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Review

Recent developments in the identification and biosynthesis of antitumor drugs derived from microorganisms

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ABSTRACT

Secondary metabolites in microorganisms represent a resource for drug discovery and development. In particular, microbial-derived antitumor agents are in clinical use worldwide. Herein, we provide an overview of the development of classical antitumor drugs derived from microorganisms. Currently used drugs and drug candidates are comprehensively described in terms of pharmacological activities, mechanisms of action, microbial sources, and biosynthesis. We further discuss recent studies that have demonstrated the utility of gene-editing technologies and synthetic biology tools for the identification of new gene clusters, expansion of natural products, and elucidation of biosynthetic pathways. This review summarizes recent progress in the discovery and development of microbial-derived anticancer compounds with emphasis on biosynthesis.

1. Introduction

Cancer is the second leading cause of death globally and a major human health concern. The clinical burden of cancer is rapidly growing worldwide, with an expected annual death toll of up to 28.4 million by 2040, exerting tremendous physical, emotional, and financial strain on individuals, families, communities, and health systems. According to global cancer statistics, female breast cancer (11.7%) is the most commonly diagnosed cancer, followed by lung (11.4%), colorectal (10.0%), prostate (7.3%), stomach (5.6%), liver (4.7%), rectum (3.8%), cervix uteri (3.1%), esophagus (3.1%), and thyroid (3.0%) cancers. Lung cancer has the highest mortality rate (18%), followed by colorectal (9.4%), liver (8.3%), stomach (7.7%), female breast (6.9%), colon (5.8%), esophagus (5.5%), pancreas (4.7%), prostate (3.8%), and rectum (3.4%) cancer [1]. Therefore, there is an urgent clinical need globally for the discovery and development of effective anticancer agents.

Microorganisms have been important resources for compounds and secondary metabolites which have greatly contributed to the development of medical agents and treatments for over the last 150 years [2]. Microorganism metabolites are an important source of antitumor agents, with ongoing attempts to discover novel effective antitumor drugs from microbes. Compounds derived from microorganisms include a variety of structural types, including proteins, polysaccharides, anthracyclines, organic acid esters, terpenes, alkaloids, macrolides, and enediynes [3]. Many candidates have been used in clinical practice and have become essential antitumor therapies.

This review discusses recent progress in the development of antitumor drugs from microorganisms, including their biological sources, antitumor activities and mechanisms, gene cluster properties, and biosynthetic pathways. Furthermore, we describe recent developments in gene editing technologies that allow the activation of silent biosynthetic gene clusters to mine novel secondary metabolites. Reconstruction of biosyn-

Abbreviations: 4-MHA, 4-methyl-3-hydroxyanthranilic acid; NPRS, non-ribosomal peptide synthetase; BLM, Bleomycins; PKSs, polyketidesynthases; AHBA, 3-amino-5-hydroxybenzoic acid; HDAC, histone deacetylase; EPI, epirubicin; JNK, c-Jun N-terminal kinase; AMPK, AMP-dependent protein kinase; mTORC1, mammalian target of rapamycin complex 1; ROS, reactive oxygen species; MTM, Mithramycin; SP1, specificity protein 1; MTM-NPs, MTM nanoparticles; MA, Macrolide antibiotics; RAPA, Rapamycin; PI3K, phosphatidylinositol 3 kinase; LDM, Lidamycin; ADC, antibody-drug conjugates; CLM, Calicheamicin; ADA, adenosine deaminase; dATP, 5′-deoxyadenosine triphosphate; PRPP, phosphoribosyl pyrophosphate; GA, Geldanamycin; Hsp90, heat shock protein 90; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; 17-DMAG, 17-dimethylamino-ethylamino-17-demethoxygeldanamycin hydrochloride; AT, acyltransferase; KS, ketone synthase; ACP, acyl carrier protein; KR, ketoreductase; DH, dehydratase; ER, enol reductase; MT, methyltransferase; BGC, biosynthetic gene clusters; FAC, fungal artificial chromosomes; LNM, Leinamycin.

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thetic gene clusters has allowed the generation of high-yield strains that have improved the output of industrial processes.

2. Microbial-derived antitumor drugs

Natural products represent valuable resources in the discovery and development of antitumor drugs [4,5]. Microorganisms have been widely used in the discovery of novel drug candidates. In recent years, researchers have obtained a range of compounds with antitumor activities from microorganisms, many of which have been used therapeutically and achieved remarkable results (Table 1).

2.1. Peptides

Herein we describe several classic antitumor antibiotics with the structural characteristics of polypeptides. In addition to standard peptide bonds, peptide antibiotics may contain glycosidic bonds, ester bonds, lactone rings, or fatty acids. Other than the 20 proteinogenic α-amino acids, other non-proteinogenic amino acids can be found in peptide antibiotics. Peptide antibiotics have the advantages of small molecular weight, simple construction, easy transformation, and low risk of adverse reactions [36]. Accordingly, peptide antibiotics have been widely studied in the development of antitumor drugs.

2.1.1. Cyclic peptides-Actinomycin D

Actinomycin D is an antitumor antibiotic containing a chromophore isolated from *Streptomyces parvullus* by Waksman in 1940 [37] and was the first antibiotic with antitumor activity to be discovered [38]. At present, multiple strains producing actinomycin D have been reported, including *S. parvullus, S. griseoruber, S. avermitilis, S. antibioticus,* and *S. costaricanus* [39–41]. More than 40 actinomycins (including actinomycin C, D, G, F, Y, Z, and N-demethylactinomycins) have been purified from various microorganisms [42]. Actinomycin V has a significant growth inhibitory effect on a variety of tumor cells, including the human leukemia cell line, K562, human breast cancer cell line, MCF-7, human non-small cell lung cancer cell line, A549, and Friedel's leukemia cell line, F5-5. Further, the cytotoxicity of actinomycin V is substantially greater than that of actinomycin D [43]. Actinomycin D was first shown to have strong antibacterial activity, with its substantial antitumor activity demonstrated later [44,45]. Actinomycin D has broad-spectrum inhibitory activity against tumors, bacteria, viruses, and tuberculosis [46]. The phenoxazinone chromophore of actinomycin D can intercalate between G-C bases with the side-chain cyclic pentapeptide of actinomycin D shown to intercalate with the minor groove of the DNA double helix to increase binding stability, thereby suppressing the activity of DNA-dependent RNA polymerase and interfering with transcription [47,48]. In addition, experimental studies have shown that actinomycin D can exert antitumor effects by inducing apoptosis or altering the cell cycle. Actinomycin was first approved by the Food and Drug Administration (FDA) in 1964 and has been used since 1954 in the treatment of various cancers including rhabdomyosarcoma, Ewing's sarcoma, and choriocarcinoma [49]. Actinomycin D has also been shown to enhance the clinical efficacy of other antitumor drugs. Actinomycin D has synergistic cytotoxicity with RG7787, an immunotoxin used for the treatment of refractory pancreatic cancer and mesothelioma, in mesothelin-positive cancer cell lines, with actinomycin D leading to significant tumor regression in pancreatic and gastric cancer xenografts [50]. The actinomycin synthetic gene cluster (50 kb) includes a*cmA*, a*cmB, acmC*, and *acmD* genes, which encode ACMSI, AcmACP, ACMSII, and 4-MHA-Amp, respectively. These proteins work synergistically in the synthesis of non-ribosomal peptides [6]. The biosynthesis of actinomycin is divided into three stages (Fig. 1). The first stage involves the synthesis of 4-methyl-3-hydroxyanthranilic acid (4-MHA) through the kynurenine pathway [45,51]. In the second stage, 3-hydroxy-4-methyl-anthranilate pentapeptide lactone is generated via the assembly of non-ribosomal enzymes. The non-ribosomal peptide synthetase (NRPS) system is one of the largest enzyme systems and typically consists of multiple modules [6,52]. In the third stage, the resulting two 3-hydroxy-4-methyl-anthranilate pentapeptide lactones are condensed under the catalysis of phenoxazinone synthase to form a phenoxazinone chromophore [53]. However, heterologous expression of actinomycin D has not yet been achieved.

2.1.2. Glycopeptides-bleomycins

Bleomycin (BLM), an alkaline water-soluble glycopeptide antibiotic first extracted from *S. verticillus* by Hamao Umezawa in 1966, is a cell-cycle nonspecific drug [54]. The molecular weight of BLM is approximately 1400. Other antibiotics in the BLM family with antitumor activities include zorbamycin, tallysomycins, and phleomycins. Pingyangmycin hydrochloride is a BLM antibiotic produced by *S. pingyangi* with higher antitumor activity, lower toxicity, and wider clinical applications than most BLM antibiotics. In the middle and late 1970s, peloromycin was developed as a second-generation BLM. Compared with BLM, the concentration of peloromycin is four times greater in tumor cells with a toxicity of only one-third that of BLM. BLM is comprised of a mixture of components, predominantly A2 (50%–60%) and B2 (25%–32%) [55]. This drug was first approved by the FDA in 1973 and is currently widely used in more than 80 countries as an essential chemotherapeutic agent. BLM has a curative effect on malignant lymphoma and squamous cell carcinoma originating in the head, neck, skin, esophagus, lung, and cervix, among other locations. [56]. In addition to its antitumor activity, BLM can effectively inhibit gram-positive and gram-negative bacteria, including *Staphylococcus aureus, Shigella, Escherichia coli, Proteus, Typhoid Bacillus, Bacillus cereus, Pseudomonas aeruginosa*, and *Bacillus subtilis*. In addition, BLM has antiviral effects and induces lysogenic phage production. *In vitro*, BLM inhibits cell proliferation in prokaryotic and eukaryotic cells and prevents viral DNA replication. The antitumor functions of BLM are predominantly mediated by its direct effects on the structure and function of DNA, including the formation of DNA single-strand breaks and the inhibition of DNA replication, cell division, and proliferation, thereby inducing cell death in cells in the proliferative phase and even G_0 phase of the cell cycle. BLM typically blocks the cell cycle in the G_2 phase. Four unique sites are involved in the antitumor activity of BLM: the metal binding domain can complex with Fe^{2+} to activate molecular oxygen and selectively ligate DNA; the DNA-binding region is responsible for binding DNA and recognizing the nucleotide sequence and also contributes to the selective cleavage of DNA; (2S, 3S, 4R)-4-amino-3-hydroxy-2-methylvaleric acid is responsible for stable binding of the metal binding domain to DNA and improves the efficiency of DNA cleavage; and the disaccharide region is involved in cell surface recognition, binding to metal ions, and the generation of reactive oxygen species, thereby increasing the inhibioy effects of BLM on cellular proliferation. BLM contains a typical hybrid peptide-polyketide structure and disaccharide unit, in which the peptide-polyketide skeleton is composed of nine amino acids (serine, histidine, threonine, alanine, β-alanine, two cysteines, and two asparagines), one acetic acid, and two s-adenosine methionines. NRPSs, polyketidesynthases (PKSs), and glycosylsynthase are involved in the synthesis of BLM (Fig. 2). NRPSs and PKSs assemble 9 amino acids and one malonyl-CoA into a BLM peptide-polyketide skeleton molecule [7]. Sugar synthetase (BlmC) and sugar isomerase (BlmG) catalyze the synthesis of the BLM glycosyl group, producing two monosaccharides which then combine with the BLM peptide-polyketide framework molecule to produce decarbamoyl-BLM under the action of glycosyltransferase (BlmE) and hydroxylase (BlmF). The carbamyl transferase BlmD catalyzes the final step of BLM synthesis to produce the active BLM molecule [57].

2.1.3. Depsipeptides-romidepsin

Romidepsin (FK228) was first isolated from *Cromobacterium violaceum* in 1994 [12], with the basic structural unit of a bicyclic tetrapep-

Table 1
Representative microbial-derived antitumor drugs and candidates

Name	Chemical Classes	Source	Indication	Clinical Status	Mode of action	Biosynthetic gene cluster	Synthetase	Heterogeneous expression host	References
Epoxomicin	Peptide	*Actinomycetes strain, No. Q996-17*	Prostate Cancer, Small-cell Lung Cancer, Neuroendocrine Cancer, Non-small Cell Lung Carcinoma, Kidney Cancer	Approved by FDA	Inhibiting the ubiquitin-proteasome pathway	30 kb	PKS-NRPS	/	[6]
Actinomycin D	Cyclic peptide	*Actinomyces antibioticus*	Wilms Cancer, Ewing Sarcoma, Neuroblastomas, Trophoblastic Tumors	Approved by FDA	DNA intercalation	50 kb	NPRS	/	[7]
Romidepsin	Depsipeptide	*Chromobacte rium violaceum*	Cancers of Lung, Breast, Pancreatic, Prostate, Colorectal, Ovarian	Approved by FDA	Potent HDAC inhibitor	/	/	/	[8]
Bleomycin	Glycopeptides	*Streptomyces verticillus*	Head and Neck Squamous Cell Carcinomas, Hodgkin's Disease, Non-Hodgkin's Lymphoma, Testicular Carcinomas, Ovarian Cancer	Approved by FDA	DNA lesions	62 kb	PKS-NPRS	/	[9,10]
Mitomycin C	Indolequinones	*Streptomyces caespitosus*	Cancers of Head and Neck, Lung, Breast, Cervix, Bladder, Colorectal and Anal Carcinomas, Hepatic Cell Carcinoma, Melanoma, Stomach and Pancreatic Carcinomas	Approved by FDA	DNA intercalation	55 kb	Heterocyclic quinone	/	[11,12]
Doxorubicin	Anthracycline	*Streptomyces peucetius var. Caesius*	Leukemia, Wilms' Tumor, Neuroblastoma Hodgkin's Disease, Oral Squamous Carcinoma, Cancers of Breast, Ovarian, Bladder, Thyroid, Gastric Carcinoma, Bronchogenic Oral Squamous Carcinoma	Approved by FDA	DNA intercalation	40kb	PKS II	/	[13,14]
Daunorubicin	Anthracyclines	Streptomyces peucetius and others	Hematologic Neoplasms, Leukemia, Liver Cancer	Approved by FDA	DNA intercalation	39 kb	PKS II	/	[15]
Aclarubicin	Anthracyclines	*Streptomyces galilaeus*	Leukemia	Phase 3	Inhibiting DNA replication and repair, and RNA and protein synthesis	/	/	/	[16]
Aclacinomycin Y	Anthracyclines	*Streptomyces and Streptomyces galilaeus*	Leukemia	Phase 3	/	15 kb	PKS I & II	/	[16,17]
Epirubicin	Anthracyclines	Streptomyces peucetius and Streptomyces venezuelae	Cancers of Breast, Gastric, Esophageal	Phase 3	Interference with the synthesis and function of DNA	/	/	/	[18]
Mithramycin	Anthracycline	*Streptomyces strains*	Lung Cancer, Esophageal Cancer, Mesothelioma, Gastrointestinal Neoplasms, Breast Cancer, Sarcoma	Phase 2	Inhibiting RNA and protein synthesis	40 kb	PKS II	/	[19]
Calicheamicin	Enediyne	*Micromonospora echinospora sp.*	Novo Acute Myeloid Leukemia, Acute Promyelocytic Leukemia,	Approved by FDA	Acting as a DNA double-stranded cleaving agent	90 kb	PKS II	/	[8,20]
Lidamycin	Enediyne	*Streptomyces globisporus*	Glioma, Liver Cancer	Phase 2	/	85 kb	PKS II	/	[8]
Epothilone	Macrolide	*Sorangium cellulosum*	Cancers of Breast, Epithelial, Prostate, Renal Cell Carcinoma, Non-Small Cell Lung Cancer	Approved by FDA	Inducing tubulin polymerization, microtubule bundling, and mitotic arrest	72 kb	PKS-NRPS	*Streptomyces coelicolor*	[13,14,21]

(*continued on next page*)

Table 1 (*continued*)

Name	Chemical Classes	Source	Indication	Clinical Status	Mode of action	Biosynthetic gene cluster	Synthetase	Heterogeneous expression host	References
Rapamycins	Macrolide	*Streptomyces hygroscopicus*	Cancers of Breast, Prostate, Bladder, Rectum, Head and Neck, Thyroid, Kidney, Squamous Cell Cancer, Non-Small Cell Lung Cancer	Approved by FDA	Inhibiting cytokine production	107kb	PKS-NRPS	/	[22,23]
Elsamicin A	Aminoglycoside	*Actinomycete strain J907-21*	Mantle Cell Lymphoma, Leukemia, Non-Hodgkin's Lymphoma	Phase 2	Inhibition of DNA replication	/	/	/	[24]
Streptozocin	Aminoglycoside	Streptomyces alanosinicus, Apis cerana, and Streptomyces achromogenes	Cancers of Pancreatic, Adrenocortical, Bladder, Urothelium, Colorectal, Neuroendocrine Tumors, Mixed Gliomas, Brain Tumor	Phase 2	/	/	/	/	[25]
Tomaymycin	Aminoglycoside	*Streptomyces tenebrarius*	Leukemia, Other Myeloid Malignancies	Phase 3	/	26 kb	NRPS	/	[26]
Pentostatin	Purine nucleoside analog	*Streptomyces antibioticus*	Kidney Cancer, Hematologic Malignancies, Lymphoma, Leukemia, Mesothelioma, Adenocarcinoma of Lung, Pancreatic Neoplasms	Approved by FDA	Blocking DNA replication	34 kb	SAICARS	/	[27]
Staurosporine	Alkaloids	*Streptomyces longisporoflavus*	Breast Cancer, Lymphoma, Prostatic Neoplasm,	Phase 1	Inhibiting protein kinase	23 kb	Indolocarbazole	*S. albus* *S. lividans*	[28]
Geldanamycins	Benzoquinone	Streptomyces hygrocopicus and Streptomyces zerumbet W14	Cancers of Kidney, Prostate, Bladder, Breast, Colorectal, Ovarian, Lung, Leukemia	Phase 1	HSP90 inhibitor	70 kb	PKS I	/	[12,29]
Trichostatin	Hydroxylamine	Streptomyces hygroscopicus, Streptomyces, and other organisms	Solid Tumors, Neuroendocrine Tumors, Breast Neoplasms, Leukemia, Uterine Fibroids	Phase 3	HDAC inhibitor	41 kb	ND	*S. lividans*	[30,31]
Rebeccamycins	Indoles	*Lentzea aerocolonigenes*	Cancers of lung, Extrahepatic Bile Duct Cancer, Gallbladder, Liver, Colorectal, Breast, Kidney, Ovarian, Small Cell Lung Cancer, Lymphoma, Small Intestine Cancer, Neuroblastoma, Retinoblastoma, Sarcoma	Phase 2	Promoting double-strand breakage of DNA and inhibiting topoisomerase I	26 kb	Indolocarbazole	*S. albus* *E. coli*	[28,32]
Porfiromycin	Indolequinones	Streptomyces purpurascens and Streptomyces	Head and Neck Cancer	Phase 3	Inhibiting DNA synthesis	/	/	/	[33,34]
Salinosporamide A	Lactones	*Salinispora tropica*	Non-Small Cell Lung Cancer, Pancreatic Cancer, Melanoma, Lymphoma, Multiple Myeloma, Pediatric Cancer	Phase 2	Proteasome inhibitor	41 kb	PKS-NRPS	/	[35]

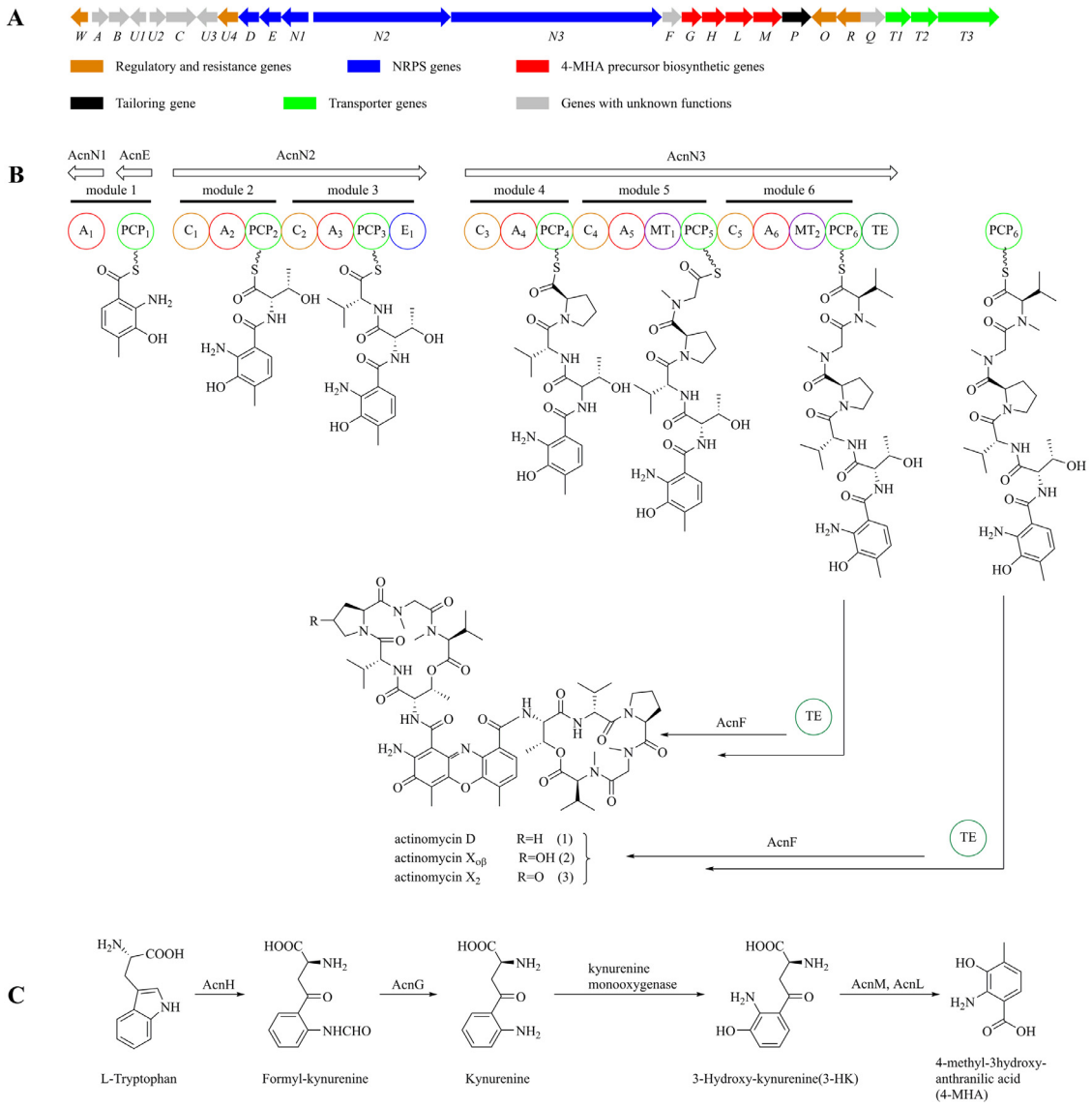

Fig. 1. (A) Actinomycin D gene cluster. (B) Proposed biosynthesis of actinomycin D. (C) Biosynthesis of the actinomycin D precursor, 4-MHA in *S. costaricanus* SCSIO ZS0073 [42]. The proposed functions of individual ORFs are indicated. The pentapeptide precursor is synthesized by the NRPS assembly line using 4-MHA as the initiating unit. The central phenoxazinone chromophore is condensed by two pentapeptide lactones under the catalysis of phenoxazinone synthase. A, adenylation domain; C, condensation; PCP, peptidyl carrier protein; TE, thioesterase.

tide disulfide shown to be formed by disulfide and ester bonds. The same year, the potential anticancer activity of romidepsin was confirmed. Romidepsin was subsequently shown to be a broad-spectrum histone deacetylase (HDAC) inhibitor in 1998. HDACs have been widely researched as potential antitumor drug targets. Romidepsin (Istodax) is an HDAC inhibitor approved by the FDA as an orphan drug in 2009 and is the second HDAC inhibitor approved for marketing in the United States. Romidepsin is predominantly used to treat progressive, persistent, and relapsed cutaneous T-cell lymphomas. Through histone deacetylation and DNA methylation, HDACs inhibit the replication of DNA resulting in a tight nucleosome structure, thereby inhibiting gene transcription, blocking the function of tumor suppressor genes, and promoting tumor formation. Romidepsin specifically binds to HDACs, inhibiting the activity of HDAC1 and HDAC2 with similar selectivity for both. Romidepsin has been shown to catalyze the deacetylation of acetylated lysine residues in histones or non-histone proteins, regulate gene expression in tumors cells, induce tumor cell differentiation, inhibit tumor cell growth, and promote tumor cell apoptosis [60,61]. Studies have shown that after romidepsin enters cells, the disulfide bond is reductively cleaved into two thiol groups under the action of glutathione to produce a reduced form of romidepsin in which the ring structure recognizes the activity pocket of target enzymes. The surface structural unit (CAP) and long-chain sulfhydryl group (ZBG) are chelated with zinc ions at the bottom of the enzyme activity pocket, and the long chain (linker) linking CAP and ZBG occupies a long and narrow hydrophobic channel in the enzyme activity pocket. Romidepsin has been shown to only have antitumor activity in its reduced form [62]. The use of romidepsin in combination therapy can improve the sensitivity and anticancer effects of other drugs. Combinatorial treatment of cancer with other drugs is the current focus of studies assessing the clinical applications of romidepsin [63]. Romidepsin has been used in combination with proteasome inhibitors, MEK inhibitors, cytotoxic drugs, and conventional anti-leukemia/lymphoma drugs for the treatment of leukemia, lymphoma, and relapsed/refractory multiple myeloma. The biosynthetic pathway of romidepsin involves the biosynthetic gene cluster, *dep*, of romidepsin which was cloned for the first time in 2007 (Fig. 3) [64]. The

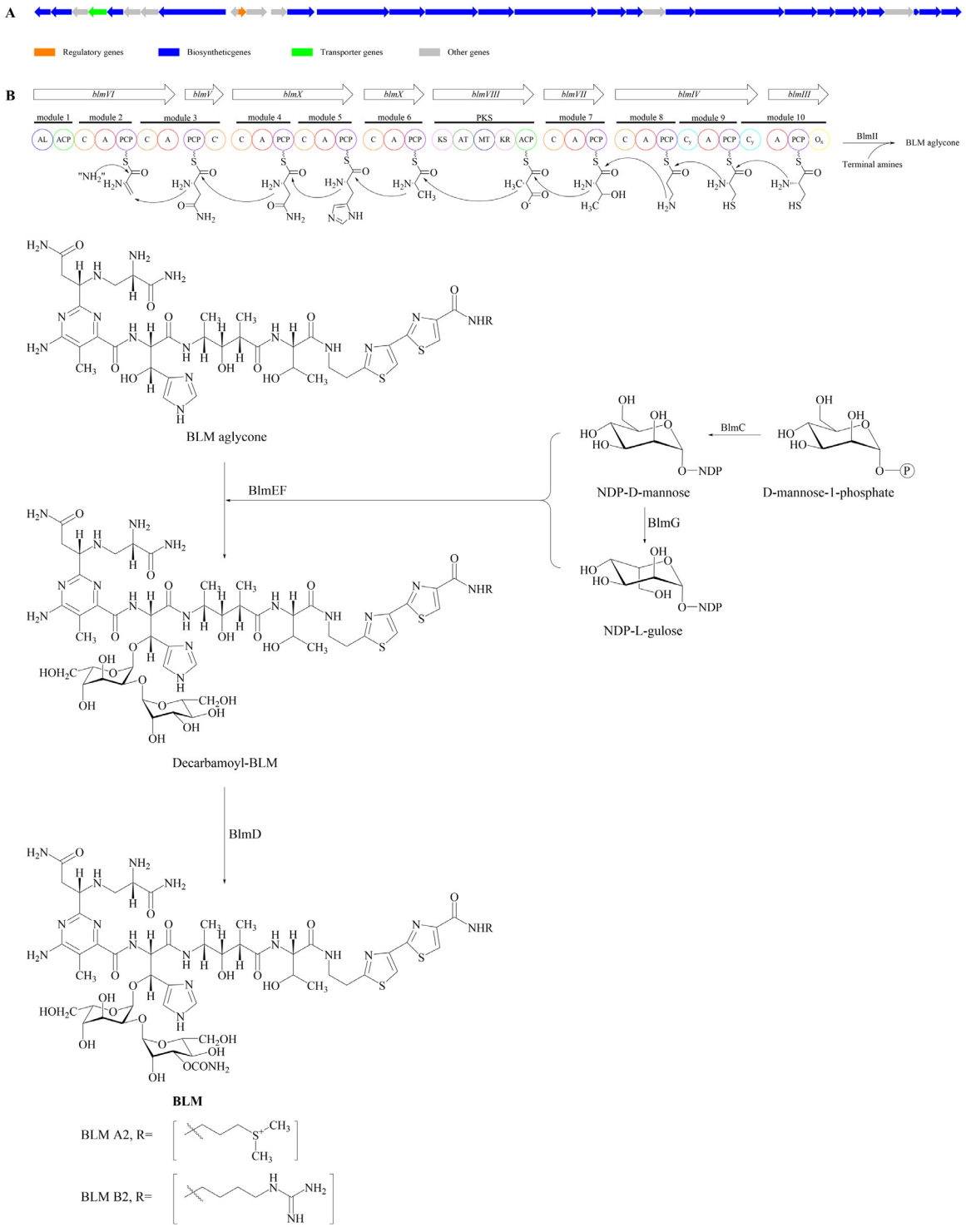

Fig. 2. (A) Organization of the bleomycin gene cluster with proposed functions. (B) Biosynthetic pathway from *S. verticillus* [7,58,59]. The BLM peptide-polyketide skeleton molecule is assembled with 9 amino acids and one malonyl-CoA under the action of NRPSs and PKSs. A sugar molecule then reacts with the peptide-polyketide core to form decarbamoyl-BLM under the catalysis of BlmE and BlmF. Finally, the decarbamoyl-BLM is converted to active BLM under the activity of BlmD. A, adenylation; ACP, acyl carrier protein; AL, acyl CoA ligase; AT, acyltransferase; C and C', condensation; Cy, cyclization; KR, ketoreductase; KS, ketosynthase; MT, methyltransferase; Ox, oxidation; PCP, peptidyl carrier protein.

dep gene cluster contains three NRPS genes; *depA, depD*, and *depE*, as well as two PKS genes, *depB* and *depC*. The encoded protein catalyzes the formation of the romidepsin backbone structure comprising 7 compound units. DepA contains NRPS module 1, which is responsible for activating cysteine as a starting unit. DepB and DepC, i.e. PKS modules 2 and 3, sequentially load two two-carbon units derived from malonyl-CoA. DepD contains NRPS modules 4 and 5, which sequentially activate and load valine and cysteine. DepE contains NRPS modules 6 and 7, which in turn activate and load anhydrothreonine and valine. The C-terminal thioesterase domain of DepE is responsible for the cyclization and release of the product. Finally, DepH catalyzes the formation of intramolecular disulfide bonds via sulfhydryl groups. In 2011, romidepsin

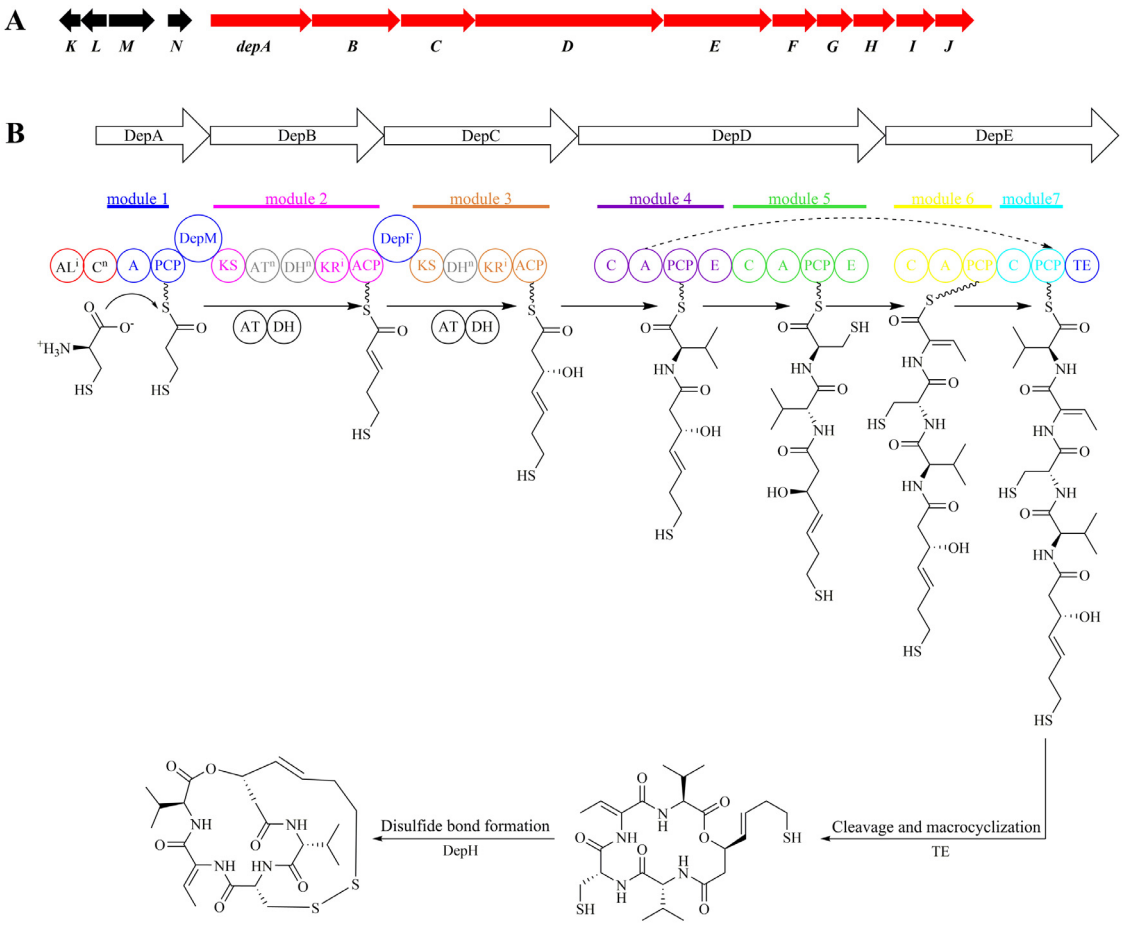

Fig. 3. (A) The romidepsin biosynthetic (dep) gene cluster. (B) Proposed biosynthetic pathway [64]. Romidepsin biosynthesis is initiated by the activation of a cysteine and undergoes chain extension catalyzed by a hybrid NRPS-PKS assembly line. Romidepsin is finally formed under the action of TE and DepH. A, adenylation; ACP, acyl carrier protein; AL, acyl CoA ligase; AT, acyltransferase; C, condensation; KR, ketoreductase; KS, ketosynthase; PCP, peptidyl carrier protein; TE, thioesterase; E, epimerase.

was heterologously expressed in *E. coli* using synthetic biology techniques [65].

2.2. Indolequins-mitomycin C

Mitomycin C is an antitumor antibiotic derived from *S. caespitosus* that has a broad anti-carcinoma properties [66]. The molecular weight of mitomycin C is 334.33. The mechanism of action of mitomycin C is predominantly mediated by activating DNA alkylation and crosslinking through the G-G interchain bond [67]. Mitomycin C selectively inhibits DNA synthesis, thereby inhibiting cell mitosis and resulting in an abnormal increase in the number of chromosomes [68]. Mitomycin C can also inhibit cell proliferation and cause cessation of the cell cycle in the S phase and G_2/M phases to promote cell apoptosis. As the one of the most commonly used chemotherapeutic drugs in the world, mitomycin C has been used as a component of combination chemotherapy for various tumors including gastrointestinal cancers, bladder cancer, head and neck sarcoma, esophageal carcinoma, pancreatic cancer, and other cancers since 1974 [68]. In addition, mitomycin C is increasingly used to improve outcomes of ophthalmology and otolaryngology surgery due to its anti-fibrotic effects. In recent years, studies have shown that mitomycin C can inhibit mitosis in fibroblasts to inhibit proliferation and has long-lasting anti-fibrosis effects after short-term exposure. In the synthesis of mitomycin C, 3-amino-5-hydroxybenzoic acid (AHBA) is the specific precursor of its aromatic chromophore group (Fig. 4). In addition, mitomycin C contains D-glucosamine, L-citrulline, and methionine. Precursor molecules are assembled into active mitomycin under the action of: MitB, a glycosyltransferase; MitE, a CoA ligase; MitR, which catalyzes C-C bond formation; and MmcS, which catalyzes *O*-carbamoylation of homologous enzymes. However, the mechanism by which these precursor molecules are assembled has not been fully elucidated.

2.3. Anthracyclines

Anthracyclines are antineoplastic antibiotics with broad anti-carcinoma properties. Anthracyclines are commonly used in chemotherapy and are considered some of the most effective anticancer drugs, whether used as a single agent or in combined therapy [69]. Anthracycline antitumor drugs can intercalate with DNA [70,71], suppressing DNA replication and inhibiting topoisomerase II, leading to DNA breakage and damage. Anthracyclines cause cell cycle arrest to induce apoptosis, promote the generation of free radicals causing DNA damage and lipid peroxidation which damages cell membranes, and combine with cell membranes or metal ions to reduce enzyme activity.

2.3.1. Doxorubicin

Doxorubicin was originally extracted by Farmitalia from cultures of *S. peucetius var. caesius* in 1969 and shown to have anticancer properties [72,73], with a molecular weight of 543.5. Doxorubicin has a wide anticancer spectrum and high treatment index. In addition to leukemia, doxorubicin has been used for the treatment of liver cancer, breast cancer, gastric cancer, kidney cancer, and lymphomas [74]. The clinical use of doxorubicin is limited by cardiotoxicity and other adverse effects.

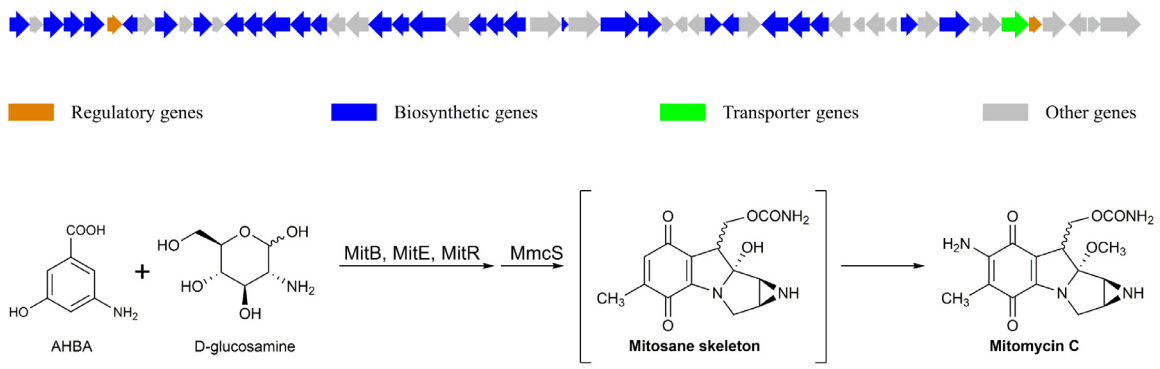

Fig. 4. The biosynthetic gene cluster of mitomycin from *S. lavendulae* and its proposed biosynthetic pathway [10]. Mitomycin C is produced as a mitosane skeleton from AHBA and D-glucosamine under the under the action of: MitB, a glycosyltransferase; MitE, a CoA ligase; MitR, which catalyzes C-C bond formation; and MmcS, which catalyzes *O*-carbamoylation of homologous enzymes.

To identify anthracycline antitumor drugs with improved efficacy and lower toxicity, scientists have screened thousands of analogs with potential antitumor activity. Currently, epirubicin (EPI) and idarubicin (4-demethoxydaunorubicin) are the most widely used doxorubicin analogs in clinical practice. Doxorubicin and daunorubicin are the most effective and commonly used antitumor drugs. Aside from these four common antitumor drugs, a few other anthracyclines have been approved for clinical use, including pirarubicin, aclarubicin, and mitoxantrone. The main mechanism of action of doxorubicin is DNA intercalation. Through free diffusion or active transport by membrane proteins such as SLC22A16 as carriers, doxyrubicin enters the cell and accumulates in the nucleus [75] followed by the insertion into double-stranded DNA resulting in DNA breakage and interference with DNA replication. Another effect of doxorubicin is inhibition of topoisomerase II, which can transiently break DNA double strands to regulate DNA topology. After doxorubicin is inserted into DNA, it forms a ternary complex with topoisomerase II and DNA which prevents the reassembly of broken DNA, thereby altering the expression of a range of proteins and inducing apoptosis [76,77]. In addition to suppressing DNA synthesis by regulating p53, doxorubicin can also induce cell cycle arrest and apoptosis. By disrupting the activity of p_{34}^{cdc2}/cyclin B, doxorubicin arrests the cell cycle in the G2/M phase and inhibits cell proliferation. In addition, doxorubicin can regulate the expression of Fas/FasL on the cell membrane surface and enhance the activation of caspase-9 and caspase-3, thereby promoting the transmission of intracellular apoptotic signals and leading to cell death [78–80]. Activation of p53, c-Jun N-terminal kinase (JNK), and AMP-dependent protein kinase (AMPK) inhibits the mammalian target of rapamycin complex 1 (mTORC1) and induces downregulation of the anti-apoptotic protein, Bcl-2, and upregulation of the pro-apoptotic protein, Bax, eventually leading to apoptosis [81]. Doxorubicin increases the generation of reactive oxygen species (ROS) in cells. The production of ROS can promote apoptosis, with Gajewski *et al.* demonstrating that DNA bases are damaged by ROS generated in response to doxorubicin. Doxorubicin and daunorubicin are synthesized by a 40 kb gene cluster [82]. The only reported strain that can produce doxorubicin is *S. peucetius* ATCC 27952. The biosynthesis of doxorubicin in *Streptomyces* involves 24 key genes and can be divided into 3 steps (Fig. 5): the generation of ε-rhodomycinone, which is catalyzed by the PKS II gene cluster comprises eight polyketide synthases of dpsABCDEFGY [13,83,84]; generation of dTDP-L-daunosamine, with α-D-glucopyranose-1-phosphate as the starting substrate, catalyzed by dnmL, dnmM, and dnmU to dTDP-4-dehydro-β-L-rhamnose in a series of continuous catalysis [16]; finally, ε-rhodomycinone and dTDP-L-daunosamine are glycosylated to form rhodomycinone D [85] which is then methylated, decarboxylated, and hydroxylated to form doxorubicin. Doxorubicin can also be synthesized by a semi-synthetic chemical reaction; however, the biosynthesis method is safer, more stable, more environmentally friendly, provides higher yields, lowers the requirement for raw materials, reduces production costs, and is more suitable for large-scale industrial applications.

2.3.2. Mithramycin

Mithramycin (MTM), also known as prucamycin, is an anthracycline antitumor antibiotic with an aureoic acid structure which can be extracted from various strains of *Streptomyces* [87,88] and with a molecular weight of 1083.1. Other aureoic acid antibiotics include durhamycin A, UCH9, olivomycin A, and chromomycin A3 [89]. MTM is used clinically in the treatment of testicular embryoma, glioma, Paget's disease of the bone, and tumor-related hypercalcemia [90]. Specificity protein 1 (SP1) is a member of the Sp/Krüppel-like factor family of transcription factors containing zinc fingers which can bind to GC-rich sequences in the promoter regions of target genes to regulate cell metabolism, growth, and death. SP1 is overexpressed in a range of cancer types and is involved in tumor growth, angiogenesis, invasion, and metastasis [91,92]. Accordingly, SP1 is a potential target for tumor therapy [93,94]. Recent studies have demonstrated that MTM can bind to the GC region of genes, form a stable complex with DNA, and competitively inhibit the binding of SP1 to the regulatory elements of its target genes, such as VEGF, c-Myc, c-Src, XIAP, and survivin [95,96]. MTM is a specific inhibitor of SP1 that directly reduces expression levels of SP1 [92,97]. Moreover, MTM nanoparticles (MTM-NPs) can inhibit the growth of pancreatic cancer BxPC-3 xenograft tumors with a tumor inhibition rate of 86%, which is 51% higher than that for the same dose of glareomycin. Both MTM and MTM-NPs can reduce the expression of the oncogene, c-Myc, and decrease protein levels of CD47 [98]. The synthetic gene cluster of MTM is 42,371 bp. The biosynthetic pathway of MTM has been studied extensively and begins with the condensation of acetyl-CoA mediated by PKS II, which is modified to form 4-demethylpremithracinone (Fig. 6) [99]. Second, 4-demethylpremithracinone is glycosylated by dTDP-α-D-olivose, dTDP-α-D-oliose, and dTDP-α-D-mycarose. Glucosyl transferases, mtmGIII and mtmGIV, first catalyze the addition of dTDP-α-D-oliose, dTDP-α-D-oliose, and dTDP-α-D-mycarose to position 2 [100], and the other two dTDP-α-D-olivoses are attached to position 6 by mtmGI and mtmGII [101]. Simultaneously, position 9 of 4-demethylpremithracinone is C-methylated [99]. Thus, premithramycin B is synthesized. Finally, mtmOIV oxygenase catalyzes the formation of the premithramycin B lactone [101,102]. After spontaneous decarboxylation, mtmW ketoreductase catalyzes the reduction of the 4′-keto group to form MTM [89].

2.4. Macrolides

Macrolide antibiotics (MA) are a class of antibiotics commonly used in clinical practice. MA are polyketide antibiotics composed of a multicarbon lactonic ring attached to one or more deoxysugars. Common MA refers to a 14–16-membered ring MA, such as erythromycin derivatives

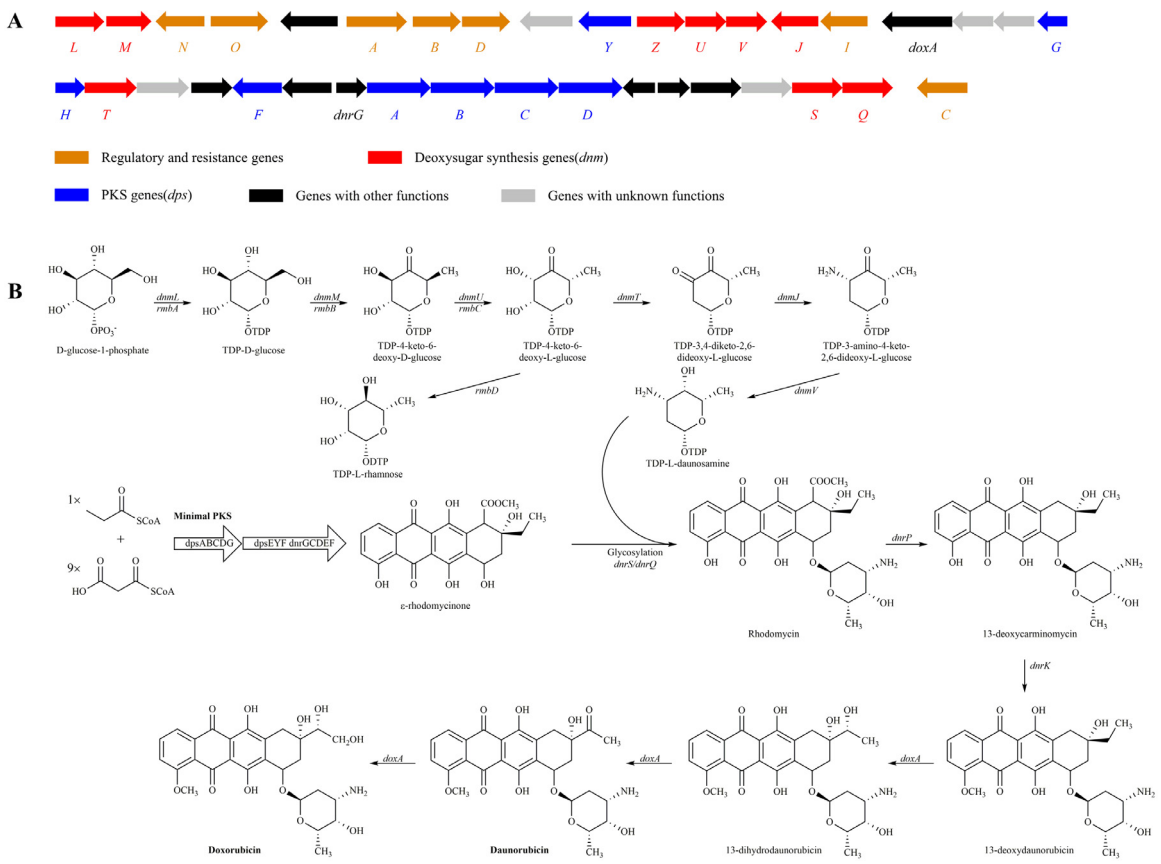

Fig. 5. (A) The doxorubicin biosynthetic gene cluster. (B) Proposed doxorubicin biosynthetic pathway [86]. The biosynthesis of DXR is completed in three stages: the biosynthesis of ε-rhodomycinone with propionyl-CoA and malonyl-CoA as the starting substrates catalyzed by PKS II modules; generation of dTDP-L-daunosamine with α-D-glucopyranose-1-phosphate as the starting substrate catalyzed by dnmL, dnmM, and dnmU; and glycosylation followed by post-modifications.

and acetyl spiramycin. MA are antibacterial agents with rapid activity that have been in use in clinical practice for more than 40 years. The antitumor activity of MA has received increasing attention recently [103].

2.4.1. Epothilone

Epothilone is a class of antitumor drugs secreted by *Sorangium cellulosum* So ce90, with a 16-membered lactone macrocycle as the centrosome [104]. Eight derivatives of epothilone have been reported to date; epothilones A, B, C, D, E, F, G, and H, in which the active substances are epothilones A and B. Epothilone can bind to the *β* subunit of the microtubule dimer, resulting in the polymerization of tubulin to prevent shortening of the spindle filament. Thus, the cell remains in the anaphase stage of cell division, thereby inhibiting mitosis. The mechanism of epothilone is similar to that of paclitaxel as both are microtubule stabilizers; however, epothilone differs from paclitaxel in structure. The structure of epothilone is simpler than that of paclitaxel and considered superior to paclitaxel in terms of safety, water solubility, antitumor activity, and synthesis method, with epothilone demonstrating improved drug resistance. In recent years, an increasing number of structural types of epothilone compounds have been reported. The structures of these novel epothilone compounds have been predominantly obtained by chemical semi-synthesis or total synthesis using natural epothilone as the substrate. Epothilones can be roughly divided into three generations according to the chronological order of their synthesis [39]. The first generation are natural products including epothilone A and B; the second generation are chemical semi-synthetic epothilones derived from the first generation of epothilone analogs, mainly ixabepilone (BMS247550), KOS-1584, and 21-amino-epothilone; and the third generation are fully synthetic analogs represented by sagopilone [105]. The semi-synthetic analog, ixabepilone (BMS-247550), has been approved by the FDA for the treatment of breast cancer. In addition to ixabepilone, epothilone B (EPO906), ixabepilone (BMS-247550), sagopilone (ZK-EPO), BMS-310705, KOS 862 (epothilone D), KOS 1584, and KOS 193 are currently the subjects of ongoing clinical research. The clinical utility of several analogs of epothilone is currently being evaluated, including desoxyepothilone F, 21-aminoepothilone (BMS-310705), and 26-fluoroepothilone B [106–108]. The epothilone biosynthetic gene cluster is 56,019 bp [14,21] and includes *epoA, epoP, epoB, epoC, epoD, epoE*, and *epoF*, which together encode five polyketide synthases (EPOSA, EPOSB, EPOSC, EPOSD, and EPOSE), one non-ribosomal peptide synthase (EPOSP), and one cytochrome P450 cyclooxygenase. The synthesis of epothilone has four stages (Fig. 7): ①synthesis of the initial thiazole ring with PKS and NRPS cooperating to form a methylthiazole ring; ②extension of the polyketide chain, the skeleton is extended by a two-carbon unit through a module unit with modifications which has eight modules forming a 16-membered skeleton of epothilone; ③cyclization and release of the polyketide chain under the action of the thioesterase domain (TE) at the end of module 8 encoded by *epoE*, the synthesis of the chain is terminated, self-cyclizes, and epothilone C or epothilone D is released; ④post-modification of the product with the P450 epoxidase encoded by *epoF* oxidizing the unsaturated double bond between 12C-13C to form an epoxy ring, which oxidizes epothilone C or epothilone D to epothilone A or epothilone B [109,110]. Owing to the slow growth rate, unclear genetic background, and lack of effective genetic operators of *C. cellulosum*, the heterologous expression of epothilone in a host with faster growth and easier genetic manipulation is subject of ongoing research [111,112]. In 2000, Tang *et al.* divided the epothilone biosynthesis gene cluster derived from SMP44 into two parts, cloned both

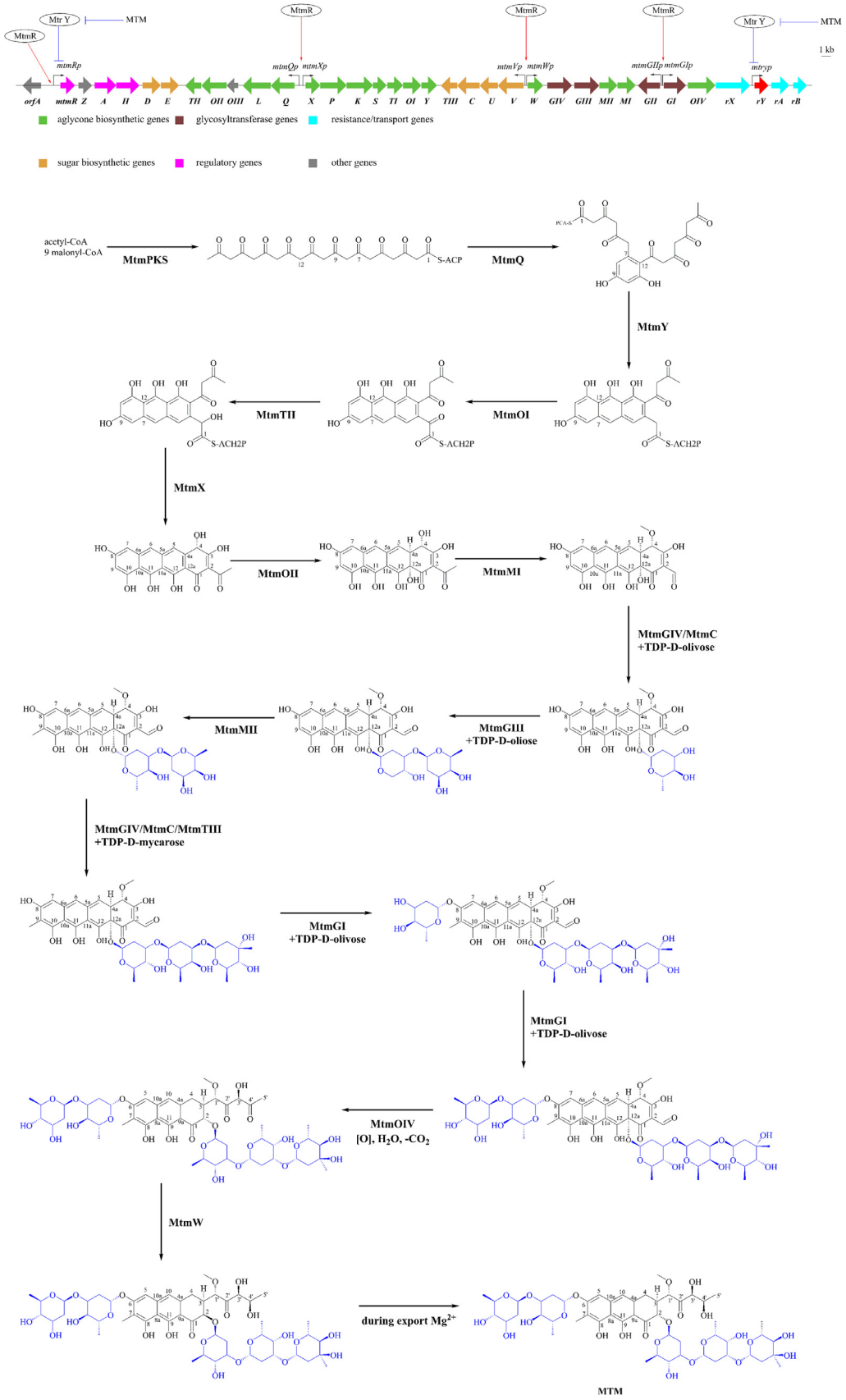

Fig. 6. The gene cluster of mithramycin from *S. argillaceus* ATCC 12956 and the proposed biosynthetic pathway [86]. The biosynthesis of MTM starts with one acetyl-CoA and nine malonyl-CoA extender units catalyzed by PKS II synthase, MtmP (KSα), MtmK (KSβ), and MtmS (ACP) to produce the decaketide chain which is cyclized and aromatized by aromatase to form the polyketide intermediate. Then, five deoxysugars are added sequentially to the C-12a-O and C-6-O position of premithramycinone under the action of MtmGIV, MtmGIII, and MtmGI. Finally, mtmOIV oxygenase catalyzes the formation of premithramycin B lactone. After spontaneous decarboxylation, mtmW ketoreductase catalyzes the reduction of the 4′-keto group to form mithramycin.

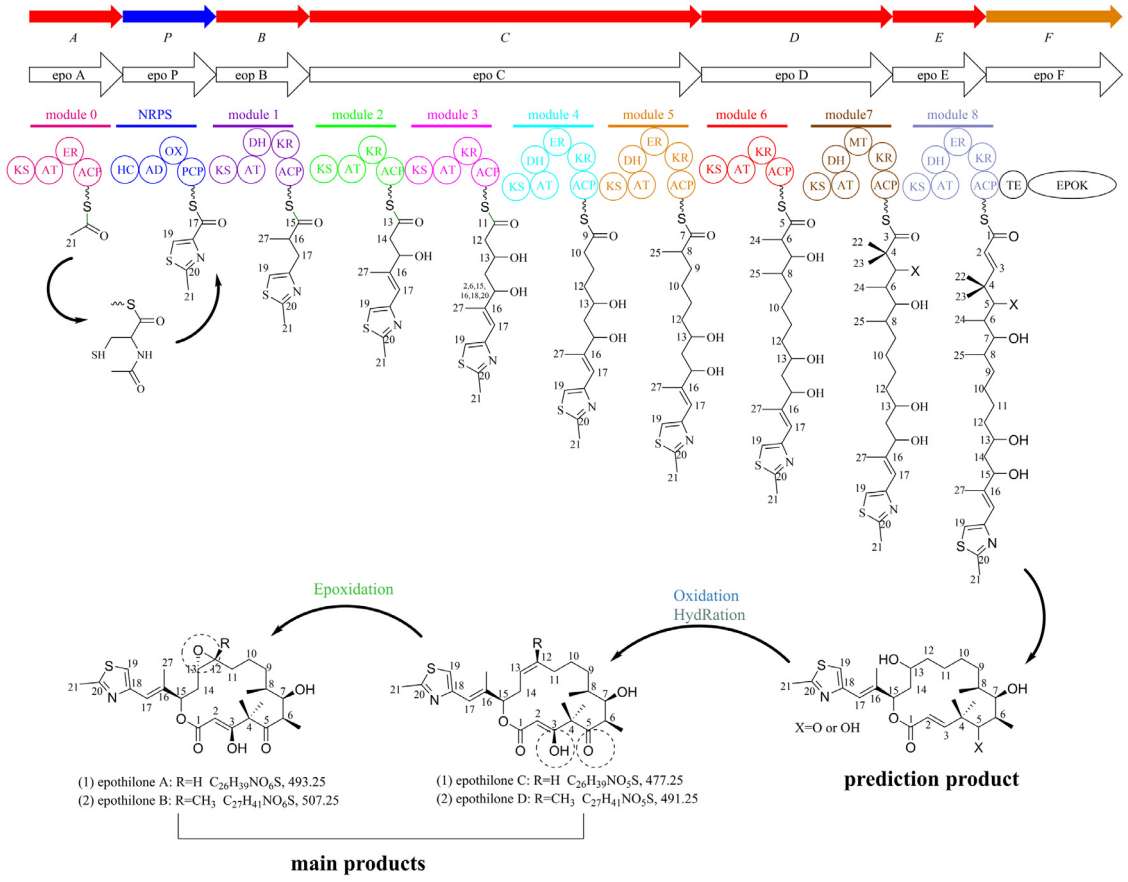

Fig. 7. The epothilone gene cluster and its proposed biosynthetic pathway [14]. The biosynthesis of epothilone has four stages: synthesis of the initial thiazole ring, extension of the polyketide chain, cyclization and release of the polyketide chain, and post-modification of the product. A, adenylation; ACP, acyl carrier protein; AT, acyltransferase; ER, Enoyl reductase; C, condensation; KR, ketoreductase; KS, ketosynthase; PCP, peptidyl carrier protein; TE, thioesterase; DH, β-hydroxyacyl dehydratase; Ox, Oxidation.

parts into two separate plasmids, and introduced them into *S. coelicolor* CH999, representing the first heterologous expression of epothilone (50–100 μg/L) [111]. Subsequently, Julien *et al.* (2002) achieved heterologous expression in a closely related host, *Myxococcus aureus*, by homologous recombination (100 μg/L) [112,113]. In 2006, Mutka *et al.* achieved the heterologous expression of epothilone in *E. coli* (< 1 μg/L) [114]. In 2008, Park *et al.* heterologously expressed epothilone in *S. venezuelae* DHS2001(0.1 μg/L) [115].

2.4.2. Rapamycin

Rapamycin (sirolimus, RAPA) is a nitrogen-containing triene MA that was isolated from *S. hygroscopic* AYB-944 in 1975 [116,117]. Rapamycin has antifungal, antiproliferative, and antitumor effects with a molecular weight of 914.2 [118]. In September 1999, the FDA approved rapamycin as an immunosuppressant for the prevention and treatment of renal transplant rejection. In 2003, rapamycin was approved by the FDA as a stent-coating drug for the treatment of coronary restenosis, and the FDA approved the C-43 derivative of rapamycin, temsirolimus, for advanced renal cancer in 2007. In 2009, the FDA approved the rapamycin derivative, everolimus, for the treatment of advanced renal cancer in patients that had failed treatment with sorafenib and sunitinib. In the same year, rapamycin was reported to prolong the lifespan of old mice, causing a sensation worldwide. It was then reported that rapamycin has a range of beneficial functions including improving heart function, treating osteoporosis, and possibly treating Alzheimer's disease. In recent years, several *in vitro* and *in vivo* experiments have shown that rapamycin can significantly induce tumor cell apoptosis and inhibit tumor cell growth and proliferation in the treatment of liver cancer, lung cancer, ovarian cancer, breast cancer, small cell lung cancer, and others cancer types [119,120]. The advantages of rapamycin are that it can selectively block signal transduction without damaging normal cells with few side effects. Currently, there is significant research interest in the development of rapamycin as a component of combination drug therapy. Phase I and II clinical trials of rapamycin as cancer treatment are currently in progress (NCT02423954, NCT02891603, and NCT02509468). Mammalian target of rapamycin (mTOR) is an atypical serine/threonine protein kinase located in the cytoplasm [121]. The protein structure of mTOR is highly conserved. mTOR belongs to the phosphatidylinositol 3 kinase (PI3K) protein kinase family and can stimulate signaling pathways in response to energy signals and growth factors to regulate cellular proliferation, growth, and differentiation, and the cell cycle [121]. Abnormal activation of the mTOR signaling pathway is directly related to the formation, development, and metastasis of various tumor types [122]. Rapamycin can form a rapamycin-FKBP12 complex with FKBP12 to inhibit mTOR activity. After mTOR activity is inhibited, the biologically inactive ribosomal S6 protein kinase (p70S6K) becomes unable to effectively phosphorylate its targets leading to the inhibition of mRNA translation and expression by translation elements encoding ribosomal proteins, translation elongation factors, and translation initiation factors that are controlled by p70S6K, thereby affecting protein synthesis. Thus, rapamycin functions by regulating downstream phosphorylation of a range of proteins related to translation and transcription, thereby affecting the expression of genes associated with tumor formation [123–125]. The organic synthesis of rapamycin is limited due to its complex molecular structure causing yields to be very low. At present, rapamycin production is mainly achieved

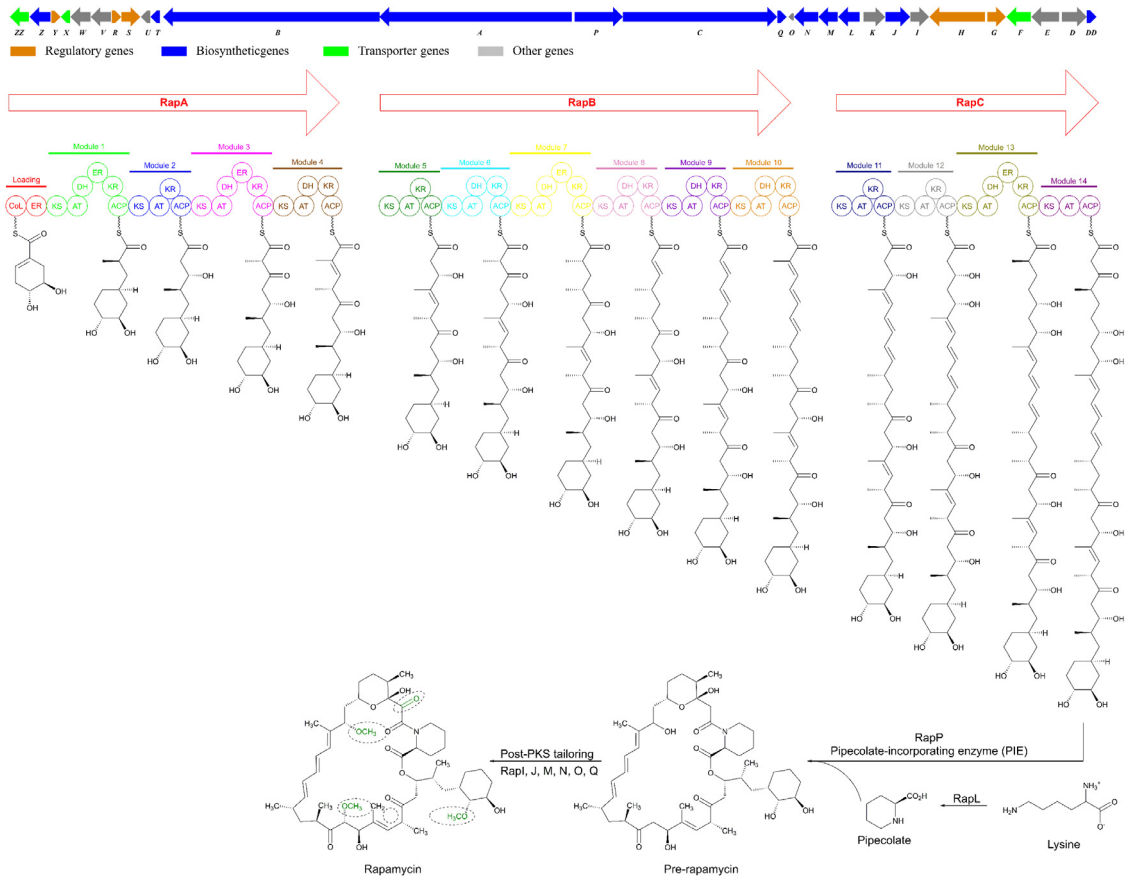

Fig. 8. Gene cluster and biosynthetic pathway of rapamycin [130]. The biosynthesis of the polyketide chain is initiated using reduced derivatives of shikimic acid used as a starter unit, which is extended sequentially by condensation steps with seven acetate and seven propionate. The polyketide skeleton is attached by a pipecolate moiety through a NPRS, which also catalyzes the cyclization of macrolactone ring to produce prerapamycin. Then, rapamycin is formed by sequential catalysis by polyketide synthase (PKS) including oxidation steps catalyzed by cytochrome P450 monooxygenases and O-methylation steps catalyzed by S-adenosylmethionine-dependent methyltransferases.

via microbial fermentation. There are two main rapamycin-producing microbial strains, *S. hygroscopics* AYB-944 and *Actinoplanes* sp. N902-109 [116,117]. Similar to other MA, rapamycin can also be synthesized through the polyketide peptide pathway, which can be divided into four steps (Fig. 8). The first step involves the initiation of rapamycin synthesis with the reduced derivatives of shikimic acid acting as starting units. The conversion process involves a 1,4-conjugation elimination reaction (dehydration), reduction, isomerization, and reduction. A starting unit containing a saturated cyclohexane ring is formed, which retains the integrity of cyclohexane [22]. Then, polyketide chain elongation occurs with coenzyme A acting as a carrier of the carboxylic acid precursor. After condensation with the carboxylic acid precursor, a series of catalytic reactions form malonyl coenzyme A and methylmalonyl-CoA. The condensation reaction is completed under the catalysis of the three polypeptide domains of RAPS1, RAPS2, and RAPS3, and the polyketide chain is extended [126,127]. The next step is the cyclization of the polyketide chain, which occurs through a specific mechanism. NRPS is the core enzyme in the biosynthesis of polyketides and can promote the cyclization of polyketide chains. L-lysine cyclodeaminase (RapL) catalyzes the conversion of L-lysine to L-pipecolic acid, and NRPS catalyzes the binding of L-pipecolic acid to the polyketide chain skeleton of rapamycin to achieve cyclization of the polyketide chain. Through this process, an intermediate for rapamycin biosynthesis is formed. After further modifications, a complete rapamycin structure can be obtained. The last step is modification and is performed in four steps. The first step is C39 methoxylation, which is catalyzed by methyltransferase (RapI); the second step is the production of ketone, which is produced by C9 through cytochrome P450 monooxygenase (RapJ) catalytic oxidation; the third step is the methoxylation of the hydroxy group on C16 by methyltransferase (RapM); and in the fourth step, C27 catalyzes hydroxylation by cytochrome P450 monooxygenase (RapN). The resulting product is methylated by methyltransferase (RapQ). After these four steps, complete rapamycin is obtained [128,129].

2.5. Enediynes

Dienediyne antibiotics are a novel class of antitumor antibiotics with special molecular structures and unique mechanisms that have potential utility in a broad range of applications. The active center of dienediyne contains a characteristic enediyne structure. Enediyne antibiotics, including lidamycin, calicheamicin, and neocarcinstatin, have good inhibitory activity against a range of tumor types, particularly hematological tumors.

2.5.1. Lidamycin

Lidamycin (LDM) is a novel antitumor antibiotic isolated from *S. globisporus* C-1027 [131]. LDM has bacteriostatic effects against most gram-positive bacteria but is ineffective against gram-negative bacteria and mycobacteria. LDM consists of an active aromatic chromophore (AE) and acidic carrier protein (LDP) [132]. AE is the active part of LDM that exerts an antitumor effect and contains a 9-membered cyclic enediyne. The function of the LDP is to protect the AE. LDM is cytotoxic to a range of tumor cells *in vitro* and *in vivo*, with greater toxicity than most commonly used chemotherapeutic drugs. The mechanisms of

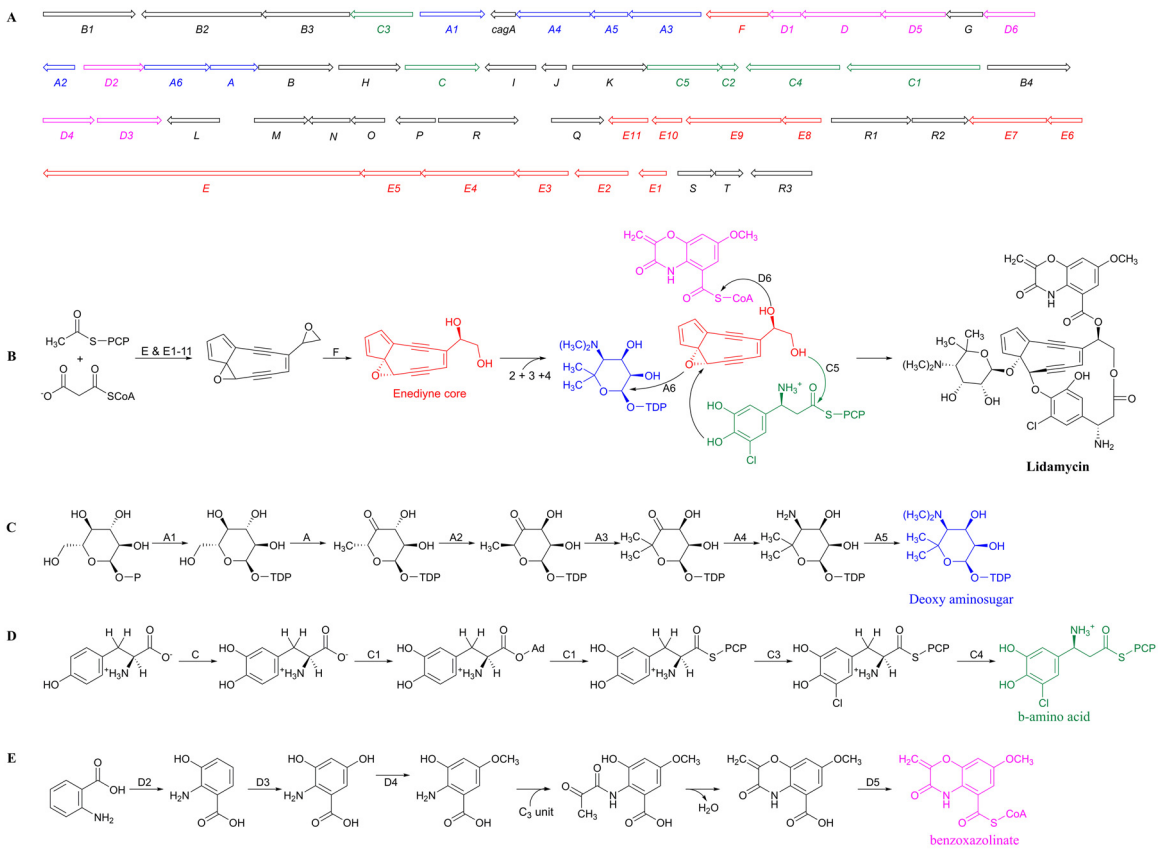

Fig. 9. (A) Organization of the lidamycin biosynthesis gene cluster. Hypothetical biosynthetic pathways for the enediyne core (B), deoxy aminosugar (C), *β*-amino acid (D), benzoxazolinate (E) and their convergent assembly strategy [146]. Gene *E* encodes PKSs; *E6, E7*, and *E9* encode various oxidoreductases; *E10* encodes thioesterase; *F* encodes epoxide hydrolase; *E1–E5, E8*, and *E11* have unknown functions. Gene *A-A6* is responsible for the biosynthesis of deoxy aminosugar, which contains a TDP-glucose 4,6-dehydratase (*A*); a TDP-glucose synthetase (*A1*); a TDP-4-keto-6-deoxyglucose epimerase (*A2*); a C-methyl transferase (*A3*); an amino transferase (*A4*); an N-methyl transferase (*A5*); and a glycosyl transferase (*A6*). Gene *C-C5* is responsible for the biosynthesis of *β*-amino acid, which contains a phenol hydroxylase (*C*); NRPSs (*C1, C2, C5*); a halogenase (*C3*); and an aminomutase (*C4*). Gene D-D6 is responsible for the biosynthesis of benzoxazolinate, which contains anthranilate synthase (*D* and *D1*); monoxygenase (*D2*); a P-450 hydroxylase (*D3*); an O-methyl transferase (*D4*); a CoA ligase (*D5*); and an acyltransferase (*D6*).

action of LDM are diverse depending on the tissue origin and genetic background of tumor cells and include: binding to the minor groove of the DNA double helix causing DNA strand breakage and debasing, inhibiting tumor angiogenesis [133,134]; inducing tumor cell apoptosis, cleavage, and senescence-like phenotypes [135–139]; interfering with cell cycle and arresting cells in G1 or G2/M phase [134]; and interfering with the activity of signaling pathways related to tumor growth, such as K-ras, Akt, NF-κB, and MAPK signaling pathways [140,141]. The targeting of antibody drugs against tumor cell surface antigens represents an effective method of drug therapy. Riuxan was the first antitumor antibody drug approved by the FDA in 1997 for the treatment of B-cell non-Hodgkin lymphoma. To enhance the killing activity of antibody drugs on tumor cells and the selectivity of conventional chemotherapy drugs, doxorubicin, daunorubicin, and BLM have been coupled with antibodies to produce antibody-drug conjugates (ADC). ADCs can specifically recognize tumor antigens, enter target cells through receptor-mediated endocytosis, and release molecular “warheads” to selectively kill tumor cells. Both the killing activity of ADCs against tumor cells and their activity against tumor target cells are higher than conventional drugs [141,142]. The above mechanisms underlie the use of lidamycin as a “warhead” for ADC. Studies have confirmed that the ADC or peptide conjugates formed by lidamycin with antibodies and peptides, such as conjugates between CD30 antibody and LDM and peptide conjugates formed by EGFR/MMP-2 and LDM, have greater efficacy than monoclonal antibodies *in vivo*. These mechanisms also allow targeting of tumor cell surface antigens [143,144]. The enediyne core of LDM is biosynthesized by a polyketide pathway involving PKS SgcE through acetyl-CoA and seven malonyl-CoA units in an iterative process (Fig. 9). The linear polyunsaturated intermediate is then modified by the associated enediyne cluster enzyme to obtain an appropriate enediyne core [145]. In addition to the 9-enediyne core, LDM contains three distinct structural units; deoxyamino sugars, *β*-amino acids, and benzoxazolinate moieties [146]. Deoxy amino sugars and *β*-tyrosine are derived from glucose-1-phosphate and L-tyrosine, respectively [145], and the benzoxazolinate moiety is produced by a novel biosynthetic pathway that starts with chorismate [147]. However, the synthetic pathway of benzoxazolinate moieties has yet to be experimentally validated.

2.5.2. *Calicheamicins*

Calicheamicin (CLM), extracted from *Micromonas aculeatus* in 1986, is a small-molecule lipid-soluble compound belonging to the ten-membered cycloenediyne class of antitumor antibiotics with no protein component and relatively high stability. CLM has potent toxicity against gram-positive and gram-negative bacteria, with an antibacterial activity 4000-fold higher than doxorubicin. The molecular weight of CLM is 1500. CLM comprises a rigid bicyclic core with two hydroxyl side chains and includes four deoxyamino sugars and an orsellinic acid derivative, which are attached to the enediyne core by glycosyltransferase [148]. CLM is an efficient DNA-cleaving agent. The auxiliary group (oligosaccharide fragment) in the CLM molecule acts as the recognition site for targeting and contributes to binding of the enediyne core of CLM to the minor groove of the DNA double helix, which causing DNA breakage

and tumor cell death. The killing activity of CLM on tumor cells is reported 1000-fold greater than that of doxorubicin, which can be used as a high-efficiency "warhead" in ADC drugs. Currently, CLM is used as a "warhead" in clinically used ADC drugs including gemtuzumab ozogamicin (MYLOTARG, in clinical phase of trials) and inotuzumab ozogamicin (BESPONSA, approved in the US in 2017). Thus, CLM is an effective "warhead" molecule that can be conjugated to different antibodies to construct novel ADC drugs [149,150]. The biosynthetic pathway of CLM begins with L-proline adenylation by CalN2, which then replaces AMP with CalN3SH (Fig. 10). CalN1 catalyzes the formation of two double bonds in the prolyl ring to produce an α-ketopyrrole derivative. The PKS process uses α-ketopyrrole-S-CalN3 as the starter unit with four methylmalonyl and two malonyl units to produce the polyketide chain of CLM. The synthesis of 3-hydroxyanthranilic acid is catalyzed by CalB1 to CalB4 with PEP and E4P. The amino group of 3-hydroxyanthranilic acid then nucleophilically attacks the bond between the polyketide chain and releases it from the acyl carrier protein (ACP). A series of undetermined reactions then occur to produce CLM.

2.6. Purine nucleoside analogs-Pentostatin

Pentostatin was first discovered in 1974 by Woo *et al.* as a white crystalline purine-mimicking antitumor drug isolated from soil microorganism *S. antibioticus.* In 1995, Warner-Lambert further optimized the separation and purification process of pentostatin and established an efficient pentostatin extraction method, with an extraction purity of 99.7%. In 1998, the FDA officially approved pentostatin (trade name, Nipent) for the treatment of chronic lymphocytic leukemia, hairy cell leukemia, and other diseases [152]. Pentostatin is a purine-like antimetabolite with antitumor activity mediated by inhibition of adenosine deaminase (ADA). ADA catalyzes the irreversible deamination of intracellular adenosine and deoxyadenosine, thereby regulating intracellular adenosine levels. Pentostatin inhibits the activity of ADA, resulting in substantial intracellular accumulation of deoxyadenosine and 5′-deoxyadenosine triphosphate (dATP), with the accumulation of dATP eventually inducing cell death. This process may be related to the inhibition of DNA or RNA synthesis. In addition, combination therapies comprising pentostatin and other drugs have become commonly used clinical treatments for malignant tumors [17]. For example, the combination of pentostatin and venetoclax is used in the treatment of acute lymphoblastic leukemia [153] and the combination of pentostatin, cyclophosphamide, and rituximab has significant efficacy in the treatment of lymphoma [154]. In 2017, Chen *et al.* introduced the gene cluster, *pen*, into *S. aureochromogenes* CXR 14 and achieved the heterologous synthesis of pentostatin [155]. This gene cluster is 10.5 kb and contains 10 genes termed *penA–penJ*. PenA, PenB, and PenC are key enzymes in pentostatin biosynthesis, which jointly mediate pentostatin synthesis. PenA was identified as an ATP phosphoribosyltransferase homologous to HisG responsible for the coupling of phosphoribosyl pyrophosphate (PRPP) to dATP in the histidine metabolic pathway. *In vitro* experiments have shown that PenB is a short-chain dehydrogenase A member of the enzyme family responsible for the final step of pentostatin synthesis, while PenC is a homologue of SAICAR synthase. The function of PenC has yet to be elucidated. In 2020, Ren *et al.* demonstrated the purification of PenA using only dATP as a substrate, with PenA identified as the rate-limiting enzyme in the pentostatin biosynthetic pathway (Fig. 11) [156].

2.7. Benzoquinones-Geldanamycin

Geldanamycin (GA), a benzoquinone antibiotic, was first isolated from *S. hygroscopicus var. geldanus var. nova* in 1970 and was the first heat shock protein 90 (Hsp90) N-terminal inhibitor to be identified [157]. GA has various biological activities including antibacterial, antiprotozoal, antitumor, antiviral, immune regulation, and anti-inflammatory effects. GA has also been shown to regulate the activity of epithelial nitrogen oxygenase. Of these effects, the potent antitumor activity of GA has attracted widespread attention from scientists [158]. The crystal structure of the Hsp90-GA complex revealed that GA is a competitive inhibitor of ATP. GA can compete with ATP and specifically bind to the ATP-binding site of Hsp90, thereby inhibiting Hsp90 function [159]. Hsp90 is a protein molecular chaperone widely and abundantly present in mammalian cells that has important effects on the cell cycle, cell growth, cell survival, apoptosis, and carcinogenesis. The inhibition of Hsp90 function by GA directly leads to the rapid degradation of many important proteins in tumor cells, thereby inhibiting the growth of tumor cells or triggering tumor cell death. Studies have shown that the expression of Hsp90 [160] in tumor cells is much higher than that in normal cells, and the binding capacity of GA to Hsp90 in tumor cells is 100% higher than that in normal cells, thereby greatly improving the specificity of GA against tumor cells [161,162]. To date, hundreds of studies have been conducted on GA and its derivatives and more than 500 derivatives of GA have been developed. 17-allylamino-17-demethoxygeldanamycin (17-AAG) is a GA derivative that is produced by replacing the methoxy group at the 17th position of GA with an allylamino group. The biological activity and toxicity of 17-AAG are substantially greater than GA [163]. Phase III clinical trials of 17-AAG have been completed [14]; however, the efficacy of 17-AAG in the treatment of advanced tumors is limited by poor solubility and off-target toxicity. To achieve the best therapeutic effect, 17-AAG is currently administered in combination with other anticancer regimens. 17-AAG has also been found to slow the development of encephalomyelitis in mice by impairing T-cell function. 17-AAG also disrupts HIV protein expression by inhibiting Hsp90, with *in vivo* targeting of Hsp90 also shown to interfere with norovirus replication [164]. 17-dimethylamino-ethylamino-17-demethoxygeldanamycin hydrochloride (17-DMAG) is a derivative obtained by replacing the methoxy group at the 17th position of GA with N, N-dimethylethylamine. 17-DMAG has improved oral bioavailability and antitumor activity compared to that of 17-AAG [165]. A phase I clinical study of 17-DMAG has recently been completed [166]. 17-DMAG can increase number of CD8-positive T cells, thereby reducing proteinuria. IPI-493 is a derivative of GA in which the 17th methoxy group is substituted with an amino group. Compared with that of GA, IPI-493 has greatly improved biological activity and toxicity [167]. IPI-493 has strong antitumor potential against gastrointestinal stromal tumors. IPI-493 has substantial antitumor activity when administered alone and its therapeutic efficacy can be further enhanced when used in combination with imatinib or sunitinib. Moreover, the combinatorial use of Hsp90 and tyrosine kinase inhibitors can potentially overcome resistance to imatinib or sunitinib in patients with intestinal stromal tumors [168]. IPI-504 is a water-soluble hydroquinone hydrochloride derivative of 17-AAG with improved affinity and water solubility compared to 17-AAG [169]. IPI-504 was once considered the most promising inhibitor derived from GA; however, phase I to phase III clinical trials in patients with multiple myeloma, non-small cell lung cancer, breast cancer, and gastrointestinal stromal tumors either failed to achieve the expected efficacy or observed higher mortality in the treatment group. Accordingly, clinical trials of IPI-504 were terminated. Andreas *et al.* cloned the biosynthetic gene cluster of GA in 2003 with an approximately 60 kb size comprising 16 genes including PKS, precursor synthesis, post-modification enzymes, and regulatory genes (Fig. 12) [29]. AHBA, the initial unit of GA biosynthesis, is produced from glucose in a process predominantly catalyzed by aminoDHQ synthase, aminoDAHP synthase, aminoDHQ dehydrogenase, and AHBA synthase [170]. Another important biosynthetic region is the PKS module. In the geldanamycin biosynthesis gene cluster, *gdmAI, gdmAII*, and *gdmAIII* encode seven modules of PKS, namely acyltransferase (AT), ketone synthase (KS), ACP, ketoreductase (KR), dehydratase (DH), enol reductase (ER), and methyltransferase (MT). First, the initiation unit is catalyzed by an acyl ligase to form an acyl-adenylate intermediate, which loads AHBA onto ACP0 [10], followed by the transfer of the initiation element from ACP0 to KS1 and the 2-methylmalonyl extension element is bound to ACP1

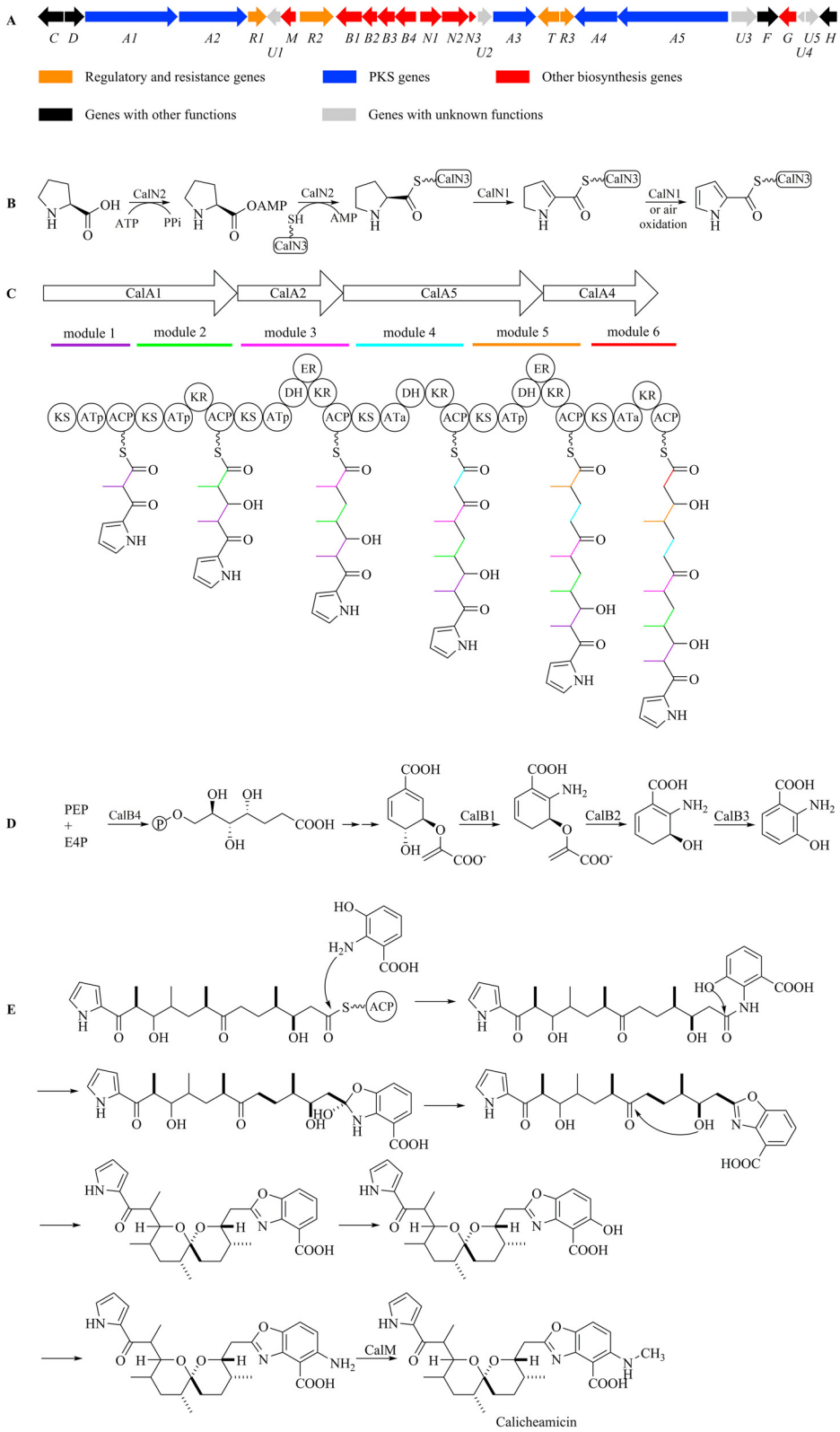

Fig. 10. (A) Calicheamicin gene cluster. Proposed biosynthetic pathways for pyrrole (B), polyketide (C), benzoxazole moieties (D), and generation of the benzoxazole ring and further maturation (E) of CLM [151]. (A) The proposed functions of individual ORFs are indicated. (B) L-proline is adenylated by CalN2 and then takes place of the adenyl group with CalN3SH. CalN1 oxidizes the prolyl ring to produce the α-ketopyrrole derivative. (C) Then, the PKS assembly line starts with α-ketopyrrole-S-CalN3 and uses four ethylmalonyl and two malonyl units to generate the polyketide backbone of CLM. (D) The precursor 3-hydroxyanthranilic acid is catalyzed by CalB1 to CalB4. Finally, the benzoxazole ring is generated and undergoes further maturation to produce CLM. CalB1, anthranilate synthase; CalB2, isochorismatase; CalB3, 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase; PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; ADIC, 2-amino-2-deoxyisochorismate; DHHA, trans-2,3-dihydro-3-hydroxyanthranilic acid; HA, 3-hydroxyanthranilic acid.

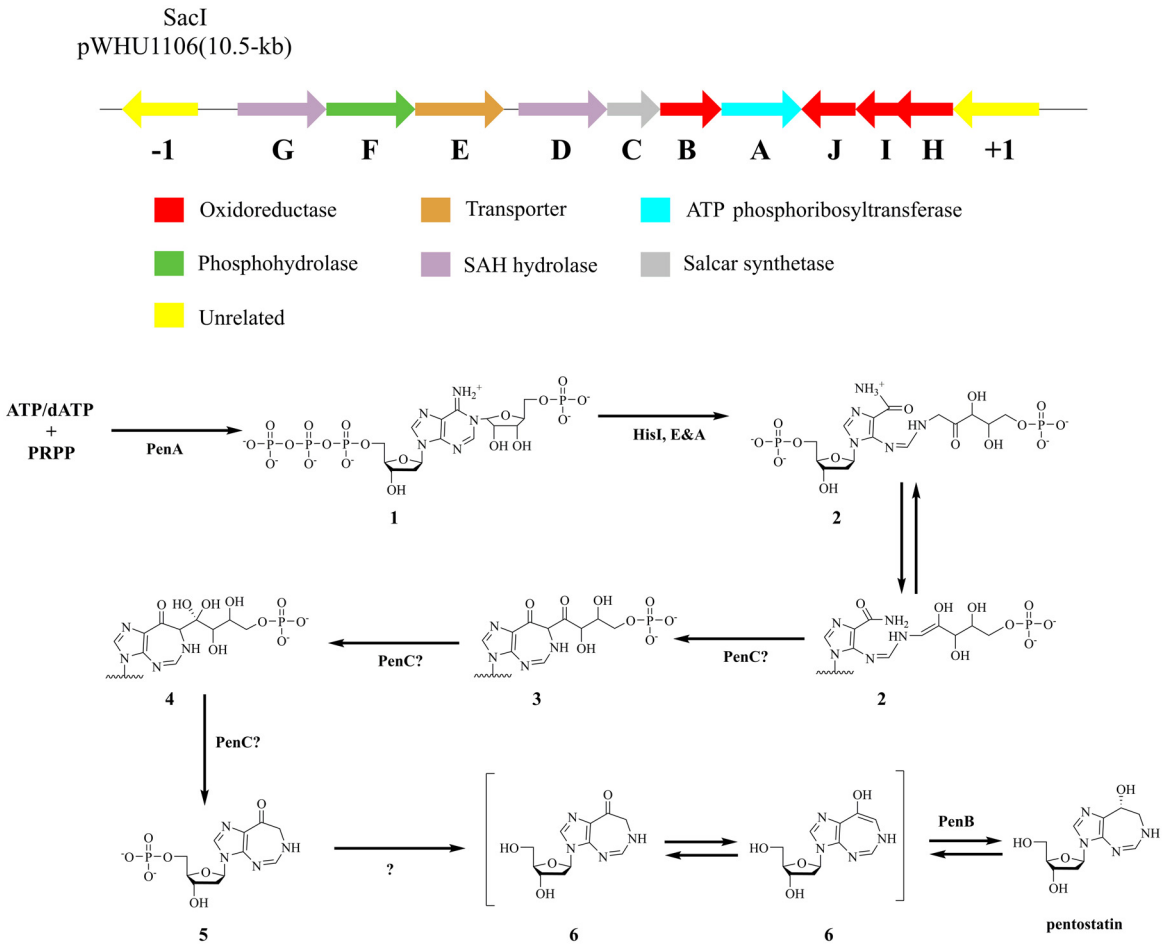

Fig. 11. The gene cluster of pentostatin and its proposed biosynthetic pathway [155]. PenA is responsible for the condensation of dATP/ATP and PRPP to produce compound 1 that is sequentially converted to compound 2 under the catalysis of three enzymes; HisI, HisE, and HisA in the histidine metabolic pathway. Compound 2 undergoes a number of steps catalyzed by PenC to generate compound 5. PenB catalyzes the last step of pentostatin synthesis.

[10]. Simultaneously, the extension element is added to ACP via AT catalysis and KS catalyzes the reaction between the initiation and extension elements, with the initiation unit transferred from KS to the extension element of ACP to form a new polyketide chain [171]. After the synthesis of the polyketide chain, amide synthase, encoded by the *gdmF* gene, catalyzes the condensation of the polyketide chain to synthesize the precursor of GA followed further reactions catalyzed by the *gdmM*-encoded monooxygenase, *gdmN*-encoded carbamoyltransferase, and *gdmP*-encoded oxygenase [20, 171-173], resulting in the final synthesis of GA. However, the catalytic sequences of these enzymes have yet to be fully elucidated.

3. Strategies and technologies for the discovery of natural products

Many antitumor drugs are derived from microbial metabolites. Hence, the identification of natural microbial products may contribute to drug discovery and development. Traditional research methods for identifying microbial natural products typically involve three steps. The first is the isolation of culturable strains from the environment. Then, natural products are extracted and purified by fermentation. Third, candidate compounds are screened for further evaluation (Fig. 13A). However, the identification of sufficient numbers of bioactive natural products while avoiding the rediscovery of known compounds is technically challenging due to the large numbers of natural products discovered over the last two centuries [174].

Therefore, there is a need for new strategies for the development of of novel drugs from natural products (Fig. 13B). The primary aim is to develop more tools and technologies to identify novel natural products to contribute to compound libraries for hit screening. The identification and modification of biosynthetic pathways in original strains to yield sufficient amounts of target products has been technically challenging as many microorganisms are hard to culture and genetically manipulate in laboratory settings. Thus, bioinformatics tools have been used to reveal biosynthetic gene clusters (BGCs), with heterologous expression of these BGCs in suitable hosts contributing to the discovery of new natural products. As genomics have proved invaluable in the identification of new natural products, bioinformatics tools represent a powerful approach for the identification of BGCs that potentially encode previously undiscovered natural products [175]. Restriction enzymes and CRISPR technology can then be used to isolate target BGCs and insert them into target vectors through self-recombination repair in the heterologous host. As an example, Fu *et al.* reconstructed epothilone gene clusters using Red/ET technology and Bian *et al.* introduced these clusters into *Burkholderiales* DSM 7029 for heterologous expression. Following optimization of fermentation conditions and genetic modification of host bacteria, the total yield of epothilone was increased 75-fold (307 μg/L) [176,177]. In addition, Li *et al.* spliced the epothilone gene cluster of *Sorangium cellulosum* So0157-2 distributed on two library clones through homologous recombination and then integrated the entire gene cluster into different *M. xanthus* clones by transposition. The fermentation yield of epothilone in the *M. xanthus* ZE9 mutant improved to approximately

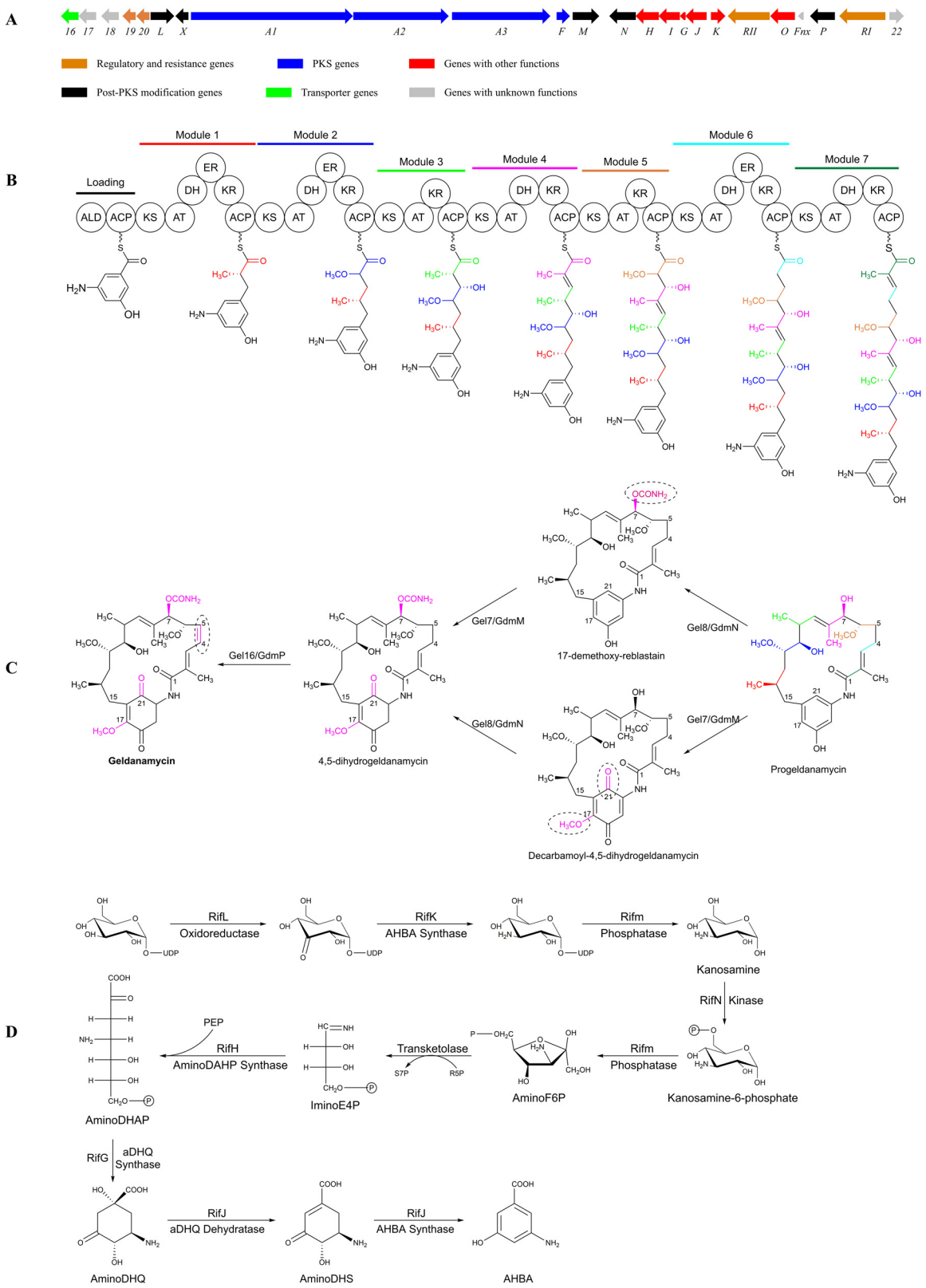

Fig. 12. (A) The geldanamycin gene cluster. (B, C) Proposed synthetic pathway of geldanamycin [171]. (D) AHBA formation in *A. mediterranei* [170]. (A) Proposed functions of individual ORFs are indicated. (B, C) The hypothetical pathway for GA biosynthesis begins with production of the intermediate, progeldanamycin, from AHBA by the PKS assembly line with seven chain-elongation units followed by formation of GA by post-PKS modifications. (D) The precursor AHBA is generated by several primary metabolic enzymes including aminoDHQ synthase, aminoDAHP synthase, aminoDHQ dehydrogenase, and AHBA synthase.

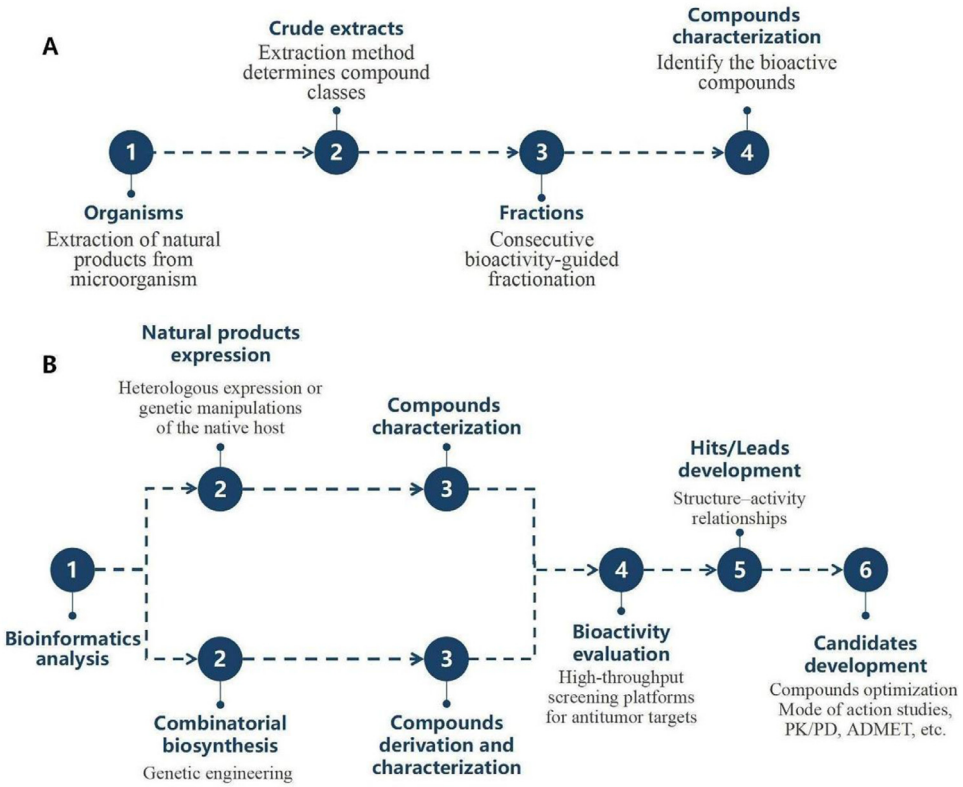

Fig. 13. (A) The steps of traditional natural product drug discovery. (B) Novel strategies for the discovery of microbial-derived antitumor drugs [184,185].

1 mg/L, reaching the highest level of heterologous expression reported [178].

Furthermore, combinatorial biosynthesis is rapidly developing as a result of flourishing genetic engineering technologies and bioinformatics, which provides a strategy for the precise discovery of target natural products with structural diversity and potential biological activities. For example, Kudo *et al.* [179] optimized the rapamycin biosynthetic pathways by deleting and replacing modules in the BGCs using CRISPR/Cas9 and Gibson assembly technology, thereby obtaining a series of rapamycin derivatives with improved activity and lower toxicity. In addition, Shen *et al.* [180] successfully identified 49 bacterial strains carrying potential genes encoding leinamycin-type compounds by mining bacterial genomes from public databases then identifying and characterizing new natural products belonging to the leinamycin family including two novel compound types named guangnanmycins and weishanmycins. As many well-known antibiotics, such as rapamycin and leinamycin, are considered lead compounds in the discovery of antitumor drugs, the use of rational combinatorial biosynthesis approaches for the discovery of their respective derivatives using bioinformatics and gene editing may provide a novel source of promising drug leads.

In addition, more natural products with potential antitumor activity could be obtained using phylogenetic-based methods in genome mining. The structural diversity of natural products is the result of the continuous evolution of BGCs. Molecular phylogeny is a commonly used technique for tracking the evolutionary footprint of a specific gene sequence and determining its evolutionary relationship to homologous sequences. The basic idea of phylogeny-oriented discovery of new natural products is based on the co-evolution of a biosynthetic gene and its respective biosynthetic gene cluster, which can be used as a phylogenetic marker to represent the evolutionary path of its entire biosynthetic gene cluster and determine the degree of novelty of natural products [181]. Anthracyclines are a class of natural products with antitumor activities, of which the representative molecule, doxorubicin, has been used in clinical anticancer chemotherapy for more than 30 years [182]. The gene cluster, AZ129, was identified from the metagenome and found to have a set of biosynthesis genes similar to the known anthraquinone compound, steffimycin BGC, in a phylogenetic analysis [183]. Heterologous expression of the AZ129 gene cluster in *Streptomyces albus* resulted in a novel natural product, arimetamycin A, with greater antitumor activity than doxorubicin in tumor cell proliferation assays *in vitro* [183].

Regarding future directions for the discovery of antitumor drugs and lead compounds from natural products, high-throughput drug screening platforms appears to represent an important approach for the rapid identification of potential hits. The design of screening methods and relevant instruments is key to the establishment of high-throughput drug screening platforms. The development of probes or sensors for different targets and key signaling pathways involved in antitumor mechanisms may provide more efficient screening methods, thereby increasing the identification of bioactive natural products of interest. Hence, approaches combining microbiology, synthetic biology, and pharmacology may accelerate the discovery of natural products with superior pharmacological properties. In addition, it may be more beneficial to determine the structure of new compounds identified from natural sources before bioactivity screening, thereby avoiding the rediscovery of known compounds. Structure-activity relationships (SAR) are useful theoretical models for predicting the biological activities and physicochemical properties of compounds in terms of drug discovery and development, which may increase the identification of new antitumor compounds from microorganisms. The continued development of bioinformatics tools may help determine the relationships between BGCs characteristics, structures, and activities and generate theoretical models for further prediction of compounds of interests.

The rapid development of gene editing techniques, bioinformatics, combinatorial biosynthesis approaches, and microbial platforms based on synthetic biology are expected to facilitate efficient synthesis and innovative discovery of microbial natural products. The application of high-throughput drug screening platforms and the combined use of conventional and advanced tools in pharmaceutical science

may accelerate the discovery of novel natural products with antitumor properties.

4. Conclusion

The present review summarizes anticancer agents and candidates identified as natural compounds in microorganisms and provides descriptions of their pharmacological properties, mechanisms of action, and biosynthesis. These drugs play important roles in cancer treatment. The majority are DNA intercalation agents, such as actinomycin D, doxorubicin, mitomycin C, and daunorubicin, which intercalate with double-stranded DNA to cause DNA breaks and disrupt DNA replication and transcription. In addition, the chemotaxonomies and biosynthesis of these moleculres are summarized. Most of these compounds are derived from *Streptomyces* and *Actinomyces*, and their biosynthesis can be generally divided into four steps: synthesis of precursor substances; elongation of carbon chains mediated by PKS, NRPS, or PKS-NRPS pathways; intramolecular or intermolecular condensation reactions; and subsequent modifications. The biosynthetic pathways of some antitumor drugs, such as epothilone and rapamycin, have been fully elucidated. However, further studies are required to reveal the biosynthesis pathways of several antitumor drugs, such as mitomycin C, MTM, and pentostatin. Epothilone, trichostatin, staurosporine, and rebeccamycin have been expressed in heterologous hosts, which may allow microbial chassis engineering and gene cluster refactoring to generate high-yielding strains for industrial cost reduction.

Furthermore, we discuss several issues related to microbial-derived drug discovery and development. The expansion of bioinformatics tools and models may have utility in predicting and identifying BGCs, providing information on unknown compounds with potential antitumor activity. New technical advances in gene editing, such as CRISPR/Cas and Red/ET, may promote the rapid discovery of microbial secondary metabolites and the elucidation of their respective biosynthetic pathways. Moreover, the combination of bioinformatics and gene editing may provide a combinatorial biosynthesis approach to the design artificial gene clusters for increasing target compound diversity and yields. Taken together, the approaches described above are expected to allow increased discovery of novel microbial natural products with antitumor activity. Further, the combination of screening methods, pharmaceutical tools, and bioinformatics may allow optimization of screening methods for novel drugs and lead compounds.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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