Cellular Protection Against the Antitumor Drug Bleomycin

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1 Introduction

Bleomycin is a basic hydrophilic antibiotic isolated as a metal complex from the culture medium of the microorganism *Streptomyces verticillus* [1, 2]. Bleomycin comprises of several species differing only in the terminal amine moiety (Fig. 1), with bleomycin-A_2 being the most abundant form [2–5]. By the late 60s, substantial evidence had accumulated showing that bleomycin can diminish the growth of experimentally induced tumors in animal models, and dramatically decrease the size of human tumors [6–10]. It has been postulated that bleomycin mediates the cell killing by directly attacking the DNA [11, 12]. This notion rapidly gained support from subsequent independent studies showing that bleomycin triggers the induction of lysogenic phage in bacteria, a result of DNA damage, and induces mitotic recombination and mutations in many model systems including the budding yeast *Saccharomyces cerevisiae*, *Aspergillus*, and *Drosophila* [13–18]. Later studies also showed that bleomycin can induce micronuclei formation and chromosome aberrations in human lymphocytes [19]. The accumulated findings strongly suggest that bleomycin mediates its effect as a chemotherapeutic agent primarily by damaging the DNA [20–23]. However, additional studies showed that RNA is also damaged by bleomycin, raising the possibility that, besides DNA, RNA could be a major target [24].

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L. Panasci et al. (eds.), *Advances in DNA Repair in Cancer Therapy*,
Cancer Drug Discovery and Development 72, DOI 10.1007/978-1-4614-4741-2_9,
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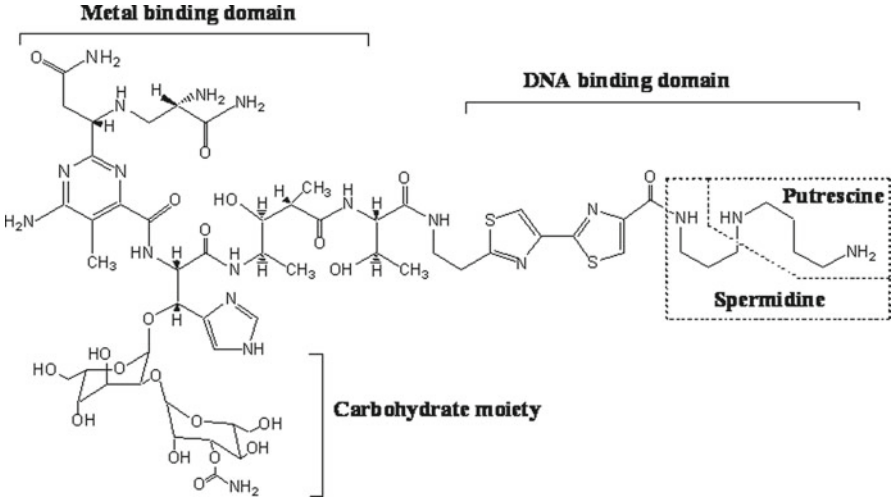

Fig. 1 Structure of the antitumor drug bleomycin depicting the three domains

Bleomycin is widely used in the clinic as a mixture (blenoxane), consisting primarily of the isomers bleomycin-A_2 and bleomycin-B_2, as well as several additional minor species including bleomycin-A_5 [4]. It is used only in combination therapy with a number of other antineoplastic agents such as etoposide [4, 25, 26]. Bleomycin is most effective against lymphomas, testicular carcinomas, and squamous cell carcinomas of the cervix, head, and neck [27, 28]. One useful property of bleomycin is that it does not appear to cause myelosuppression, a phenomenon that leads, for example, to the decrease of cells responsible for immune response, as compared to other cytotoxic antineoplastic drugs [28, 29]. Moreover, bleomycin is eliminated rapidly from the circulatory system by renal excretion. At least half of the drug is cleared from the blood within 2–4 h, except for patients with impaired renal function [29]. Like many other antitumor drugs, bleomycin also manifests clinical limitations. For example, at high doses (i.e., >400 U or ~235 mg), bleomycin can induce pulmonary fibrosis, a condition characterized as a diffuse disease of the lung parenchyma that can cause respiratory insufficiency leading to fatal hypoxemia [30, 31]. The exact mechanisms by which bleomycin induces pulmonary fibrosis is not known, but findings from several experimental animal models suggest that the onset of the disease is triggered by lipid peroxidation [32, 33]. Another common factor that limits the clinical application of bleomycin is tumor resistance [28]. So far, a clear mechanism has not yet emanated to account for tumor resistance towards bleomycin. While several possible mechanisms are likely to involve (i) decreased drug uptake, (ii) increased drug extrusion, (iii) enhanced repair of bleomycin-induced DNA lesions, and (iv) increased inactivation of bleomycin [34–38], recent studies provide strong evidence to support the former possibility [34–38]. This has been aided by the advent of fluorescently labeled bleomycin (F-BLM) and the identification of a transporter that mediates uptake of F-BLM. It seems logical that the existence

of a transporter would serve as a key regulatory step to control drug entry and that defects in this process is likely to engender striking resistance towards this chemotherapeutic agent, which are elaborated in detail below.

2 Structure of Bleomycin

The structure of bleomycin consists of three functional domains, including a metal binding domain, a DNA binding domain, and the carbohydrate moiety (Fig. 1). The metal domain also binds to molecular oxygen, in addition to the minor groove of DNA. This domain is thus largely responsible for the anti-tumor activity of bleomycin. It has a flexible requirement for metal ions, as it is capable of binding to both redox-active transition metal ions such as iron and copper and non-redox active metal ions such as zinc, cobalt, and cadmium [39–44]. The metal ion plays two roles in bleomycin-induced genotoxicity, i.e., one is to facilitate contact between bleomycin and DNA, and the other is to activate oxygen such that a reactive radical species is generated [20, 40, 41, 45–47]. Among the metal ions, cobalt forms the most stable complex with bleomycin. Despite this, iron is the metal ion predominantly used in clinical preparations of bleomycin, as it enhances the production of DNA lesions [47, 48].

The function of the two other domains of bleomycin is not clearly established. The DNA binding domain bears a bithiazole group required for DNA binding and for sequence-selective DNA cleavage [22, 49, 50]. In some species of bleomycin, such as bleomycin-A_5, the DNA binding domain also contains the chemical composition of polyamines, and thus, this form of the drug is considered to be a polyamine analogue [51]. The role of the carbohydrate moiety of bleomycin is far more elusive. Removal of the carbohydrate moiety from bleomycin does not alter the resulting deglycobleomycin ability to cleave DNA, excluding a role for this region in incising DNA [52]. No additional studies have been conducted with deglycobleomycin to examine whether it is capable of entering cells, or causing cell killing.

To date several chemical modifications have been introduced to alter the structure of bleomycin, but so far none has led to a more potent antitumor activity without the ability to cause pulmonary fibrosis. Thus, enhancing the antitumor effect of bleomycin will likely rely on modulation of cellular molecules that would improve, for example, uptake of bleomycin or its interaction with nucleic acids in cancer cells.

3 Bleomycin Induced DNA Lesions

Bleomycin can enter into mammalian cells, through an active transport pathway (see below), where a fraction of the drug reaches the nucleus to inflict a narrow set of DNA lesions through a multistep process [53]. In the earliest events, bleomycin

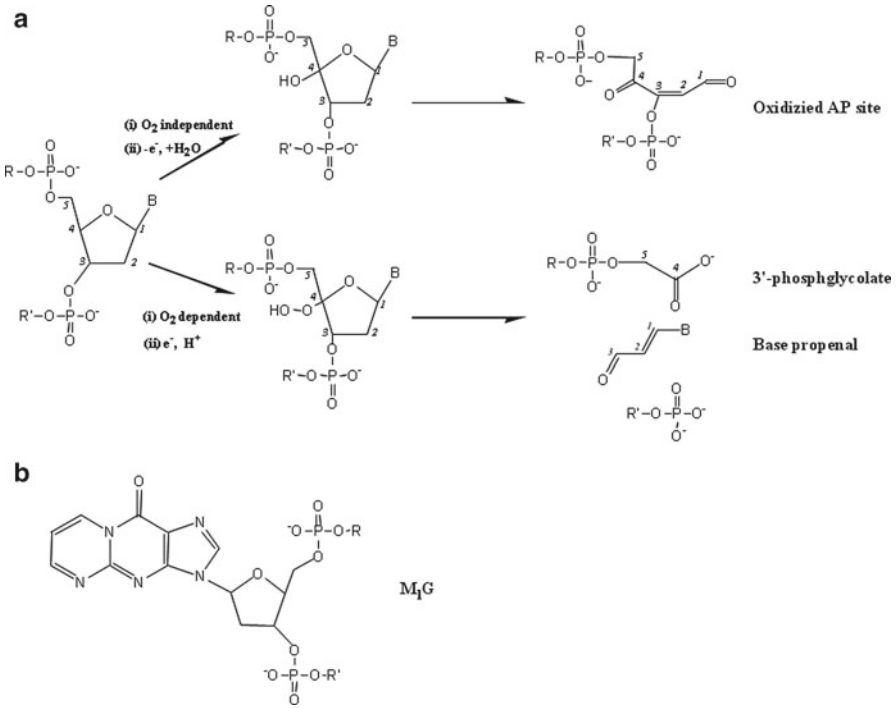

Fig. 2 Structure of bleomycin-induced DNA lesions. Production of the various types of bleomycin-induced lesions is dependent on oxygenation conditions. (**a**) In the absence of oxygen, bleomycin produces primarily oxidized apurinic/apyrimidinic (AP) site, while in the presence of oxygen it generates mostly DNA strand breaks, such as 3′-phosphoglycolate. (**b**) The M_1G lesion, 3-(2′-deoxy-β-D-erythro-pentofuranosyl)-pyrimido[1,2-a]purin-10(3H)-one, is produced by reaction of the base propenal with deoxyguanosine

binds to reduce iron (Fe II) and molecular oxygen followed by its conversion into an activated form [54]. The activated bleomycin (Blm-Fe(II)-O_2) complex then acts as an oxidant, abstracting a hydrogen atom from the 4′-carbon of deoxyribose to produce an unstable sugar carbon-radical and a single electron reduced form of activated bleomycin (Blm-Fe(III)-OH•), which can carry out multiple attacks on DNA [55–58]. The unstable sugar generated by activated bleomycin can be rearranged to generate at least four types of oxidative DNA lesions (Fig. 2). These lesions are structurally and chemically related to some of the lesions produced by ionizing radiation, and include the following: (i) *Oxidized* (*ketoaldehyde*) *apurinic/apyrimidinic* (*AP*) *sites*, where the entire base is lost, resulting in no template information for DNA polymerase [53, 59], (ii) *DNA single strand breaks* where the 3′-ends are terminated with a portion of the deoxyribose ring to form 3′-phosphoglycolate (3′-PG) which effectively blocks DNA synthesis [59, 60]. The remaining portion of the fragmented sugar, left attached to the base, exists in the

free base propenal form, and exhibits a high propensity to undergo secondary reactions to form a variety of base adducts [59, 60], (iii) *Pyrimidopurinone of deoxyguanosine* (M_1G) is the most abundant base adduct produced when the malondialdehyde moiety of the propenal base reacts with guanine [61]. The M_1G lesion is also generated by aerobic metabolism and it is detected at levels of ~5,000 adducts/cell in normal human liver [62–64]. This lesion is mutagenic in bacterial test systems [62–64], and (iv) *Bi-stranded DNA lesions*, which are produced at certain sequences, such as CGCC, when the Fe.bleomycin complex induces an AP site on one strand, and directly opposes strand break on the complementary strand [44, 65–67]. This lesion requires a single activated bleomycin molecule, which binds to both strands of the duplex DNA [44]. The bi-stranded lesions can be converted to double strand breaks following spontaneous cleavage of the AP site by primary amines (e.g., histone amine) in vivo [65–67].

The extent of formation of the various bleomycin-induced lesions depends on the redox status of the cells [68–71]. In the presence of oxygen, bleomycin produces primarily DNA strand breaks, but under low oxygen tension it forms largely AP sites in the DNA [53, 59, 65, 72]. Thus, the redox state of the cells is likely to dictate the types of DNA lesions that are generated by bleomycin. These DNA lesions are also influenced by bleomycin concentrations. At high concentrations, bleomycin releases all four bases from DNA in the order of preference thymine > cytosine > adenosine > guanine [53, 56, 73]. At lower concentrations, bleomycin exhibits significant base sequence specificity. Although bleomycin cuts mixed sequence DNAs with a disposition for GC=GT>GA>>GG, it efficiently cleaves regions of (AT)n•(TA)n and hardly at (ATT)n•(TTA)n, (ATT)n•(AAT)n, (AC)n•(GT)n, and (A)n•(T)n raising the possibility that AT rich regions of the genome are more susceptible to lesions formed by bleomycin [74–76]. The structure of DNA also plays a role in the outcome of bleomycin-induced DNA lesions [77]. DNA that is pre-exposed to other DNA damaging agents, such as cisplatin, alters the pattern of lesions produce by bleomycin [78–80]. Thus, the clinical application of bleomycin together with other DNA damaging agents is likely to produce irreparable DNA lesions.

Several studies clearly demonstrate that bleomycin-induced DNA lesions are mutagenic [81–85]. For example, introduction of bleomycin-treated vectors into mammalian cells, followed by recovery, revealed that the vectors contain high levels of base substitutions and single-base deletions [81, 82]. The base substitutions are likely to be misincorporation of nucleotides by DNA polymerase at unrepaired oxidized AP sites, while the one-base deletions may arise from incorrect repair of bi-stranded DNA lesions [81, 82]. Thus, the normal cells of a cancer patient exposed to bleomycin must rely on enzymes to efficiently repair bleomycin-induced DNA lesions to prevent the production of lethal mutations that can lead to toxic side effects and secondary tumors. Likewise, tumor cells are likely to employ even more efficient DNA repair mechanisms to evade the genotoxic effects of bleomycin.

4 Bleomycin-Induced RNA Cleavage

Several reports demonstrated that bleomycin can also cleave many different RNAs including HIV-1 reverse transcriptase mRNA, transfer RNAs, ribosomal RNA, and RNA present in RNA•DNA heteroduplex [86–91]. Incision of RNA also occurs via an oxidative pathway reminiscent of the cleavage mechanism of DNA [92, 93]. Furthermore, RNA cleavage occurs preferentially at 5′-GU-3′ sequences similar to the site-specific 5′-GT-3′ incision observed in DNA [86, 92]. Besides these similarities, there are distinct differences between RNA and DNA with respect to cleavage with bleomycin. A notable difference is that not all RNA molecules, e.g., *Escherichia coli* $tRNA^{Tyr}$ and yeast mitochondrial $tRNA^{Asp}$, are cleaved by bleomycin [86, 92]. This observation led to the suggestion that bleomycin-induced cleavage of RNA is structure specific. Another key difference is that double stranded RNA is not cleaved by bleomycin [93]. Moreover, the extent of RNA cleavage is significantly less than that of DNA [86]. Finally, the cleavage of RNA, but not DNA, is impeded with as low as 0.5 mM Mg^{2+} ions [87]. Since Mg^{2+} ions are required to maintain most RNA structure and function, it is postulated that the Mg^{2+} ions bind to RNA at the same exact site that coincides with binding of bleomycin [87]. The exceptional selectivity for destruction of certain RNAs, even with excess non-substrate RNAs, led to the suggestion that at least one unique RNA species could be targeted for destruction by bleomycin during chemotherapy. However, the following findings stand against RNA being a therapeutic target: (i) most RNA molecules exist in multiple copies and that destruction of a few molecules is unlikely to cause cell death, unless bleomycin is able to target a specific essential RNA species present in extremely low abundance, (ii) RNA cleavage by bleomycin is inhibited by the physiological concentration of Mg^{2+} (2 mM), and (iii) cleavage of RNA is structure specific and occurs much slower than DNA [86, 87, 93]. As such, it can be inferred that DNA is the most likely target during bleomycin chemotherapy.

5 Other Cellular Targets

In addition to DNA and RNA, bleomycin can also attack the integrity of the cell wall (a complex structure composed mainly of glucans, mannoproteins, and chitin) of microbes. At high doses, or under prolonged exposure, bleomycin can create small incisions in the cell wall thereby exposing the protoplast [94, 95]. The protoplast is osmotically fragile and this can lead to plasma membrane rupturing and cell death [94, 95]. Because the sugar constituents of the cell wall have a stereochemistry at the C-5 position that is similar to the C-4 position of the deoxyribose moiety of DNA, it is believed that bleomycin destroys the cell wall via oxidative damage to the sugar [95, 96]. Another relevant target affected by bleomycin is the plasma membrane, which is believed to undergo lipid peroxidation, and this may constitute the initiation process of bleomycin-induced pulmonary fibrosis [32, 97, 98].

6 Prokaryotic and Eukaryotic Defense Mechanisms Against Bleomycin Toxicity

6.1 Cell Wall and Membrane Barriers

The highly reactive nature of bleomycin towards various cellular components, particularly DNA, suggests that organisms must employ multiple defense mechanisms to combat the deleterious effects of this drug. Some of these defense mechanisms include the barrier afforded by the cell wall and plasma membrane, proteins that bind and sequester bleomycin, and proteins that repair bleomycin-induced DNA lesions [95, 99, 100]. The contribution of each mechanism towards the protection against bleomycin toxicity is often determined by measuring the sensitivity of mutants to the drug. This type of analysis revealed that in the budding yeast *S. cerevisiae*, the cell wall appears to play a minor passive role in the protection against bleomycin toxicity. This is supported by the fact that some, and not all, cell wall-defective mutants displayed only a modest sensitivity towards bleomycin (Leduc, A and Ramotar, D., unpublished).

Previous report claimed the presence of a receptor protein that exists on the plasma membrane of mammalian and yeast cells that may mediate bleomycin internalization [99, 101]. This putative receptor (~250-kDa in size) was initially identified by its specific interaction with labeled cobalt-bleomycin complex [99]. However, no further study was undertaken to identify and characterize this plasma membrane protein, although it could hold the key to provide a rational explanation for why certain tumor types, and not others, can be reduced by bleomycin chemotherapy (see below). Aside from the prediction of a bleomycin-receptor, it is equally plausible that the plasma membrane may harbor a specific efflux pump to limit bleomycin uptake. So far, there is no direct evidence that any of the known drug efflux pumps has a role in expelling bleomycin from cells as part of a detoxification process, although a genetic approach is currently being used in this laboratory to exploit the yeast system to search for such an efflux pump.

6.2 Bleomycin Binding Proteins

The transposon Tn5, commonly used for insertion mutagenesis in many Gram-negative bacteria, was serendipitously discovered to harbor a gene *ble* that renders cells resistant to bleomycin [102–104]. Two other genes *Sa ble* and *Sh ble*, have been characterized and shown to encode proteins that are homologous to *Tn5 ble* [102, 105–107]. The *Sh ble* gene from *Streptoalloteichus hindustanus* encodes a 14-kDa protein that confers resistance to bleomycin by sequestering the drug [108, 109]. The X-ray crystal structure of Sh ble revealed that it consists of two halves that are identically folded despite no sequence similarity [109]. The structure further revealed that the Sh ble dimer binds to two molecules of bleomycin [109].

In vitro assays demonstrated that this protein prevents the action of bleomycin on DNA. At concentrations as low as 1 μM, bleomycin can completely degrade 0.2 μg of chromosomal, linear, or covalently closed circular (CCC) DNA within few minutes at ambient temperature, a process that is completely inhibited in the presence of a fivefold molar excess of the Sh ble protein [110]. It is likely that the related ble members may also function to sequester bleomycin, and possess no direct role in DNA repair as previously suggested [111, 112]. A ble-related protein is also present in the bleomycin producing strain *S. verticillus*, raising the possibility that the ble-related protein could have yet another role by sequestering bleomycin in *S. verticillus* for efficient transport to the exterior [113, 114]. To date, database searches reveal that eukaryotes do not possess the ble-related protein, and suggest that higher organisms may have evolved other mechanisms to mount a defense against bleomycin.

6.3 Bleomycin Hydrolase

Earlier studies demonstrated that bleomycin can be metabolically inactivated in normal and tumor tissues by an enzyme called bleomycin hydrolase, and that such inactivation may play a role in bleomycin resistance [115–117]. This is supported by the correlation that tissues with low levels of bleomycin hydrolase are usually sensitive to bleomycin, and tumor cells that acquire resistance to bleomycin possess higher levels of activity [35, 36, 38, 118, 119]. To better understand the role of bleomycin hydrolase, the enzyme was characterized and shown to be a thiol protease that hydrolyzes the β-aminoalanine amide moiety at the carboxyl terminus of bleomycin to generate the inactive deamido metabolite [35, 37, 118]. Using a specific thiol protease inhibitor (E64) that blocks bleomycin hydrolase activity, it was further shown that cells become more sensitive to bleomycin [120]. This finding quickly led to the isolation of the bleomycin hydrolase corresponding gene from yeast and mammalian cells [121–124]. Expression of the yeast bleomycin hydrolase gene *BLH1* in mammalian cells conferred nearly eightfold increase resistance to bleomycin, and which was blocked by the E64 inhibitor [125]. One would expect that removal of the *BLH1* gene from yeast would cause a bleomycin-hypersensitive phenotype. However, two independent studies showed conflicting data regarding the role of Blh1 in the detoxification of bleomycin in the yeast model system [121, 122]. While one study showed that *blh1Δ* mutant is mildly sensitive to bleomycin, another clearly established that the mutant is not at all sensitive [121, 122]. Additional findings revealed that *blh1Δ* mutants are not sensitive to bleomycin [126]. Moreover, overexpression of the *BLH1* gene in yeast cells confers no additional resistance to bleomycin [126]. Thus, the role of bleomycin hydrolase in producing tumor resistance is controversial. The situation is further complicated by the fact that the Blh1 protein, also called Gal6, is under the control of the Gal4 transcriptional activator [127]. Blh1/Gal6 binds specifically to the Gal4 transcription factor DNA binding site and acts as a repressor to negatively control the galactose

metabolism pathway [127–129]. On the basis of the foregoing studies, it would appear that bleomycin hydrolase has a more general role in the cells to degrade proteins, or perhaps to degrade transcription factors to regulate gene expression [130–132]. In support of this, a more recent study documented that Blh1 is one of the proteases required to process Huntington protein to generate the N-terminal cleave form thought to be involved in the pathogenesis of the disease [133]. In fact, both yeast and human BLH1 play a more direct physiological role in protecting cells against homocysteine toxicity, a risk factor for Alzheimer's disease, by hydrolyzing intracellular homocysteine-thiolactone [134, 135].

6.4 DNA Repair Pathways

Repairing of bleomycin-induced DNA lesions is likely the most crucial mechanism employed by cells to avert bleomycin-induced genotoxicity. Thus, organisms exposed to bleomycin must recruit a variety of enzymes and/or proteins to repair the diverse types of bleomycin-induced DNA lesions. While such enzymes are still being characterized in eukaryotic cells, the bacterium *E. coli* has two well documented enzymes, i.e., endonuclease IV and exonuclease III, that repair bleomycin-induced DNA lesions [100, 136, 137]. Both enzymes possess (i) a 3′-diesterase that removes 3′-blocking groups (such as 3′-phosphoglycolate) at strand breaks, and (ii) an AP endonuclease that cleaves AP sites. These enzymatic activities regenerate 3′-hydroxyl groups that allow DNA repair synthesis by DNA polymerase [137–139]. *E. coli* mutants lacking both endonuclease IV and exonuclease III are severely impaired in the removal of bleomycin-induced DNA lesions, and, as a consequence, display extreme hypersensitivity to bleomycin [137]. Between the two enzymes, endonuclease IV plays a more predominant role in repairing bleomycin-damaged DNA [140]. This is supported by two independent studies, the first showing that mutants deficient in endonuclease IV are substantially more sensitive to bleomycin than exonuclease III-deficient mutants [137, 141]. The second study demonstrated that purified endonuclease IV is more active at processing bleomycin-induced DNA lesions in vitro, as compared to purified exonuclease III [140].

While the *E. coli* studies were in progress, the first eukaryotic homologue of endonuclease IV, called Apn1, was discovered in *S. cerevisiae* [142, 143]. Surprisingly, yeast mutants lacking Apn1 are not sensitive to bleomycin, leading to the prediction that yeast may use alternative enzyme(s) to combat the genotoxic effects of bleomycin [143, 144]. Consequently, a rigorous search was initiated for a possible auxiliary enzyme(s) in yeast that might repair bleomycin-induced DNA lesions. One approach exploited the power of biochemistry to detect enzymatic activities that would process lesions along defined DNA substrates. In one case, a highly sensitive assay was developed consisting of a double stranded DNA substrate where one strand (*[^{32}P]-labeled) bears a single-strand break terminated with 3′-phosphoglycolate (Fig. 3). This biochemical assay identified an extremely weak 3′-diesterase in total extracts derived from an *apn1Δ* mutant (i.e., lacking the major

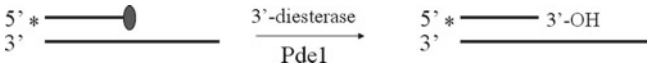

Fig. 3 Depiction of an oligonucleotide DNA substrate bearing a 3′-phophoglycolate terminus. The 3′-phosphoglycolate (*oval shape*) is produced by bleomycin and requires processing by a 3′-repair diesterase in order to regenerate a 3′-hydroxyl group for DNA polymerase activity. Labeling (*asterisk*) the 5′-end with ^{32}P allows detection of the processed product by polyarcylamide gels

3′-diesterase/AP endonuclease activity of Apn1). The weak activity removed the 3′-phosphoglycolate (PG) from the labeled DNA strand to produce 3′-OH (Fig. 3) [145]. The enzyme, called Pde1, was partially purified and also found to have an AP endonuclease in addition to the 3′-diesterase activity [146]. Immediately following this report, the gene (*APN2*/*ETH1*) encoding Pde1 was isolated by two independent laboratories, and the deduced amino acid sequence was found to share 19 % identity with the *E. coli* exonuclease III [146, 147]. Thus, Pde1/Apn2/Eth1 is the yeast homologue of *E. coli* exonuclease III. The most surprising finding is that yeast mutants lacking both Apn1 and Pde1 (Apn2/Eth1), if at all, showed very mild sensitivity to bleomycin (D.R., unpublished). However, the *apn1Δ pde1Δ* double mutants are exquisitely sensitive to the alkylating agent methyl methane sulfonate, which produces natural AP sites, as opposed to oxidized AP sites generated by bleomycin [53]. It is therefore possible that the 3′-phosphoglycolate and the oxidized AP site lesions produced by bleomycin are inaccessible or refractory to cleavage by the 3′-diesterase/AP endonuclease activities of either Apn1 or Pde1 in vivo. If so, yeast may possess yet other "backup" enzymes to initiate the repair of bleomycin-induced DNA lesions. This possibility is supported by the discovery of the hPNKP gene encoding the human polynucleotide kinase, which possesses two enzymatic activities, a kinase that phosphorylates the 5′-hydroxyl group of DNA and a strong 3′-diesterase activity that repairs oxidative DNA lesions in *E. coli* [148]. hPNPK is unrelated to any of the known 3′-diesterase/AP endonuclease belonging to the endonuclease IV or exonuclease III family, but it may share a related active site [100, 149]. A gene (*TPP1*) encoding a yeast homolog of the human hPNKP has been subsequently isolated, but gene knock out of *TPP1* alone has no effect on bleomycin sensitivity, unless TPP1 is also deleted in a background lacking both Apn1 and Apn2 [148, 150].

The repair of bleomycin-induced DNA lesions is not restricted to enzymes with the ability to cleave AP sites or remove 3′-blocking groups, as other DNA repair pathways also participate in the repair process. In yeast, the recombination and the post-replication DNA repair pathways, respectively, represented by the Rad52 and Rad6 proteins are involved in the repair of bleomycin-induced DNA lesions [151, 152]. These two pathways also repair a wide spectrum of other DNA lesions including those generated by the alkylating agent methyl methane sulfonate, 4-nitroquinoline-1-oxide (which forms bulky DNA adducts), and γ-rays. The *rad52Δ* and *rad6Δ* mutants are hypersensitive to a large number of DNA damaging agents including bleomycin [153–156]. On the basis of cell killing and growth kinetic analyses, both Rad52 and Rad6 showed different contribution to the repair of

bleomycin-induced DNA lesions [152]. At bleomycin concentrations ranging from 10 to 15 μg/ml in culture media the Rad52 pathway is required to repair bleomycin-induced DNA damage, while at higher concentrations (15–30 μg/ml culture media), the Rad6 pathway plays a more prominent role [152]. This disparate response can be explained if distinct lesions are generated in yeast cells in a manner that depends on the bleomycin dose.

A few studies also implicated the involvement of other proteins in the repair of bleomycin damaged DNA. For example, the Ku proteins, a heterodimer composed of a 70-kDa subunit and a 80-kDa subunit that is involved in nonhomologous end joining of DNA, is implicated in the repair of bleomycin-induced DNA lesions [157, 158]. However, a number of laboratories could not convincingly confirm the earlier findings that Ku heterodimer-deficient yeast mutants (*hdf1Δ hdf2Δ*) are sensitive to bleomycin [157, 158] (Masson, J-Y and Ramotar, D., unpublished). This discrepancy may be related to the yeast strain background used in the initial studies [157, 158]. Proteins that remodel the chromatin structure are also involved in protecting the genome from the genotoxic effects of bleomycin. It has been shown that either bleomycin or methyl methane sulfonate can activate the Mec1 kinase in yeast, leading to direct phosphorylation of serine 129 of histone H2A [159]. A mutation (S129A) that prevented the phosphorylation of H2A causes cells to be hypersensitive to both bleomycin and MMS [159]. The investigators proposed that phosphorylation of H2A is required to relax the chromatin to either allow gene expression to facilitate repair, or to permit access of repair proteins and other factors directly to the DNA lesions [159].

While it is clear that DNA repair plays an important role in the protection against bleomycin-induced DNA lesions, there is no direct evidence that the overproduction of DNA repair proteins can contribute to enhance bleomycin resistance in cells. At least in yeast, overproduction of some of the DNA repair proteins described above does not confer bleomycin resistance to parental cells [144, 156]. This is in discord with one of the earlier predictions that tumor resistance to bleomycin may be attributed to elevated DNA repair activities [38]. Irrespective of whether DNA repair activities are subsequently discovered to be elevated in bleomycin resistant tumors, any attempts to promote the antitumor potential of bleomycin should take into consideration the possibility of diminishing the DNA repair capacity of tumor cells.

7 Transport of Bleomycin into Yeast and Mammalian Cells

The first evidence for a possible transporter for the uptake of bleomycin into cells came from a study showing that the plasma membrane of yeast and mammalian cells contained a protein that binds to bleomycin carrying labeled cobalt [^{57}Co] [99]. Further characterization of this plasma membrane protein was hindered as it requires the use of [^{57}Co]-bleomycin. As such, an alternative approach was devised that employs coupling the dye fluorescein to bleomycin-A_5 followed by the purification of the conjugated drug (F-BLM) using high performance

liquid chromatography [160]. The resulting purified F-BLM retained the ability to inflict damage to the DNA in a manner analogous to the native bleomycin-A_5 [160]. Moreover, yeast mutants that are defective in the repair of bleomycin-A_5-induced lesions exhibit sensitivity to F-BLM, indicating that this modified drug has the capability of entering the cell and damaged the DNA. In fact, F-BLM enters parent yeast cells in a concentration- and time-dependent manner and raises a distinct possibility that a plasma membrane transporter exists to allow bleomycin entry into the cell [160]. If this is indeed the case, yeast mutants lacking the transporter function are expected to be resistant to bleomycin. This notion prompted the search for a possible transporter of bleomycin starting with yeast as a model system because of the ease, for example, of identifying genes belonging to the same functional pathways by various high throughput analyses [161]. To find the bleomycin transporter, a collection consisting of ~ 4,000 yeast haploid mutant each lacking a nonessential gene was screened for those showing sensitivity or resistance to bleomycin [162]. This large scale approach revealed over 200 mutants displaying remarkable sensitivity to bleomycin and are deleted for genes encoding proteins belonging to several functional groups including DNA repair and chromatin structure, transcription, and cell cycle [163]. A large group of genes belong to the vacuolar pathway, highlighting the importance of the vacuoles in detoxifying bleomycin [160, 164]. Amongst these sensitive genes none has been characterized in any details with respect to potential target for therapy.

Of importance, the large scale screen revealed five mutants displaying sharp resistance to bleomycin as compare to the parent [163]. Amongst these mutants one lacked the *AGP2* gene and exhibited the greatest resistance (~3,000-fold more) to bleomycin, but not to other chemotherapeutic drugs such as cisplatin, camptothecin, and etoposide [163]. Of all the hypersensitive and resistant mutants, at least 76 are deleted for genes encoding proteins that share significant level of identity with a human protein [163]. Thus, it appears that both yeast and human cells may conserve the same biological processes to regulate the toxicity of bleomycin. Since one of the objectives of the large scale screen is to identify a plasma membrane transporter that when deleted causes resistance to bleomycin, most of the ensuing analyses have been directed toward the molecular characterization of the phenotypes associated with deletion of the *AGP2* gene.

7.1 Yeast Agp2 Is a Transporter of Bleomycin

AGP2 encodes a 67.2-kDa plasma membrane protein that shares significant homology with the amino acid transporter family. This transporter is involved in the uptake of L-carnitine, which serves as a carrier for acetyl-CoA, from the peroxisome to the mitochondria for complete oxidation [165]. Cells lacking Agp2 are defective in mediating the uptake of F-BLM [163]. This has been supported by epifluorescence

analysis revealing that there was no detectable staining of F-BLM in the *agp2Δ* mutants as compared to the parent [163]. F-BLM uptake could be effectively restored in the *agp2Δ* mutants by the reintroduction of the *AGP2* gene, strongly indicating that Agp2 is the main channel to allow entry of bleomycin into the cell [163]. It is no surprise then that coincubation of parent cells with L-carnitine sharply reduced the uptake of F-BLM into the cells. More importantly, overproduction of the transporter stimulated the uptake of F-BLM and selectively sensitized the cells to killing by bleomycin, a consequence of substantially elevated damage to the chromosomal DNA [163].

7.2 Human hCT2 Is a Transporter of Bleomycin

On the basis of the above studies in yeast, it seems reasonable to postulate that mammalian permeases with the ability to transport L-carnitine could be a candidate to mediate uptake of bleomycin. At least, two high affinity L-carnitine transporters hCT2 and OCTN2 are present in humans [166, 167]. hCT2 is expressed mainly in the bone marrow and testis, while OCTN2 is expressed in multiple tissues [166]. As testis expressed the utmost levels of hCT2 and that testicular cancer is highly responsive to bleomycin therapy raise the possibility that there might be a correlation between hCT2 expression and bleomycin response [168]. Indeed, RT-PCR analysis confirmed that the testicular cancer cell line NT2/D1expressed high levels of hCT2, while its expression is undetectable in the colon carcinoma cell line HCT116, and only weakly detected in the breast cancer MCF-7 and the lung fibroblast LL47 cells [169]. In fact, the testicular cancer cell line NT2/D1 displayed more than 300-fold more sensitivity to bleomycin than the colon cancer cell line HCT116, suggesting that the higher sensitivity of NT2/D1 is related to the higher expression level of hCT2 [169]. This differential response between the two cell lines was not observed if the cells were challenged with other genotoxic chemotherapeutic agents such as cisplatin [169].

Since hCT2 has been shown to be a high affinity transporter of L-carnitine [166], it seems no surprise that L-carnitine acts as a competitive inhibitor and block the uptake of bleomycin if both gain entry into the cells via the same transporter [169]. In fact, L-carnitine protected the testicular cancer cell line from the genotoxic effects of bleomycin [169]. These observations led to the prediction that if hCT2 expression is downregulated, it would block entry of F-BLM and cause the cells to become resistant to bleomycin. Indeed, using siRNA technology that specifically depleted the expression of hCT2 sharply reduced the uptake of labeled L-carnitine into NT2/D1 cells; highlighting the effectiveness of the siRNA. Furthermore, the diminished expression of hCT2 conferred upon these cells resistance to bleomycin, but not to other anticancer agents such as cisplatin [169]. It seems logical that the complete loss of hCT2 expression, such as *hct2*$^{-/-}$ homozygous null cells, would make cells even more resistant to bleomycin. However, these latter cells are not available to test this possibility [169].

A further prediction from the above findings is that cell lines devoid of hCT2 expression, such as the colon cancer cell line HCT116, when forced to express the transporter should be sensitized to bleomycin. The transient transfection of a plasmid designed to drive hCT2 expression as a hCT2-GFP fusion protein from the CMV promoter directed the production of an ~93 kDa protein that localized to the plasma membrane [169]. This fusion protein stimulated the uptake of F-BLM into the HCT116 cells [169]. These HCT116 cells overexpressing hCT2-GFP showed enhance sensitivity to bleomycin, and not to cisplatin, as compared to cells carrying only the GFP portion [169]. These findings are in agreement with the notion that hCT2 has a specific role in transporting bleomycin into the cell [164].

Currently, Hodgkin's lymphoma patients are being treated with bleomycin, although a fraction of these patients remain unresponsive to the drug [170]. To check if this is correlated with a reduced level of the transporter, hCT2 expression level was determined in a panel of established Hodgkin's lymphoma cell lines that include Namalwa, Raji, Daudi, H2, DHL16, RL, and SR. Interestingly, amongst these cell lines only H2 expressed the highest level of hCT2 and displayed significant sensitivity to bleomycin, as compared to the other cell lines with low levels of hCT2 [169]. Thus, lymphoma patients expressing high levels of hCT2 are likely to show favorable clinical respond towards bleomycin.

8 Summary and Perspectives

So far, much of our understanding of the various mechanisms leading to bleomycin resistance emanate from the yeast *S. cerevisiae*, mainly because of the availability of ready-to-use experimental tools. Based on current information, it would appear that the principal defense mechanism against bleomycin involves entry of the drug into cells (Fig. 4). The observations that the levels of the hCT2 transporter correlate with the sensitivity of cells towards bleomycin, strongly suggests that it could play a key role in specifically regulating cellular resistance to bleomycin. Thus, it can be inferred that high hCT2 activity levels in tumor cell samples would be indicative of responsiveness towards bleomycin-therapy, while low hCT2 activity would correlate with drug resistance. As such, hCT2 could be a determining factor for patients' response to treatment regimens consisting of bleomycin. To date, no studies have been performed to closely examine if hCT2 is regulated and to explore ways to stimulate its expression, for example, with small molecules or hormones [171]. Such efforts would have broad implications by enhancing the uptake of bleomycin in tumors such as breast, colon, and ovarian which are generally refractory to the drug therapy and may be due to the poor expression of hCT2.

In short, it is reasonable to propose that the major mechanism leading to bleomycin resistance occurs at the level of drug uptake and that mutations impairing the transporter activity could exist and easily explain why the remaining fraction of testicular cancer patients are resistant to bleomycin therapy.

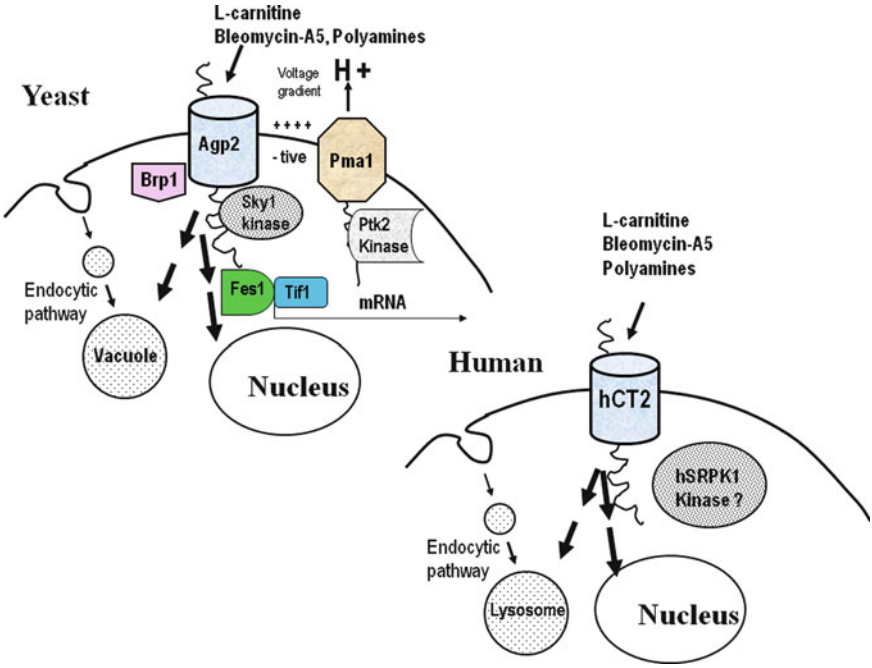

Fig. 4 A model illustrating the transport and detoxification pathway of BLM-A5 in yeast and human cells. In yeast, the drug enters the cell via the transporter Agp2, and its activity might be influenced by the kinases Ptk2 and Sky1. Following uptake, BLM-A5 is channeled to the vacuole for detoxification, as well as to the nucleus to destroy the DNA. Interruption of the endocytic pathway to the vacuoles leads to hypersensitivity towards BLM-A5. In humans, hCT2 is responsible for transporting BLM-A5, into the cells. hSRPK1 may regulate hCT2

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