efficiency and specificity for the sequence CpG.[229] Similar to the other DNA-damaging agents such as CAL, **19** is reductively activated, which converts the molecule from a noncyctotoxic prodrug into a short-lived and highly reactive quinone methide.[230] In this activated form the compound lacks the methoxide substituent and aziridine ring opening exposes an electrophilic C1, a highly reactive species termed mitosene. Covalent DNA cross-links are then rapidly formed via attack at both the C1 and C10 positions (Scheme 5).[231]

Activation of **19** can occur chemically or via enzymatic activation by intracellular flavin reductases and can proceed by either one- or two-electron reduction (Scheme 5).[232] The one-electron reduction of **19** produces the semiquinone anion radical intermediate (**23**), which is regenerated to the parental compound **19** under aerobic conditions by electron transfer to molecular oxygen, generating a superoxide radical anion. Alternatively, a second electron can be transferred to **23** to form the aforementioned mitosene species. Due to its potential to undergo activation through a variety of different enzymes in aerobic and hypoxic conditions, **19** is a natural agent for the approach termed bioreductive chemotherapy.[233] This treatment is dependent upon tumors rich in reductive activation proteins and, thus, is most successful in certain cancer lines including colon, breast, lung, and head cancer. Additionally, bioreductive chemotherapy can be employed to selectively attack carcinomas since it capitalizes on the differences in oxygen content and cellular pH between normal tissue and tumor tissue.[234] In particular, hypoxic cells of solid tumors that are resistant to most chemotherapeutic agents create an environment which favors the reductive processes that are needed for the activation of **19**. Therefore, **19** displays greater toxicity to oxygen-deficient cells in comparison to their oxygenated counterparts—a quality that makes **19** a valuable antitumor drug able to attack hypoxic regions of solid tumors.

The discovery of the alkaloid FR900482 from *Streptomyces sandaensis* in 1987 revealed a structurally related mitomycinoid with a mode of action analogous to **19**.[235] This representative of a new class of antitumor agents displays markedly lower hematotoxicity,[236] and its semisynthetic derivative FK317 has shown promising activity in human clinical trials. Due to their greatly reduced toxicity and superior DNA cross-linking activity, these compounds may have the potential to replace **19** in the clinic.[237]

Scheme 5



5.2. Clinical Resistance

Despite the high efficacy of **19** in the treatment of solid tumors, acquired or intrinsic drug resistance of tumor-cell populations is also responsible for refractory malignant target tissue and the limited utility of this antineoplastic drug. Several factors have been implicated to be involved in the acquisition of MTM C resistance in mammalian cells. These include the deficiency of activating enzymes such as an NAD(P)H oxidoreductase, DNA repair processes, and increased drug efflux.[238] Whereas these resistance mechanisms have also been reported for other drugs, cells that become specifically insensitive to **19** might also employ a distinct detoxification mechanism. In recent years it has been hypothesized that tumor cells become resistant to **19** by reoxidizing the reduced, cytotoxic intermediate of **19** to the parent drug through a redox mechanism. This assumption was prompted by the observation that a 54-kDa flavoprotein from *S. lavendulae* is able to confer **19**-resistance to Chinese hamster ovary (CHO) cells.[239] This protein is called MCRA (mitomycin C resistance associated) and renders the **19** producer insensitive to high concentrations of its own toxic product.[240] In *S. lavendulae* MCRA acts as a hydroquinone oxidase and protects DNA from cross-linking by oxidizing the toxic **19** hydroquinone (**22**, Scheme 5). That this mechanism is oxygen dependent in *S. lavendulae* is in accordance with the observation that expression of MCRA in CHO confers profound resistance only under aerobic conditions but not under hypoxia.[239] Nontransfected **19**-resistant mammalian cell lines that were developed by stepwise exposure to increasing **19** concentrations also showed **19** resistance only in the presence of oxygen. Furthermore, overexpression of **19**-activating enzymes such as the NAD(P)H oxidoreductase restored **19** sensitivity in both transfected and drug-selected cell lines.[241] All these observations lend further support to the idea that **19** resistance in tumor cells not only is derived from increased drug efflux, DNA repair, or differential rates of reduction/activation, but also involves an oxygen-dependent resistance mechanism possibly analogous to that established by MCRA.[242] Unlike the MTMs and, in particular, **19**, clinical trials for the FR900482 class are in process, and specific biological implications with respect to resistance to these antitumor drugs should be available soon.

5.3. Resistance by the Producing Organisms

To protect itself from the harmful effects of **19**, *S. lavendulae* developed several mechanisms to ensure self-resistance. Three genes have been identified so far to be involved in the cellular protection against **19** in this MTM-producing organism. The previously mentioned *mcrA* gene is located outside of the MTM C biosynthetic gene cluster and encodes a flavoprotein (MCRA) which is able to convey MTM C resistance upon the heterologous host *S. lividans*.[240] In addition to MCRA-based MTM C regeneration, a second gene (*mrd*) was located within the MTM C gene cluster.[83] It encodes a small soluble protein (Mrd)[84] that is able to confer 30-fold enhanced MTM C resistance if heterologously expressed in *E. coli*. Further characterization revealed that Mrd was able to reversibly bind and sequester **19** with no observable antibiotic modification.[243] However, Mrd was shown to require NADH to exert its protective drug-binding function. This NADH requirement led to the discovery that Mrd can, in fact, act as a weak activator of **19** through the Mrd-dependent generation of 1,2-*cis*-1-hydroxy-2,7-diaminomitosene, a compound that is produced in the reductive **19** activation cascade.[243] The finding of a **19**-activating activity appeared to be conflicting with the previously identified protective effect of Mrd. However, the reductive reaction catalyzed by Mrd is slow and results in a prolonged association of **19** and its corresponding reduced product with the protein. Therefore, reduction represents a prerequisite for binding of **19** to Mrd and rapid removal of the drug through a specific transport protein Mct (encoded by the third resistance gene). Mct displays extensive amino acid sequence similar to several antibiotic-exporting proteins. Heterologous expression of both *mct* and *mrd* in *E. coli* indicated that both proteins together form an efficient drug-binding export system.[244]

As it was mentioned in section 2.3, the Mrd-resistance protein features on the structural level an interesting monomeric tandem $\beta\alpha\beta\beta\beta$ motif.[84] Tandem $\beta\alpha\beta\beta\beta$ motifs are characteristic of a large family comprising several other proteins with diverse functions such as the BLM-resistance protein from *S. verticillus* (BlmA) and the methymalonyl−coenzyme A epimerase from *Propionibacterium shermanii*. In the context of this review, it is important to note that despite the remarkable structural differences between their ligands, Mrd and BlmA share substantial structural similarity and may implicate the $\beta\alpha\beta\beta\beta$ motif as a signature for the general drug-sequestering-resistance paradigm. It remains to be determined whether there is a relationship between this structural family of sequestering proteins and the β-sheet-rich enediyne apo-proteins and/or CalC.

6. Perspective

In the context of the DNA-damaging agents discussed (BLMs, enediynes, and MTMs), one can derive a fairly unique distinction among the mammalian mechanisms of resistance in contrast to the mechanisms employed by the drug-producing prokaryotic counterparts. For example, eukaryotic BLM resistance relies upon BLM hydrolase and DNA repair enzymes, while these higher organisms are devoid of the predominant prokaryotic-resistance components (BLM-binding proteins, acetyltransferases, and BLM-specific transporters). In a similar fashion, MDR appears to be the predominant mammalian resistance mechanism for enediynes, while sequestration, self-sacrifice, and, to a lesser extent, efflux lend to the self-preservation of enediyne-producing prokaryotes. The case for the MTMs is perhaps less clear as the often observed phenomenon of aerobic drug resistance in human cancer cell lines could be attributed to an MCRA-analogous reoxidization process. Such correlations may lead to new perspectives in terms of drug development. For example, naturally inactivated acetyl−BLMs from the producing organ-

ism may serve as reasonable prodrugs which, upon tumor-cell-dependent deacetylation, present the active DNA-cleavage agents. Alternatively, the naturally occurring prokaryotic cytotoxin-binding proteins (e.g., BlmA, Mrd, enediyne apo-proteins) could be rationally engineered to display tumor-targeting elements, and these complexes subsequently could be produced via direct fermentation, thereby enhancing tumor specificity of these highly reactive drugs and eliminating the current need for tedious mAb-conjugation strategies. In addition, as we continue to learn more about the specific mechanisms of tumor resistance to these agents, sensitizing agents (e.g., BLM hydrolase inhibitors, DNA repair inhibitors, MDR inhibitors, MTM oxidative regeneration inhibitors) can be specifically incorporated into chemotherapeutic regimens to enhance efficacy. Armed with this information, physicians may also begin to profile patients to individually tailor chemotherapeutic treatment. For example, although the exact mechanism is not clear, a receptor protein appears to exist on the plasma membrane of mammalian and yeast cells that may mediate BLM internalization.[245−247] Increasing the receptor production in tumor cells or patient profiling for overproduction of this putative BLM-receptor gene may assist in a predetermination for patients most amenable to BLM chemotherapy.[24] As gene therapy comes of age, one might even imagine delivering specific resistance proteins (e.g., CalC) to 'normal' tissues to serve as chemoprotective agents.

Within the drug-producing organisms, understanding the mechanisms of resistance, efflux, and regulation is critical to the continued success of these reagents. For example, overexpression of genes encoding drug binding and/or efflux proteins and/or simple affinity labeling of the inherent binding proteins may radically enhance production levels and/or purification strategies. Pathway engineering toward novel variants is also at the mercy of resistance and efflux elements. Moreover, a molecular-level understanding of the drug-binding protein interactions is essential to (i) fine tune intracellular release of the parent drugs within a tumor (e.g., as in the case of chromoprotein enediynes), (ii) potentially engineer de novo drug carriers (e.g., proteins capable of accommodating drugs other than the natural metabolite),[248,249] (iii) rationally incorporate tumor-directing elements, and (iv) possibly understand the subtle distinctions and/or evolution between catalysis and binding (e.g., as in the case of Mrd or possibly even CalC). Such projected research areas will continue to present exciting challenges of significant therapeutic value for years to come.

7. Abbreviations

ABC	ATP-binding cassette
ADEPT	mAb-directed enzyme prodrug therapy
AHM	4-amino-3-hydroxy-2-methylpentanoic acid
AML	acute myeloid leukemia
AP	apurinic/apyrimidinic
BCRP	breast-cancer-resistant protein
BLM	bleomycin
CAL	calicheamicin
CHO	Chinese hamster ovary
CR	complete remission
mAB	monoclonal antibody
MCRA	mitomycin C resistance associated protein
MDR	multi-drug resistance
MRP	multi-drug-resistance-associated proteins
MRSA	methicillin-resistant *S. aureus*
MTM	mitomycin
NCS	neocarzinostatin
PBA	pyrimidoblamic acid
PEM	polymorphic epithelial mucin
Pgp	P-glycoprotein transporters
PLM	phleomycin
TLM	tallysomycin

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