Expert Opinion

Antitumor conjugates with polyamine vectors and their molecular mechanisms

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Importance of the field: A polyamine conjugate is a special polyamine derivative composed of polyamine vectors appended directly or by a linker to a cargo with specific biological functions. In recent years, extensive researches have emphasized the fact that polyamine conjugates acting as promising antitumor candidates are becoming increasingly important in the polyamine field.

Areas covered in this review: Two key subjects are illustrated in this review. First, various drug-polyamine conjugates and relevant structure–activity relationships are discussed with a focus on the molecular recognition of polyamine transport system (PTS). Second, the design of polyamine conjugates is following a rational mechanism-based strategy. Therefore, it is critically important to understand the intrinsic properties of PTS on the cell membrane, enhanced pharmacological effects of polyamine vector on cellular components, and resulting comprehensive signaling networks.

What the reader will gain: A general design strategy of polyamine conjugates as well as recent progress in both fundamental mechanism studies and preclinical therapies are provided for the readers.

Take home message: The multiple functions of polyamine moieties in objective conjugates furnish broad development space for more efficacious antitumor agents.

Keywords: antitumor agents, molecular mechanism, polyamine conjugates, polyamine transport system

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1. Introduction

Polyamines, putrescine (PUT), spermidine (SPD) and spermine (SPM), a group of biogenic small molecules existing abundantly in mammalian cells, possess intriguing functions in cell growth, differentiation and other physiological processes [1]. Owing to the established close association between upregulated polyamine levels and tumor growth, numerous multidisplinary efforts with the ultimate goal of developing polyamine-based antitumor drugs have been focused on the polyamine pathway, through which the intracellular polyamine concentration is tightly regulated [2]. From a medicinal chemistry perspective, there are several strategies in polyamine-based drug design (**Figure 1**), following which some kinds of agent with functions including the inhibition of the polyamine biosynthesis and import, and the elevation of polyamine catabolism and export, have been developed.

The initial attempts on the design of specific enzyme inhibitors at different steps of the polyamine cycle are aimed at decreasing the polyamine concentration, and correcting the cell stasis and/or cell death. For example, α-difluoromethylornithine (DFMO), an irreversible inhibitor of ornithine decarboxylase (ODC), was originally designed, in the late 1970s, as an antitumor drug to block the polyamine biosynthesis [3]. However, most of these mechanism-based inhibitors failed to

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Article highlights.

- The strategies of antitumor drug design based on polyamines are discussed briefly from the viewpoint of medicinal chemistry. The polyamine conjugates targeting the PTS for efficient drug delivery are attracting more and more attention.
- Without the precise molecular structure of mammalian PTS, two indirect methods, CHO/CHO-MG and DFMO/SPD experiments, are often used to evaluate the PTS recognition.
- Thus far, diverse polyamine conjugates, structurally similar to some natural polyamine products, have been designed to probe the structure–activity relationships, and several characteristic properties of PAT have been determined based on a series of biochemical assays.
- Other than PTS on the cell membrane, polyamine conjugates can also target cellular molecules, and induce cell cycle arrest and apoptosis.
- The clinical trials of F14512, one spermine conjugate as an antitumor agent, together with some spermine conjugates as a fluorescent probe to select patients suitable for F14512, will provide a new avenue for the development of antitumor polyamine conjugates.

This box summarizes key points contained in the article.

translate to success in the clinic, partially because cells harness multiple compensatory mechanisms to maintain homeostasis in polyamines, such as the uptake of polyamines from external circumstances by means of the active polyamine transport system (PTS) [4]. From the late 1980s until now, a wide variety of polyamine analogues such as MDL72527 and DENSPM with polyamine backbones modified by various simple alkyl or aryl groups have been synthesized as potential antitumor agents. Typically, these polyamine analogues, which enter cells readily through the same PTS as native polyamines, showed therapeutic effects by interacting with diverse cellular effector sites in the polyamine pathway [2]. Comprehensive reviews of polyamine-based drugs noted above have been published recently [5].

The latest trend in polyamine drug discovery is to utilize PTS as a target apart from prior polyamine biosynthetic and catabolic enzymes [5,6]. One new hypothesis is to develop PTS inhibitors such as ORI1202, a lipophilic polyamine conjugate, which can block the polyamine import from external sources [7]. It seems that the downregulation of polyamine uptake by ORI1202 cannot inhibit the cell growth effectively. However, the combined administration of such polyamine transport inhibitors and known polyamine biosynthesis inhibitors depletes the cellular polyamine concentration more efficiently, which may in turn lead to better clinical response [8].

Another contrary idea in PTS utilization is from the fact that fast growing tumor cell types often have more active PTS, which facilitates the entry of exogenous polyamines. More importantly, some structurally modified polyamines could be transported into cells as imitations of native polyamines by means of the same transporter, which indicates that polyamines may serve as a potential vector to deliver multiple antitumor agents to cells depleted of polyamines [9]. As such, hybrid molecules combined by polyamines and these toxic cargoes may have improved therapeutic efficacy because of the elevated cellular import.

Many antitumor drugs in clinical use are poorly tumor selective. Therefore, their therapy often causes high incidences of adverse effects, which has given great momentum to improving the tumor selectivity, efficiency and safety. One feasible and less consuming approach is to attach the drug to a molecular vector. The drug-vector conjugate is thus endowed with the properties of the vector. There are many types of small molecular vector, including steroids, cationic lipids, and so on. Both natural and synthetic polyamines are also identified to be such vectors [10]. Indeed, antitumor agents conjugated with smart polyamine vectors may have elevated affinity for cancer cells, and reach the targeted tissues more specifically. The dual utilization of the properties of known antitumor agents and polyamines provides a promising avenue for the investigation of targeted tumor chemotherapy [11-13]. The intent of this review is twofold: to discuss the design of antitumor polyamine conjugates transported into cells by means of the PTS, and to address the preliminary molecular mechanism of such conjugates.

2. Polyamine transport system

To meet their demand for growth, cells get sufficient polyamines from two kinds of channel: endogenous biosynthesis and exogenous import. Indeed, proliferating cells have been proved to have a more active PTS than resting ones. Although the phenomenon of polyamine uptake through specific PTS has been known for many years, the gene and its molecular structure of the mammalian PTS are still unclear [14]. Nevertheless, multifaceted research has elucidated some general features of PTS, which have been identified to be carrier-mediated, time-, temperature-, pH-, energy- and concentration-dependent, and saturable [6,15-19]. In addition, different PTSs may exist according to cell types. Some cells, such as L1210 murine leukemia cells [20], seem to utilize a single transporter for all three polyamines, whereas two kinds of transport system are found in many other cells, one having a priority for PUT, and the other for SPD and SPM [21,22].

There is also some important progress on the critical aspect of how PTS delivers polyamine or polyamine-like molecules across the lipophilic cell membrane. Two different entry mechanisms have been proposed recently in mammalian cells. The first is receptor-mediated endocytosis, in which polyamines interact with heparan sulfate on a molecule of glypican-1 located at the outer side of the cell membrane, and then are internalized into the cells through endocytosis followed by subsequent dissociation from the receptors [23,24]. The second is a classical membrane transporter-mediated

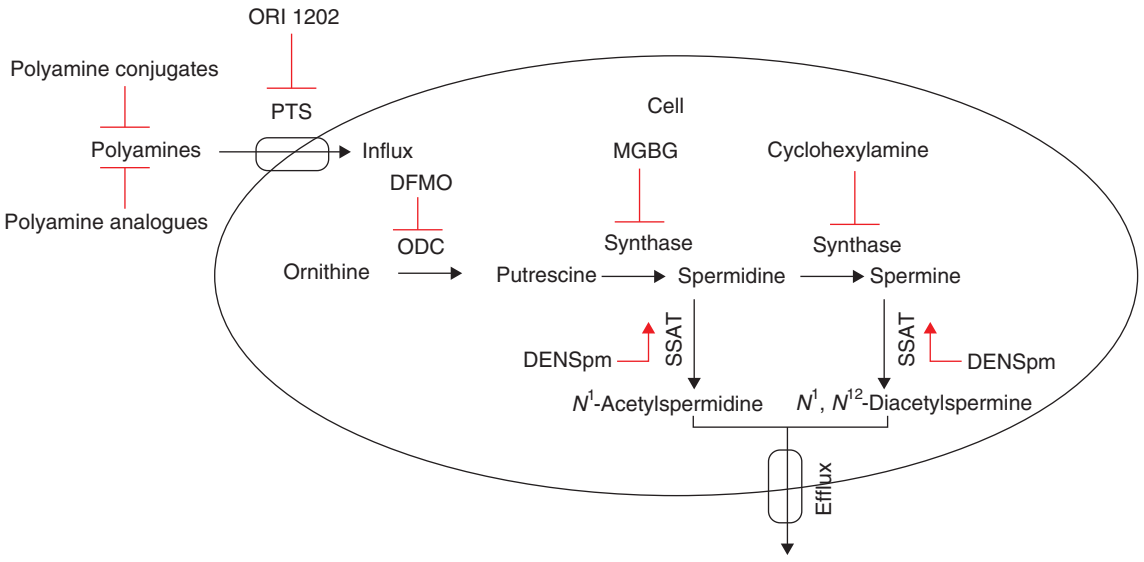

Figure 1. The strategy in drug design based on polyamines.

internalization, which requires an electronegative membrane potential, for this transport process is Na^+-independent [25].

The non-stringent structural requirement of PTS for substrates has prompted drug design on drug-polyamine conjugates during the last two decades. At present, two indirect methods are accepted to evaluate the transport of polyamine conjugates by means of the PTS in mammalian cells. First, PTS-deficient cell lines together with corresponding parent counterparts, such as CHO-MG and CHO cell lines, have been adopted to demonstrate PTS recognition of polyamine conjugates. The mutant cells without a PTS generally import smaller amounts of polyamines as well as toxic drug-polyamine compounds than their parent cells with an active PTS. For example, the IC_{50} values of many polyamine-anthracene conjugates were significant lower in CHO cells than in CHO-MG cells [11-13]. Second, DFMO/SPD experiments furnish another indirect approach for PTS targeting. Inhibition of ODC by DFMO blocks the intracellular polyamine biosynthesis and leads to a significant increase in polyamine uptake from exogenous sources. Therefore, the cells treated with DFMO should intake the toxic polyamine conjugates more readily. Conjugates thus exert elevated activity against DFMO-treated cells. On the contrary, SPD could compete with the polyamine conjugates for the PTS on the cell membrane, and consequently reduce the uptake of conjugates, which in turn would reduce the cell death. Xie and co-workers demonstrated that the IC_{50} values of polyamine conjugates will reduce or increase in the presence of added DFMO or SPD, respectively. The respective intracellular concentration of these conjugates, detected as fluorescence intensity using a high-content screening (HCS) system, is enhanced by DFMO and attenuated by SPD, providing strong evidence for DFMO/SPD experiments [26,27].

3. Design of polyamine-drug conjugates

3.1 Natural products with polyamine moieties

The polyamine segments are generally found in many natural products that exert important bioactivities, such as anti-infection, antitumor, and so on. For example, PhTX-433 and Arg659 (**Figure 2**) are isolated from *Philantus trianguluma.* These natural polyamine derivatives are of particular interest because of their ability specifically and potently to antagonize NMDA receptor function in mammalian cells [28,29]. Natural motuporamines, which are isolated from *Xestospongia exigua,* a sea sponge collected from the outer reef of Motupore Island, Papua New Guinea, contain a large hydrophobic heterocycle appended to a polyamine motif (**Figure 2**). These natural motuporamines are reported to possess anti-invasive properties on tumor cells [30,31]. Squalamine, a fascinating aminosterol isolated from the dogfish shark *Squalus acanthias,* is a cationic steroid coupled by an anionic bile salt with spermidine (**Figure 2**) [32]. It could inhibit angiogenesis and tumor growth in multiple animal models [33], and has entered into clinical trials [34]. In conclusion, these natural polyamine products can be viewed as privileged paradigms and biologically validated leads for the following design of cargo-vector polyamine conjugates.

3.2 Antitumor drugs conjugated with polyamine vectors

Highly proliferating cells, such as tumor cells, have upregulated PTSs for the uptake of exogenous polyamines, and therefore they can accumulate polyamines more effectively than normal cells [5]. Polyamine-drug conjugates that structurally mimic the natural polyamines could be preferentially carried into cells by means of the PTS. Therefore, using the polyamine backbone as a potential carrier for drug delivery

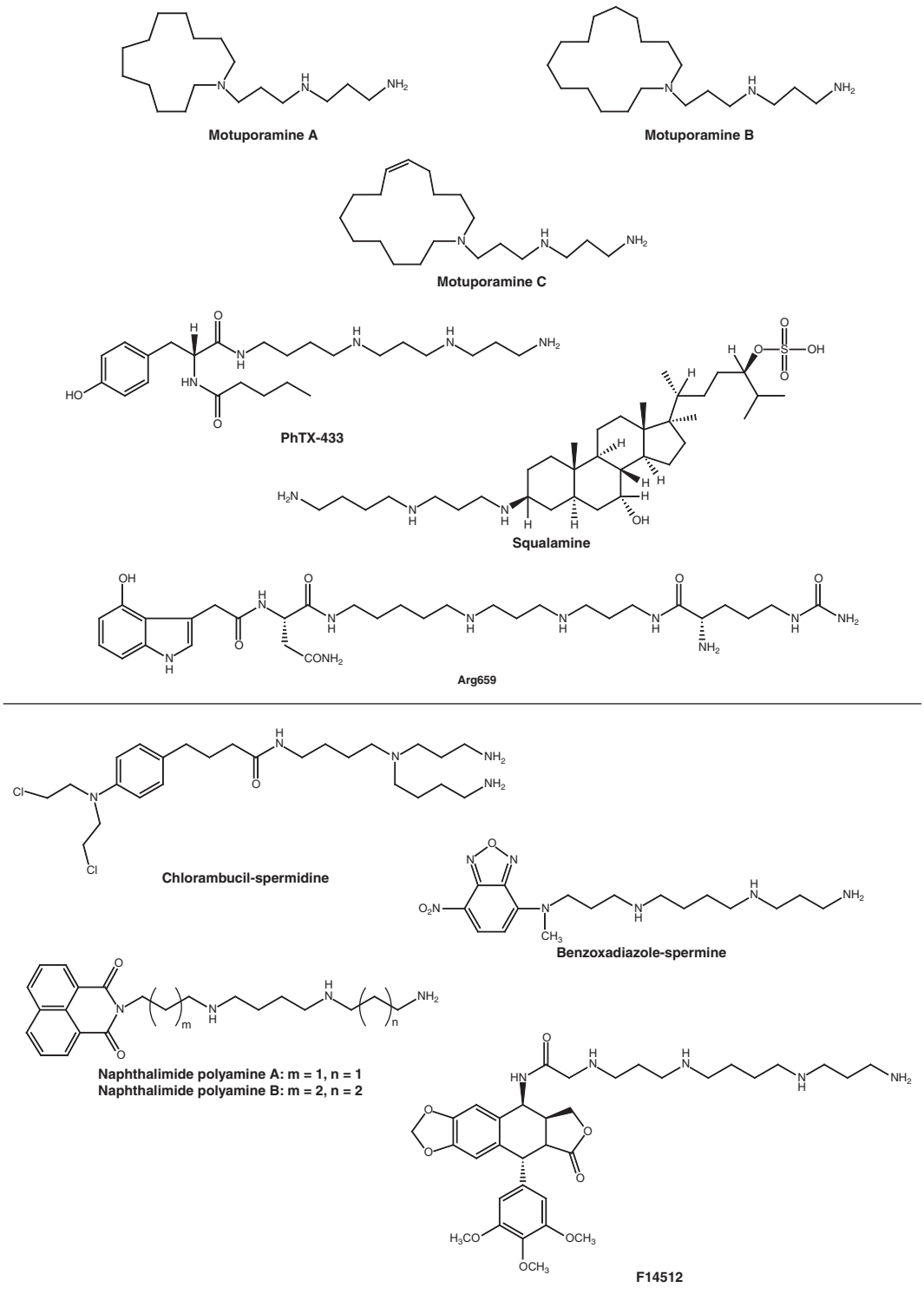

Figure 2. Structures of natural polyamine products and cargo-polyamine conjugates.

into cancer cells has been proposed as a valuable strategy to increase the selectivity of anticancer agents. In addition, the polyamines are organic cations, and have many interactions with anions within the cell. For example, polyamines cause both condensation and important conformational changes within DNA [35]. It is thus rational that various DNA targeting agents could behave as cargo candidates because the resulted cargo-polyamine conjugates should have improved DNA targeting properties owing to the extra polyamine motifs.

Over the past decades of research, polyamine skeletons such as PUT, SPD, SPM and various non-native polyamine chains have been considered as potent cell delivery vectors, facilitating selective uptake of pharmacophore by tumor cells by means of the PTS [5]. The polyamine-drug conjugates could exert additive biological activities in tandem with the polyamine moieties. In practice, cytotoxic drugs such as chlorambucil [36], nitroimidazoles [37], aziridines [38], acridines [39], enediyenes [40], anthracenes [13], naphthquinones [41], camptothecin [42] and protoberberine [43] have been reported as conjugating with deferential polyamine molecules. All these conjugates are DNA-interacting agents used to develop a general strategy by exploiting the dual contributions of polyamine motifs: the efficient transport mechanism and the extra affinity for DNA.

Chlorambucil is a commercial nitrogen mustard that serves as a DNA alkylating agent in the treatment of chronic lymphocytic leukemia and solid tumors. As mentioned above, the chlorambucil-spermidine conjugate should have improved therapeutic properties due to the added polyamine vector (**Figure 2**) [37]. Indeed, the cytotoxicity of the conjugate was 35 times more cytotoxic than chlorambucil, and the pretreatment with DFMO was ~ 7 times more cytotoxic than the conjugate alone [37]. The increase in cytotoxicity with or without DFMO was indirect evidence for the conjugate using the PTS to gain entry to the cell. Thus, it is not surprising that this conjugate is 10,000 times more active than the parent chlorambucil at causing DNA strand breaking. Although the therapeutic value was not increased because of the neurotoxicity in BALB/c mice, it did demonstrate that the PTS could potentially be exploited for drug delivery [37].

Guminski *et al.* reported a series of polyamine derivatives with benzoxadiazole, phenylxanthene tethered to a spermine moiety and investigated the chemical selectivity for recognition of the PTS (**Figure 2**) [44]. N^1-Methylspermine NBD conjugate has been proved to show a high selectivity for PTS-proficient CHO versus PTS-deficient CHOMG cells, and could be used as a tool for the selection of cells sensitive to cytotoxic compounds vectored through the PTS. Indeed, this conjugate as one fluorescent biomarker has been used in choosing patients with leukemia for therapy with F14512 in a clinical trial [45].

Antitumor agents conjugated with polyamine motifs may have elevated affinity for cancer cells and reach the targeted tissues more specifically. Tian *et al.* appended polyamine chains to naphthalimide, and evaluated for *in vitro* cytotoxicity. The typical compounds naphthalimide-polyamine A and B (**Figure 2**) showed excellent cell selectivity to cancer cells through the human hepatoma BEL-7402 and human normal hepatocyte QSG-7701 screens. In addition, naphthalimide-polyamine B could disturb the cell cycle in B16 cells. The research on caspase activity and cytochrome *c* indicates that naphthalimide-polyamine B could induce B16 cell apoptosis via both the mitochondrial and membrane death receptor pathways, and the Bcl-2 family numbers are involved in the control of apoptosis [46].

Eight polyamine perylene diimides are synthesized to evaluate the efficiency of perylene derivatives in stabilizing G-quadruplex structures and the polyamines' biological activity [47]. The results demonstrated that the number and distances of positive charges in the side chains dramatically influence the inducing of G-quadruplexes and inhibiting of telomerase. Battaglia *et al.* reported two taxol analogues bearing the linear polyamine spermine at the 7- and 10-positions of paclitaxel, and 10-deacetyl-paclitaxel cytotoxic activity was evaluated by *in vitro* growth inhibition experiments conducted on MCF7-sensitive and MCF7-R-resistant human breast cancer cell lines. The experiments showed a three order decrease in cytotoxicity with respect to paclitaxel in the sensitive cell line, whereas no activity was found towards resistant cell line [48].

The successful design of F14512, an etoposide-spermine conjugate, highlights the idea of polyamine conjugates targeting PTS (**Figure 2**). F14512 was found to be 73-fold more cytotoxic to CHO cells compared with CHO-MG cells with a reduced PTS activity. This PTS selectivity is evidenced by the rescue experiments with PUT, SPD and SPM in L1210 cells. The spermine moiety of F14512 functions both as a targeted vector for etoposide and as a subsidiary DNA anchor, which considerably enhances the drug–DNA interaction, leading to a reinforced inhibition of topoisomerase II [49]. More importantly, the experiments of F14512 targeting PTS *in vivo* were consistent with the results *in vitro*. These preclinical tests of F14512 emphasize the value of further clinical development [18].

3.3 Preliminary structure–activity research of drug-polyamine conjugates

Despite the many examples of polyamine conjugates with cytotoxic drugs or pharmacophores mentioned, systemic researches on the structure–activity relationship, which are beneficial in guiding the selection of an appropriate drug to a proper polyamine vector through an optimal linker, are still in demand. Early reports focused on conjugates with branched polyamine vectors. However, conjugates with linear polyamine vectors have been preferred in the recent years. In this regard, several groups have presented some insights into related key factors around cargo size, polyamine backbone and linker.

Through a series of conjugates coupling linear polyamines with aminoacridine and anthracene as core cargoes, the Phanstiel group has revealed structural features associated with the delivery of *N*-aryl-polyamine conjugates by means of the PTS in the established CHO/CHO-MG model cell lines [9-13]. The anthracenylmethyl-homospermidine conjugate, with a 4,4-triamine backbone, was found to have 150-fold higher cytotoxicity in CHO cells than in CHO-MG cell lines, corroborated by the cell uptake experiments in which the conjugate is delivered into CHO cells more efficiently than into CHO-MG cell lines (**Figure 3A**). Furthermore, additive DFMO could elevate potency of the

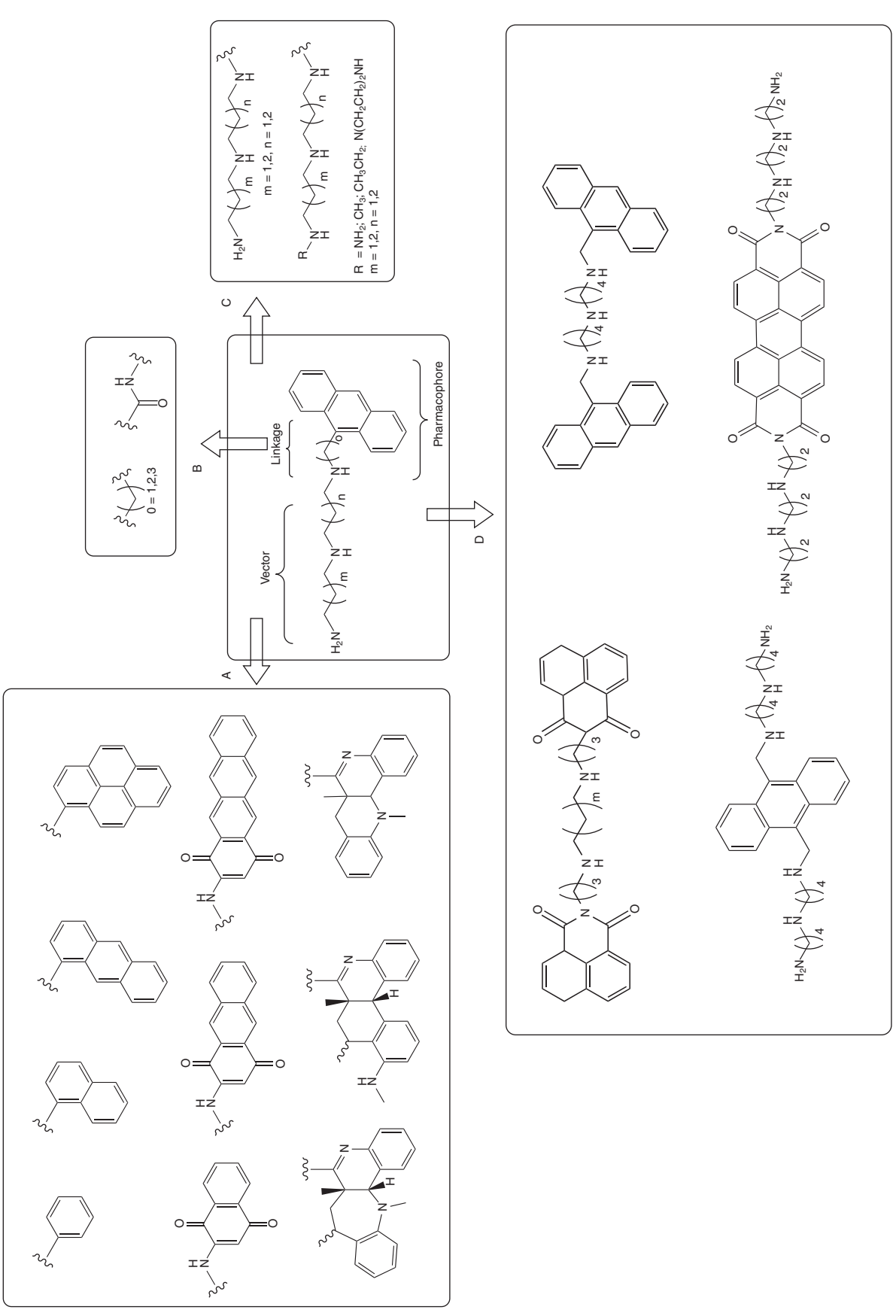

Figure 3. Models for structure–activity relationships of polyamine conjugates.

conjugate. All evidence indicated that this conjugate gains access to cells by means of nice PTS recognition. As a consequence, this homospermidine conjugate also shows nice *in vitro* preference for abnormal B16 melanoma cells from normal Mel-A melanocyte ones [9].

Continued efforts reveal that *N*-alkylation at the terminal-N position still retains modest PTS selectivity and has many effects on the biological properties. For example, *N*-alkylated polyamines have been shown to have reduced system toxicity both *in vitro* and *in vivo* (**Figure 3C**) [26,50,51]. A relatively large *N*-substituent such as a pyrene nucleus can be delivered by polyamine vectors via PTS, whereas polyamine-heterocyclic amidines showed a limited PTS-targeting ability [11-13,52]. In short, the size of the *N*-substituent (**Figure 3A**), the methylene 'linkage' units (**Figure 3B**) and the length of polyamine skeletons (**Figure 3C**) all influence PTS-mediated delivery.

Interestingly, anthracenylmethyl-spermine conjugates have a high affinity for the PTS, but they are less cytotoxic than the corresponding spermidine conjugates. One similar phenomenon is observed on aminoacridine-spermine conjugate. Deconvolution microscopy studies revealed that the indiscriminately tight binding of the spermine conjugates to the cells prohibit from their transport [11-13]. Therefore, the 4,4-triamine skeleton was confirmed as an optimal motif for cellular entry in the series of anthracenylmethyl-polyamine conjugates. Continued research revealed that the disubstituted arylene systems, with superior PTS selectivity to monosubstituted systems performed in CHO-MG/CHO trials, indicate a new direction of development (**Figure 3D**) [47,50].

Given the scope of carried drug or vector candidates, the linker and the appending point are also key factors affecting the cytotoxicity of conjugates to tumor cells and selectivity over their normal cell counterparts. There are a few reports on the liberation of the appended polyamine from the conjugate at the linker after it enters the cells. In one case, the benzyl-homospermidine compound, a non-toxic polyamine conjugate, can give free homospermidine. However, only traces of homospermidine were detected in the more toxic naphthylmethyl-homospermidine conjugate, whereas none was observed in anthracenylmethyl-homospermidine (**Figure 3A**). This polyamine vector is thus different from conventional transporters, which are supposed to be released in cells. In this regard, polyamine conjugate is actually one kind of polyamine analogue with a difference in biological functions of substituents.

Therefore, the gradually explicit relationships between polyamine and cargo pave the way for successful developments of polyamine-drug conjugates as chemotherapeutic agents. Indeed, the structure design of F14512, a promising conjugate, follows a known structure–activity relationship. The advent of similar polyamine conjugates having active PTS selectivity and significant antitumor activity both *in vitro* and *in vivo* is foreseeable with a deep understanding of structure–activity relationship.

4. Molecular mechanisms of polyamine conjugates as antitumor agents

The preceding sections have presented some aspects about how polyamine conjugates enter cells. It is also important to probe their possible antitumor mechanisms in-depth, which will assist further mechanism-based drug design. Cancer therapy interacting with a single molecular target or signal pathway is clinically successful to some extent. However, there is a general belief that cancer is a disease involving multiple deregulated genes and complex signal networks. Inhibition of one biological target is perhaps not clinically effective for the therapy of multifactorial disease, which can switch on compensatory mechanisms, as seen in DFMO. Therefore, agents regulating more targets and signal pathways are expected to have superior efficacy against a given complex pathology.

Oncogenic transformation leads to cell cycle aberration and apoptosis deregulation. Many antitumor drugs produce cytotoxicity through cell cycle arrest and apoptosis induction. Therefore, targeting cell cycle and apoptosis pathways has emerged as an attractive approach for the treatment of tumors [53]. The search for new agents that target pathological processes of human carcinogenesis has led to the discovery of small molecules that modulate cell cycle and apoptotic pathways. Indeed, only a few reports have revealed how polyamine conjugates, compared with other polyamine derivatives, kill tumor cells by means of cell cycle arrest and apoptosis induction [27]. Given the structural variety of polyamine vectors and cargoes, a universal mechanism is unexpected. However, it is reasonable that there are some common aspects derived from the similar properties of polyamine motifs.

4.1 Polyamine conjugate and DNA damage

DNA, the most vulnerable material in the cell, is the main target of most cytotoxic antitumor drugs that react either directly with DNA through reactive metabolites or indirectly through incorporation into DNA (nucleotide analogues), or by blockade of DNA-metabolizing functions such as DNA polymerases or topoisomerases [54]. Most of these drugs have a very low therapeutic window in which they are toxic to normal as well as tumor cells, and their use in clinic is thus limited [53]. Owing to the high polyamine affinity for DNA [42], it is not surprising that many DNA-intercalating agents are modified with polyamines in order to potentialize therapeutic effects and/or decrease adverse effects.

The polyamine tails support many complementary functions, that is, increasing DNA binding to reinforce Top II inhibition. Indeed, the addition of polyamine moieties to DNA intercalators increases the DNA targeting ability of the resulting conjugates. For example, the chlorambicil-spermidine compound, one of the most cited polyamine conjugates, was 4 times more potent than chlorambicil in an *in vivo* trial, whereas this conjugate was 10,000 times more active than the parent drug at causing DNA strand breaking [36]. More recently, polyamine conjugates with

naphthalimides [55], anthracenes [51] and podophyllotoxins [49] as cargoes also displayed more *in vitro* potency than parent agents, together with stronger DNA binding and Top II inhibition. These observations indicate that polyamine vectors serve not only as a molecular transporter delivering a drug to cancer cells, but also as an enhancer of the cellular component targeting, which then triggers cell apoptosis and/or cell cycle arrest.

4.2 Polyamine conjugate and cell cycle

It has been known for many years that natural polyamines are needed for cell cycle progression, so the cellular polyamine levels and ODC activity peak at specific points during cell cycle [56]. Polyamines could regulate cell cycle progression via the change of intracellular concentration, with increased putrescine levels during the S and G_2 phases, increased spermidine during the entire cell cycle, and spermine mainly during the G_1 and S phases in CHO cells (**Figure 4**, left) [57,58]. Accordingly, ODC expression is closely correlated to cell cycle and there is a biphasic increase in ODC activity with an initial increase in conjunction with the G_1/S transition and with a second increase at the S/G_2 transition [56]; so the compounds that decreased intracellular polyamine concentrations could affect cell cycle progression and induce cell cycle arrest. For example, treatment of cells with the polyamine analogue *N*1,*N*11-diethylnorspermine led to a retardation of S-phase progression [59].

Similarly, polyamine conjugates also induced cancer cell cycle arrest. The first reports revealed that an anthracene-homospermidine conjugate induced promyelocytic leukemia HL60 cell cycle arrest at G_0/G_1 phase [60], whereas it induced hepatoma BEL-7402 cell cycle arrest at S phase [61]. In addition, naphthalimide-polyamine conjugates induced hepatoma HepG2 cell cycle arrest at G_0/G_1 phase [62]. Quinone-polyamine conjugates also induced cell cycle arrest at S phase in colon cancer HT29 cell [63]. Ant4, an anthracene-putrescine conjugate, also induced HL60 cell cycle arrest at G_0/G_1 phase [64]. Notably, although some conjugates also interfere with polyamine pools, this effect is obviously inferior to that of polyamine analogues. Cell cycle might be influenced by the polyamine part if these conjugates inhibit PTS or polyamine metabolism, but most of the effects are essentially due to the conjugated drugs. Taken together, these data demonstrated that polyamine conjugates could interfere with cell cycle progression, but upstream proteins that regulate cell cycle need to be established.

Proteins such as cyclins with their associated cyclin-dependent kinases (CDKs) are involved in cell cycle regulation, and constitute the basis of molecular mechanisms [65]. At present, six different cyclin types have been identified in mammalian cells, and these molecules act at specific phases of the cell cycle. In the G_1 phase, cyclins D1, D2, D3, and cyclin E activate appropriately CDKs. Cyclin D1 forms complexes with CDK4 and CDK2; cyclin E forms a complex with CDK2; cyclin A, and cyclins B1 and B2 form complexes with CDK1 mainly in the S and G2/M phases [66]. Furthermore, a group of molecules called CDK inhibitors (CDKIs), such as p21, p27, are involved in blocking the activity of cyclin–CDK complexes and thus breaking the cell cycle progression [67,68]. Polyamines could affect cell growth and the cell cycle by integrating with cyclins, CDKs and CDKIs. Recent data demonstrated that many polyamine conjugates could decrease the concentration of putrescine, spermidine and spermine by inhibiting polyamine synthetase and/or activating metabolic enzyme. This decrease in polyamine levels was associated with a decrease in cyclin D1 levels and cell cycle arrest in the G_1 phase [5].

Interestingly, a recent study by the authors indicated that some naphthalimide-polyamine conjugates induced cell cycle arrest at G_0/G_1 phase in hepatoma HepG2 cells or G_2/M phase in HeLa cells. This effect of cell cycle arrest in hepatoma HepG2 cells was correlated with the phosphatidylinositol-3-kinase (PI_3K)/Akt/mammalian target of rapamycin (mTOR) signal pathway [62]. The mTOR signaling pathway plays a critical role in the transmission of signals for initiation of mRNA translation, protein expression and regulation of cellular growth, survival and proliferation in mammalian cells [69]. mTOR activates various downstream effectors to promote initiation of cap-dependent mRNA translation and mediate pro-mitogenic and pro-survival signals [70]. p70S6K is a member of the serine/threonine protein kinase family and is one of the downstream effectors of the PI_3K/Akt/mTOR signal transduction pathway. It phosphorylates S6 protein of the 40S ribosomal subunit and thus functions in protein synthesis and cell growth [71]. In a recent study, the authors demonstrated that the inhibition of PI_3K activity by naphthalimide-polyamine conjugates led to inhibition of HepG2 cell proliferation and induced cell cycle arrest at the G_0/G_1 phase. This effect was accompanied by the decreased expression of G1-associated proteins, including cyclin D1 and CDK4. In addition, upregulation of p27 and inhibition of phosphorylation of mTOR and p70S6K were also found. Those studies showed that naphthalimide-polyamine conjugates induce cell cycle arrest by inhibition of the AKT/mTOR signal pathway, and then affect the expression of p70S6K, CDK and CDKIs [62]. A schematic summary of polyamine conjugate-induced cell cycle arrest and its signal pathway is shown in **Figure 4** (right).

4.3 Polyamine conjugate and cell apoptosis

Many polyamine analogues produce cytotoxicity in tumor cells, and the mechanisms seem to lead to apoptosis as a common terminal point. These apoptogenic effects may depend on the depletion of cellular polyamine pools, or they may be due to the formation of toxic polyamine catabolites [72]. Apoptosis can be triggered by several stimuli and is controlled by two major pathways, namely the mitochondrial pathway and membrane death receptor pathway. In the mitochondrial pathway, mitochondria have a crucial position in apoptosis control. The loss of MMP induces cytochrome *c* release

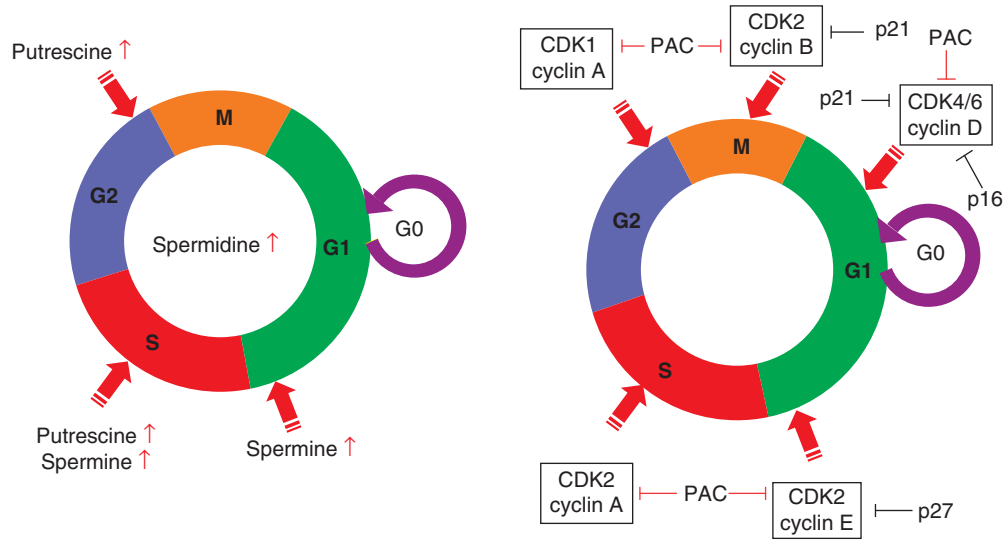

Figure 4. Cell cycle with polyamine and polyamine conjugates. Left: Polyamines are needed for cell cycle progression. Right: PAC can induce cell cycle arrest at different phases through regulating the diverse pathway, which depends on the cell type and the kind of PAC.
PAC: Polyamine conjugates.

from the mitochondria to the cytoplasm, which leads to the activation of caspase-9 and downstream cleavage of caspase-3. The membrane death receptor pathway is characterized by the binding between cell death ligands and cell death receptors and the subsequent activation of caspase-8 and caspase-3 [54].

Polyamine conjugate-induced cell apoptosis was first observed by the authors' group in the treatment of human hepatoma BEL-7402 cells, promyelocytic leukemia HL60 cells, as well as melanoma B16 cell with an anthracene-homospermidine conjugate [27,61,62]. So far, most of polyamine conjugates have been found to exert antitumor effects by means of production of cell apoptosis. However, detailed molecular mechanisms of polyamine conjugates have not been elucidated. Mitochondriotoxicity may be the pivotal factor for polyamine conjugate-mediated cell apoptosis, as many conjugates are known to cause a loss of mitochondrial transmembrane potential, followed by a release of cytochrome *c*.

Much evidence indicated that the redox state of tumor cells was an important factor in determining their susceptibility to different apoptotic stimuli [73]. In recent years, it has been found that alteration of intracellular polyamine content and production of H_2O_2 and aminoaldehydes through upregulation of polyamine catabolic enzymes, such as acetylpolyamine oxidase (APAO), could contribute to cytotoxic activity of polyamine analogues and conjugates [74]. ANTMHspd could trigger oxidative stress, induce reactive oxygen species accumulation by means of APAO activity upregulation, and then result in apoptosis via mitochondrial pathway in B16 cells [27]. Furthermore, this standpoint is also authenticated by Ant4, a putrescine-anthracene conjugate, which induces HL60 cell apoptosis by depleting intracellular polyamine content, oxidative stress and DNA damage [64].

The Bcl-2 family plays a central role in regulating the mitochondrial apoptosis pathway. More than 20 Bcl-2 family members consisting of anti-apoptosis members (e.g., Bcl-2 and Bcl-xl) and pro-apoptosis members (e.g., Bax and Bak) have been identified. Bcl-2 is an important element during apoptosis mediated by the mitochondrial pathway and has been identified as preventing cytochrome *c* release from the mitochondria. By contrast, Bax can induce the release of cytochrome *c* from the mitochondria [75]. The authors' data revealed that ANTMHspd-induced apoptosis was accompanied by upregulation of Bax and downregulation of Bcl-2 in BEL-7402 cells and B16 cells. Recently, it was also found that naphthalimide-polyamine conjugates regulated Bcl-2 family numbers, for example, downregulation of Bcl-2, p-Bad and Bcl-xL in HepG2 cells [27].

Although the genesis, growth and metaptosis of tumor are regulated by a complex signal network, some proteins may be vital in those procedures. Recently, the authors found that the molecular target of naphthalimide-polyamine conjugate is Akt, which is critical for mediating cell survival signaling and escaping from apoptosis in tumor cells [62]. Importantly, the apoptotic signal pathway of naphthalimide-polyamine conjugate was first elucidated in hepatoma HepG2 cells. Furthermore, Bolognesi *et al.* also indicated that polyamine-quinone conjugates induced colon cancer HT29 cell apoptosis by means of EGFR-mediated intracellular signaling [63]. Indeed, the existence of a pharmacophore in polyamine conjugates indicates that they may produce cellular effects that are independent of polyamine pathway.

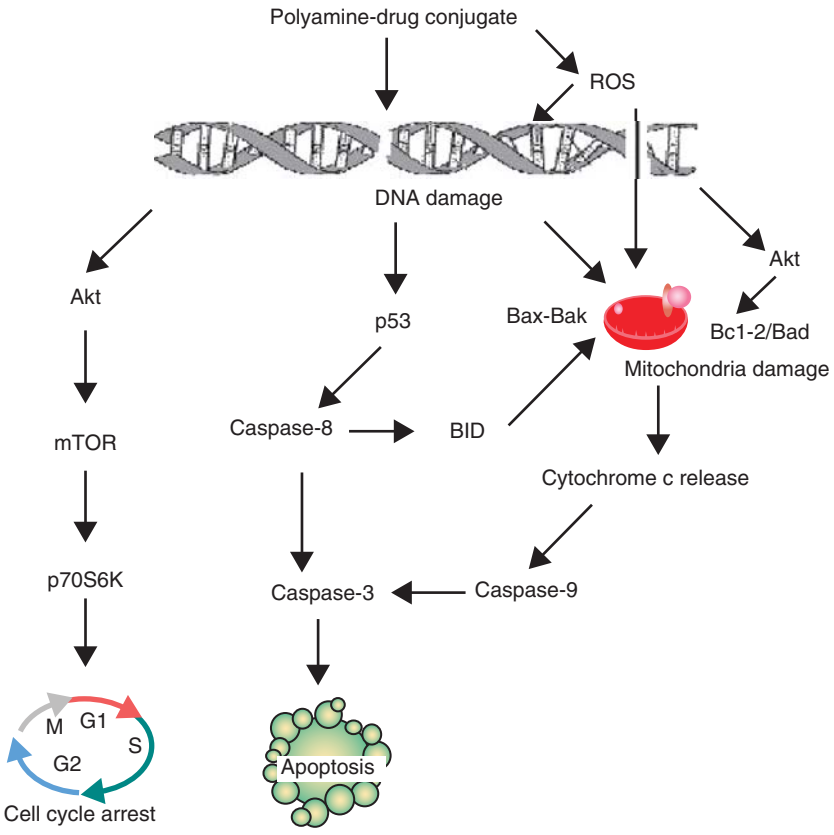

Figure 5. A schematic summary of signal pathways induced by polyamine conjugates. Polyamine conjugates can induce cell apoptosis through various signal pathways. They can induce DNA damage with or without ROS generation, and then mediate different cell events through Akt/mTOR, p53/caspase cascade or mitochondria-mediated pathway, and so on.
ROS: Reactive oxygen species.

A schematic summary of polyamine-drug conjugate-mediated signal pathways is shown in **Figure 5**.

It is well known that drugs may produce bioactivity by means of apoptosis, necrosis, autophagy, and so on. Limited reports indicate that some polyamine conjugates could induce apoptosis; however, apoptosis may not be the only cell death pathway for other polyamine conjugates. As an example, it was found that the naphthalimide-homospermidine conjugate induced B16 cell apoptosis in a different way from that of the anthracene-homospermidine conjugate [46], although these two conjugates have the same polyamine tail. This indicated that the drug part in the conjugate is a key effector in determining the total properties of this conjugate. More detailed information about the other cell death modes mediated by the polyamine conjugates should be addressed in the future. In addition, the respective roles of the drug and polyamine vector in the conjugate-mediated cell death remain an intriguing topic.

5. Conclusion

The promiscuous features of PTS for substrates have attracted lasting and fresh attention during the last two decades. However, PTS-related study was not in the mainstream in the field of polyamine-based drug design until recent years. Indeed, the non-stringent structural demand for uptake by means of the PTS of many tumor cell types provides broad opportunities for multiple polyamine-like drug screening. Limited research does furnish some insights into both drug-vector design and molecular mechanisms, whereas attempts to identify the gene of PTS in mammalian cells have not been successful. More importantly, recent progress on a podophyllotoxin-spermine conjugate, which shows obvious therapeutic improvements *in vitro* and *in vivo,* has shed light on the idea of utilizing PTS as a target. The twofold advantages with polyamines, as vectors to transport a cytotoxic cargo to tumor cells and to enhance cellular target affinity, have thus generated new avenues of polyamine investigation.

6. Expert opinion

Although existing advances in polyamine conjugates appear promising, the relatively scattered attempts to harness PTS for drug delivery are still in their infancy. Indeed, F14512 has just finished preclinical experiments, compared

with several polyamine analogues in clinical trials. Some key problems should be addressed to develop less toxic and more selective polyamine conjugates.

1) Although extensive evidence points to the fact that polyamine conjugates are delivered to cells by means of the PTS, the lack of molecular information on PTS in mammalian cells is the Achilles' heel for rational drug design. Progress in the molecular structure of PTS will certainly improve the present empirical explorations.
2) In limited cases, the role of this polyamine moiety in the conjugate appears to be inconsistent completely with the conventional function of a vector-in-drug delivery system, as some compounds with both high PTS and biological effects do not release the respective appended drug and polyamine vector in cells. Consequently, how these conjugates metabolize is an intriguing challenge to be dealt with, which is beneficial for the understanding of this confusing phenomenon.
3) Preliminary research points to the fact that polyamine conjugates may induce cell death through pleiotropic mechanisms in addition to the polyamine-mediated pathway, and thus have the advantages of multi-targeted drugs. In other words, the model of drug-polyamine is following an emerging new design strategy that an agent alone is able to manipulate multiple cellular targets or signal pathways. Undoubtedly, the detailed mechanism research contributes to the availability of polyamine conjugates as more efficacious antitumor candidates.
4) Whereas much notable cell entry by means of PTS has been observed *in vitro,* tissue uptake *in vivo,* a key indicator for potential therapeutic applications, is rarely reported. In this regard, recent progress on F14512 correlates the cell target to the tissue target nicely. In addition, with the identified synergistic functions of DFMO to polyamine conjugates *in vitro,* it is anticipated that therapeutic validation *in vivo* may provide a mode of co-administration therapy for cancer.

Declaration of interest

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