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Fungal immunomodulatory proteins: characteristic, potential antitumor activities and their molecular mechanisms

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During recent decades, >30 fungal immunomodulatory proteins (FIPs) have been found in a range of mushrooms and other fungi. Various pharmacological functions of FIPs have become important in the discovery and development of new drugs. In this review, we discuss some important factors, focusing on the use of amino acid sequence data to predict structural and physicochemical properties. We also discuss pharmacologic activities and possible mechanisms of the proteins with a focus on antitumor activities. Numerous other questions must also be addressed before FIPs can be widely accepted and used as antitumor agents.

Introduction

Fungal immunomodulatory proteins (FIPs), with a molecular weight of ~13 kDa and 110–114 amino acids, are a species of a bioactive substance obtained from some edible and medicinal mushrooms, such as *Ganoderma lucidum*, *Flammulina velutipes*, *Volvariella Volvacea*, among others, that have been summarized in previous publications [1,2]. With the development of biotechnology, more FIPs from different fungi have been found *via* biotechnology methods, such as protein purification, homologous cloning, genome mining and directed revolution (see Table S1 in supplementary material online).

In an overview of previous work, we found several papers on bioactive fungal proteins and their pharmaceutical properties, and a series of reviews have appeared that cover these aspects [1–4]. The reason fungal proteins get so much attention is that they are potential biological ingredients or functional food ingredients, and biotechnological methods can be used to produce them *in vitro* [5]. Among the numerous fungal proteins, FIPs have been demonstrated to possess some antitumor activities in modern pharmacological tests, including the inhibition of cell growth and proliferation, the induction of apoptosis and autophagy, and the reduction of invasion and migration, among others [6]. Scientific reports suggest that the antitumor effects of FIPs can be achieved by indirect immunomodulatory effects, direct tumor-cell-killing effects or a combination thereof [2]. However, these functions are closely related to their structural or physicochemical characteristics. In this review, first we compare and analyze the characteristics of the structural and physicochemical properties of FIPs and then we discuss their antitumor effects and the mechanisms of action.

Structural characteristics and physicochemical properties of FIPs

Structural characteristics

Because FIPs exhibit high homology, we applied MEME motif-based sequence analysis to seek random pattern occurrences within these amino acid sequences [7]. The sequence-based MEME analysis of these sequences showed that FIPs contained several common conserved motifs (Fig. 1). These motifs could play a vital part in the maintenance of biological structure and functions [8]. For example, Lin *et al.* found that the region consisting of 13 amino acids in the N terminus played a key part in determining the dimeric structure [9]. Although a conserved motif (Fig. 1b) had been observed, this motif was only observed in 15 out of 23 FIPs. We speculate that the amino acid sequence at the N terminus is not the key to forming dimers, but that the 3D structure is.

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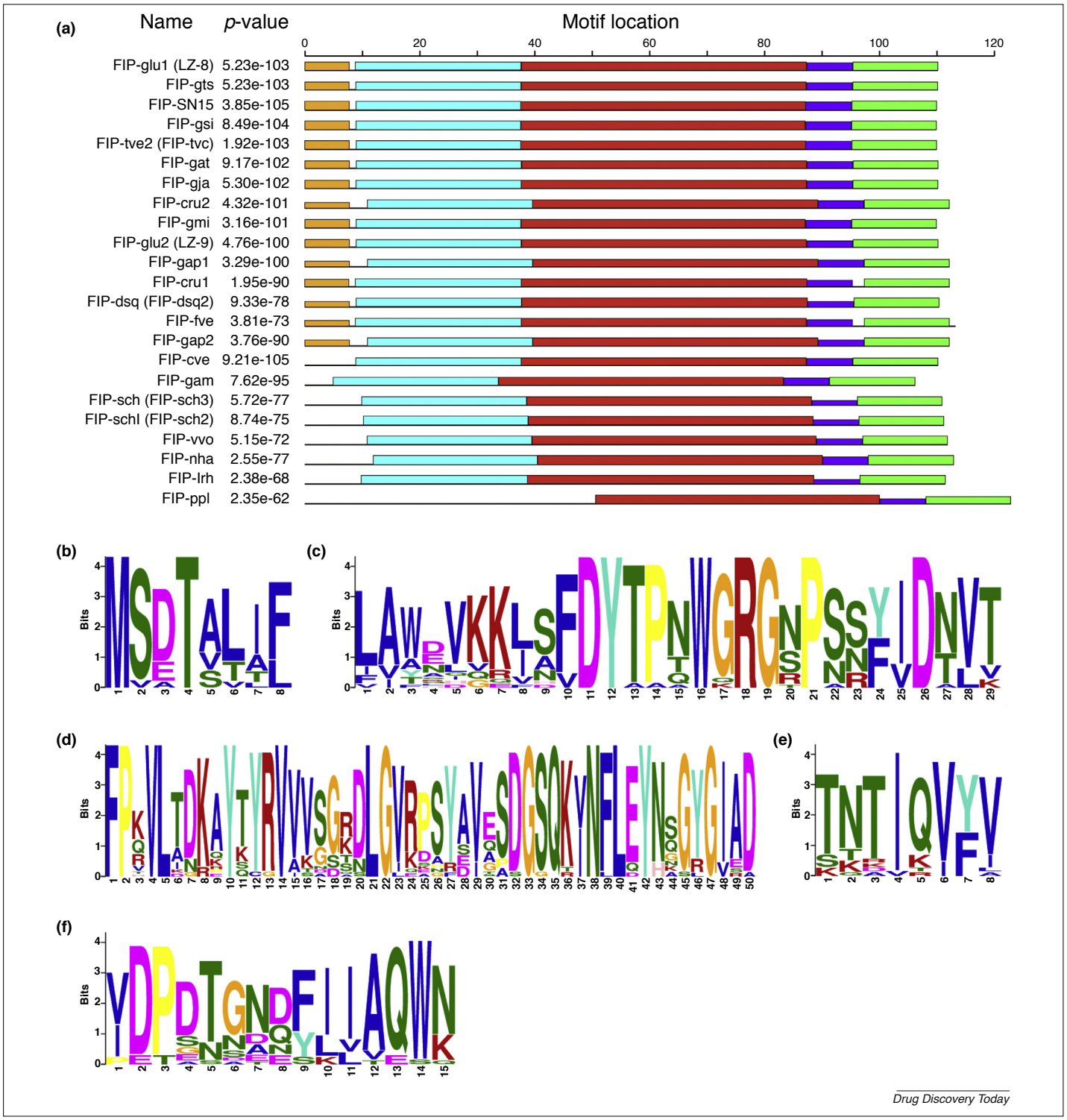

FIGURE 1

Sequence analysis of fungal immunomodulatory protein (FIP) amino acids using MEME. The full sequences of FIPs were analyzed for conserved motifs using MEME motif-based sequence analysis. The whole sequence of FIP contained five motifs. **(a)** Occurrence and location of the motif relative to the start site M. Based on the amino acid site of FIP-glu1 (LZ-8), site 1–8 is M(S/V)XTX_3(F/L) **(b)**, site 10–38 is X_9(F/V)DY(T/A)(P/A)XWXRGXPX_2(Y/F)(I/V)DX(V/L)X **(c)**, 39–88 is FPXVLX$_2$(K/R)XYX(Y/C)(R/G)VX_6LGX$_6$(V/I)X_2(D/S)G(S/G)QX(V/I)N(F/L)LXY(N/H)X(G/S)XG(I/V)X(D/A) **(d)**, 89–96 is X_3(I/V)X(V/I)(Y/F)X **(e)** and 97–111 is X(D/E)(P/T)X_9(Q/E)(W/S)X **(f)**.

Physicochemical properties

The surface hydrophobicity of proteins is related to the content of their hydrophobic amino acids and plays an important part in protein conformation, activity and protein–protein interactions [10]. To study the hydrophobicity and hydrophilicity of FIPs, we carried out a Kyte–Doolittle scale mean hydrophobicity profile analysis. Amino acid sequences of the FIPs were imported into the software BioEdit 7.2.5 with the window size set at 9 [8,11]. As

shown in Fig. 2a, hydrophobicity and hydrophilicity values were generally similar among different FIPs, but we did observe some differences among certain regions of different FIPs. The FIPs contained several obvious hydrophobicity regions. For example, the Kyte–Doolittle graph showed that FIP-glu1 (LZ-8) had six hydrophobic regions (Fig. 2b). The hydrophobic region I of most FIPs had the highest maximum hydrophobicity value. In all the FIPs, regions I and VI were similar and region II could also be found in Kyte–Doolittle graph showed in Fig. 2a and b. The main differences were with regions III and V. For example, FIP-cru2, FIP-gap2 and FIP-schl (FIP-sch2) did not have region V, and region III was not found in FIP-vvo. These differences probably cause variations in FIP structures, leading to different protein activities. For example, hydrophobicity of FIP-glu1 (LZ-8) was dramatically different from the hydrophobicity value of FIP-SN15 (Fig. 2c). Therefore, a small diversity of the hydrophobicity region between FIPs might influence the structure, and then lead to differences of activity between FIP-glu1 (LZ-8) and FIP-SN15 [12].

Electrostatic potentials are essential to the functions and activities of proteins [13]. Electrostatic interactions can influence fundamental biological processes, such as protein–protein interactions and protein conformations [14,15]. The N-terminal α-helix of the FIPs contributed to form the homodimer [9]. To study the relationship between the electrostatic potential and the homodimer, electrostatic potentials at the binding site of the homologous monomer were calculated by Discovery Studio around the FIP homodimer models [8,16]. The FIPs showed similar electrostatic potentials, although individual FIPs such as FIP-gmi were different in this respect (Fig. 3a). It is possible that these differences contribute to the formation of a unique structure, such as homotetramers, homodimers and single chains (monomers) [17]. Wang *et al.* believed that a negatively charged surface of FIP-vvo82 formed by the amino acid at position 47 could actively interact with the positively charged N-terminal α-helix, enhancing the stability of the FIP homodimer and improving immunomodulatory activity [8]. Because FIP-glu1 (LZ-8) and FIP-SN15 showed almost the same electrostatic potential except at sites 41, 46 and 58 (Fig. 3b), we speculated that these electrostatic potentials could influence the interaction of two homologous monomers.

Antitumor activities and their participation in molecular mechanisms

It has been studied that most of the reported FIPs have potential biological, therapeutic and pharmaceutical activities, especially antitumor activities. This conclusion on antitumor activities of FIPs is largely based on testing for cytotoxic activity against cancer cell lines grown either *in vitro* or using *in vivo* models (see Table S2

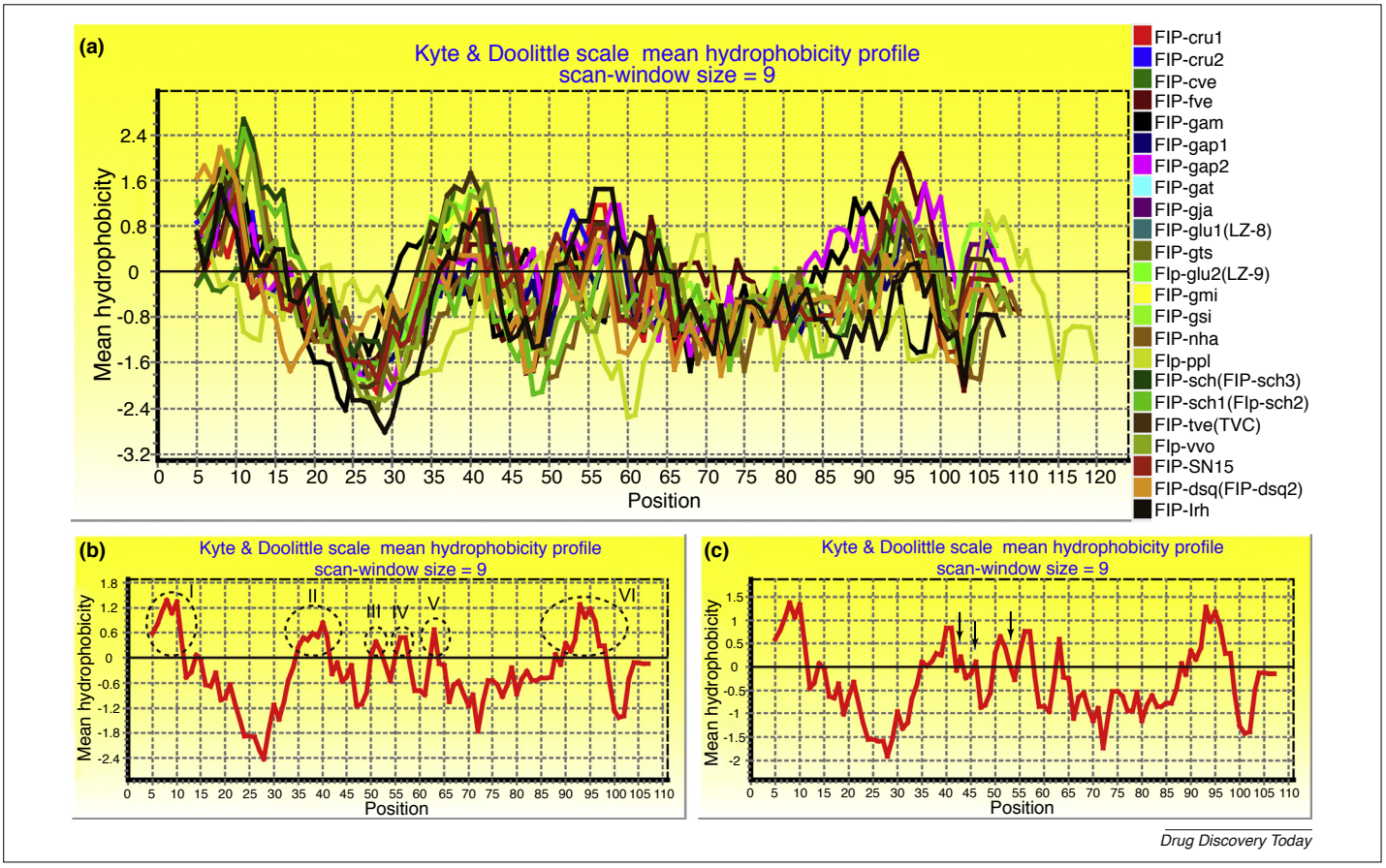

FIGURE 2

Hydrophobicity profile of fungal immunomodulatory proteins (FIPs). Positive scores denote hydrophobicity and negative scores denote hydrophilicity. **(a)** Comparison of the hydrophobicity profile of FIPs. **(b)** Hydrophobicity profile of FIP-glu1 (LZ-8), dotted circles represent hydrophobic regions of FIP-glu1 (LZ-8); **(c)** hydrophobicity profile of FIP-SN15, arrows represent amino acids with a difference of hydrophobicity/hydrophilicity between FIP-glu1 (LZ-8) and FIP-SN15.

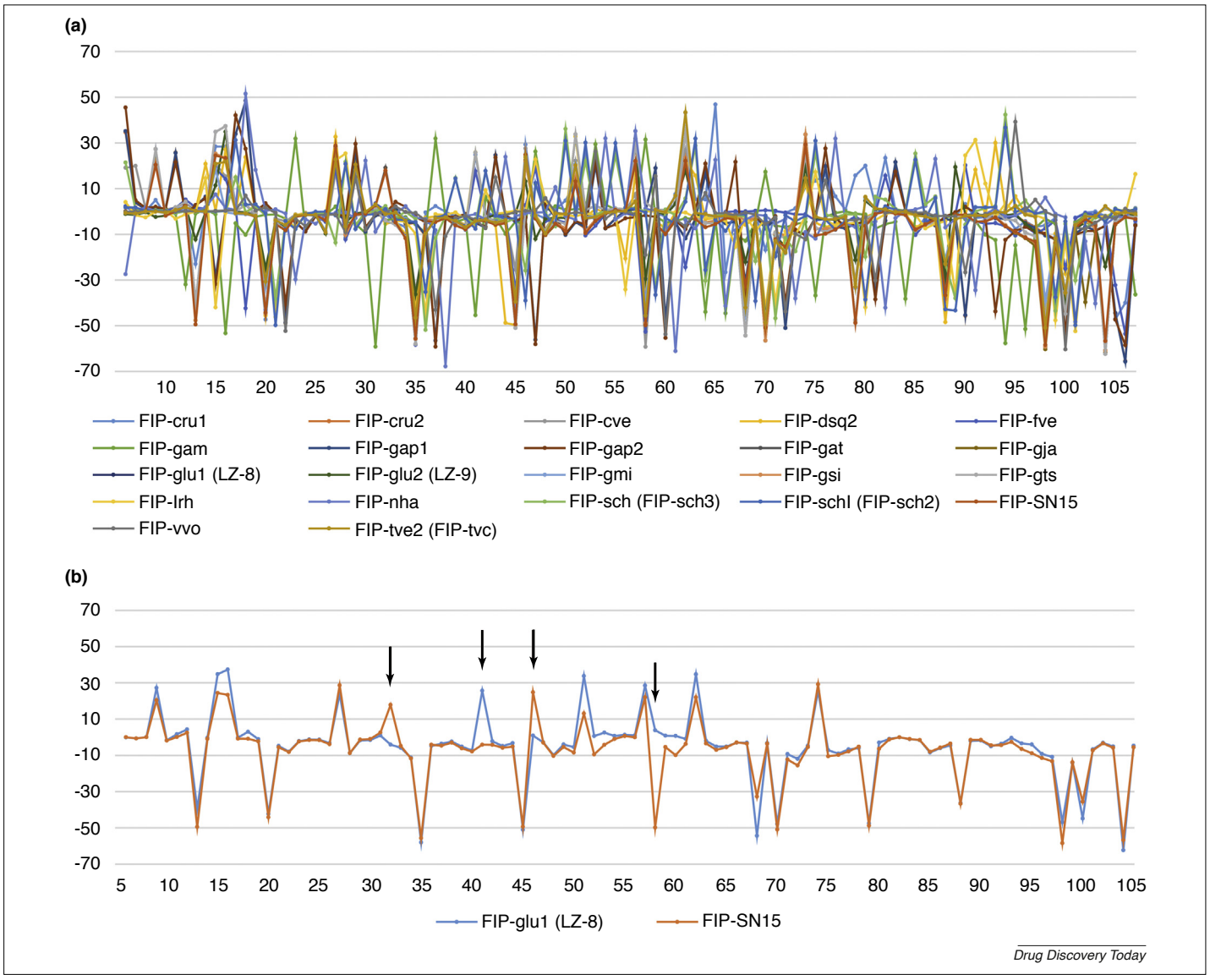

FIGURE 3

Comparison of electrostatic potential calculations of fungal immunomodulatory proteins (FIPs). **(a)** Comparison of calculated electrostatic potentials for FIPs. Most of the FIPs displayed similar electrostatic potentials at positions 7–11, 20–43, 48–55, 66–73 and 95–107. FIP-gam exhibited distinct differences at sites 23, 31, 37, 41, 70, 75 and 96 regarding electrostatic potential. **(b)** Comparison of calculated electrostatic potentials for FIP-glu1 (LZ-8) and FIP-SN15. Different sites in FIP-glu1 (LZ-8) and FIP-SN15 are marked by arrows.

in supplementary material online). Most researchers studying FIP functions hope that one of them will become a potential new class of pharmaceutical drugs. Our previous study revealed that >600 genes were involved in 96 biotic processes in FIP antitumor action [18]. However, the mechanisms of action are complicated and remain unclear. To date, it has been demonstrated that FIPs discovered from natural mushrooms or recombinant proteins exert antitumor activity through two forms: activation of the immune response of the host cells and direct cytotoxicity toward the tumor cells. Based on previous studies, the possible mechanisms of antitumor action are summarized and presented in Fig. 4.

Induction of cytokines

Interleukin (IL)-2 and interferon (IFN)-γ have crucial roles in the immune response [19,20]. It is possible that FIPs induce expression of cytokines *via* protein tyrosine kinase (PTK)/phospholipase C (PLC)/protein kinase C (PKC)α/extracellular signal-regulated kinase (ERK)1/2 and PTK/PLC/PKCα/p38 pathways. We speculated that the T cell receptor (TCR)–CD3 complex was a putative binding site or a receptor for rFIP-glu1 (rLZ-8), which is enriched on the cell membrane [21]. When TCR–CD3 activation is triggered by rFIP-glu1 (rLZ-8), a group of tyrosine kinases is activated, which in turn activates PLC [22]. rFIP-glu1 (rLZ-8)-mediated activation of the Src family of PTKs led to the activity of PLC, which phosphorylated two PKC isoforms: PKCα and PKCθ [23]. PKCα/ERK1/2 and PKCα/p38 are downstream signaling cascades of TCR-CD3/PTK/PLC, and regulate cytokine secretion [23,24]. Only the ERK1/2 pathway played a more important part, the JNK1/2 pathway did not involve IL-2 secretion, and p38 had a more significant role in

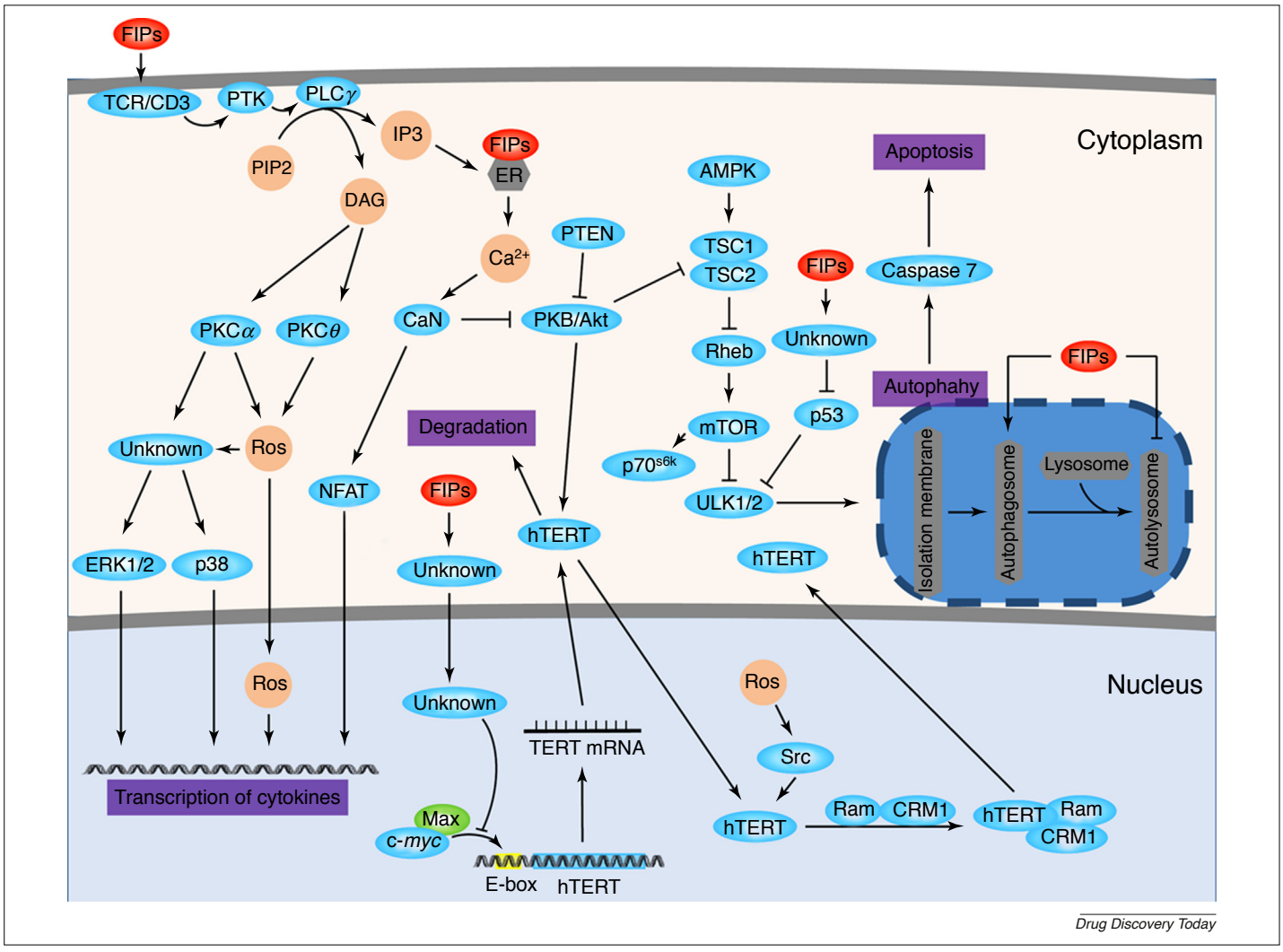

FIGURE 4

Possible mechanisms of antitumor activity of fungal immunomodulatory proteins (FIPs). Abbreviations: TCR, T cell receptor; PTK, protein tyrosine kinase; PLC, phospholipase C; PIP2, phosphatidylinositol4,5-bisphosphate; DAG, diacylglycerol; IP3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinase; ER, endoplasmic reticulum; CaN, calcineurin; NFAT, nuclear factor of activated T cells; PTEN, phosphatase and tensin homolog; PKB (Akt), protein kinase B; hTERT, human telomerase reverse transcriptase; AMPK, AMP-activated protein kinase; TSC, tuberous sclerosis; Rheb, Ras homolog enriched in brain; mTOR, mammalian target of rapamycin; ULK, UNC-51-like kinase.

rFIP-fve-induced IFN-γ secretion than in rFIP-glu1 (rLZ-8)-induced IL-2 secretion [23,24]. Cytokines might also be induced through the PTK/PKC/reactive oxygen species (ROS) pathway. PKCα and PKCθ could regulate the generation of ROS including H_2O_2 and O^{2-}, but PKCα is more efficient. Not only was ROS associated with the induction of IL-2 but H_2O_2-mediated IL-2 secretion was also partial *via* ERK1/2 and p38 [23]. Additionally, extracellular and intracellular calcium had crucial roles in IL-2 secretion. An increase in intracellular calcium could lead to the activation of the calcium/calmodulin-dependent protein serine calcineurin (CaN) [25]. Activated CaN dephosphorylated nuclear factor of activated T cells (NFAT), which in turn translocated to the nucleus and promoted the transcription of cytokines including IL-2 and IFN-γ [26]. This mechanism probably occurs *via* the calcium/CaN/NFAT pathway.

Direct antitumor effect

Inhibition of telomerase activity

Telomeres are protective structures at the end of each chromosome in eukaryotic cells and play an important part in maintaining genomic integrity [27]. Telomerase inhibition is considered to be a promising approach as an anticancer treatment [28]. Telomerase is mainly composed of telomerase reverse transcriptase (TERT) and telomerase RNA (TR) subunits. The expression and activity of human TERT (hTERT) correlate with telomerase activity, which can be regulated by multiple mechanisms [29].

FIPs are mainly involved in transcriptional and post-translational regulation. Liao *et al.* first found that rFIP-gts suppressed the telomerase activity in A549 cells *via* a *c-myc*-responsive element-dependent mechanism [30]. rFIP-gts inhibited the interaction between the E-box region of the *hTERT* promoter and the *c-myc*/Max transcription factor, suppressed *hTERT* promoter activity and then

reduced the hTERT mRNA level. This resulted in the repression of telomerase activity. However, rFIP-gts was not located in the nucleus in A549 cells [30]. Therefore, this inhibition of interaction induced by rFIP-gts is indirect. However, rFIP-glu1 (rLZ-8), the amino acid sequence of which is identical to that of rFIP-gts, gathered on the nucleus in NB4 cells [31]. It is possible that FIPs are located in a different area of different cells and exhibit unique mechanisms.

In addition to the transcriptional regulation, FIPs inhibit telomerase activity through translocation of hTERT at the level of post-translational modifications. The mechanism involves the ER/calcium/CaN/protein kinase B (PKB, also known as Akt)/hTERT signaling pathway. The localization of rFIP-gts in endoplasmic reticulum (ER) could trigger ER stress to raise intracellular calcium release [30,32,33].

The increase of intracellular calcium is a result of the activation of CaN and the dephosphorylation and deactivation of PKB (Akt), which in turn decreased the phosphorylation of hTERT and inhibited its translocation [30]. Furthermore, telomerase activity can be downregulated through the CRM1-Ran independent nuclear export machinery. FIP-glu1 (LZ-8) could induce ROS [23]. The endogenous as well as exogenous induced oxidative stress might induce phosphorylation of tyrosine707 within hTERT by the Src kinase family, and phospho-hTERT crossed the nuclear pore with exportins and Ran GTPase [34]. Additionally, rFIP-gts also promoted proteasome-mediated degradation of hTERT [30].

Induction of apoptosis and autophagy

Programmed cell death, involving apoptosis, autophagy and necrosis, plays a fundamental part in the homeostasis of tissues and organisms, where abnormal regulation is associated with cancers [35]. Nuclear calcium is an important signal in the molecular regulation of apoptosis [36]. In K562 cells, rFIP-glu1 (rLZ-8) induced the release of intracellular calcium and could enhance the activity of caspase-3, thereby promoting apoptosis [37]. After treatment with rFIP-gat, we found 669 genes expressed differentially in MDA-MB-231 cells [18]. Of these genes, such as TNFSF8, DRD1, SMPD1 and BCL-2, those related to apoptosis were upregulated. Research on these genes is important to reveal the mechanism of tumor cell apoptosis reduced by FIPs.

Autophagy, another type of programmed cell death, has dramatic roles in cancer [35]. FIPs could activate autophagosome formation and interfere with autolysosome maturation [38]. Autophagosome accumulation was thought to be the main mechanism of FIP-mediated autophagic cell death. In addition, FIP-induced autophagic cell death is probably due to the ER/calcium/CaN/PKB (Akt)/mammalian target of rapamycin (mTOR)/$p70^{S6K}$ pathway. rFIP-gmi could trigger calcium release by ER stress, PKB (Akt) was inhibited by CaN and tuberous sclerosis (TSC)1/2 was activated, resulting in the inactivation of mTOR and the induction of autophagy [32,33,38]. Moreover, rFIP-gmi increased LC3 conversion, decreased p53 expression and induced autophagy through a calcium-mediated signaling pathway coupled with a decrease in p53 expression [32]. p53 controlled autophagy by the AMP-activated protein kinase (AMPK)/mTOR-dependent pathway [39]. A plausible mechanism was that cytosolic p53 was suppressed by a factor activated by the FIPs and then the activation of the ULK1 complex induced the formation of an autophagosome [40].

In addition, FIPs also facilitated apoptosis *via* activation of autophagy [40]. rFIP-gmi potentiated cisplatin-induced apoptosis *via* LC3 conversions and the caspase 7/PARP signaling pathway [41]. We found that the transcription level of SQSTM1, also known as sequential-mediated selective autophagy 1 or p62, was increased after treatment with rFIP-gat [18]. SQSTM1 is localized in the autophagosome and is degraded by autophagy through direct interaction with LC3 [42]. The underlying mechanism needs to be studied further.

Influence of the cell cycle

Cell cycle regulation refers to the control of several regulatory points at different times. Three of the regulatory points: G_1/S, G_2/M and the junction of mitotic metaphase and anaphase, are crucial in cellular functions. This could easily lead to tumors if these points are out of control. Thus, effective control of these regulatory site points can also help to control the proliferation of cancer.

rFIP-gts stopped lung cancer cells in the G_1 phase of the cell cycle and inhibited the growth of lung cancer cells by inducing premature cell senescence [43]. A molecular mechanism revealed that FIPs induced cell-cycle arrest *via* the ribosomal protein S7 (RPS7)/murine 2 (MDM2)/p53 signaling pathway [44,45]. rFIP-glu1 (rLZ-8) from *Saccharomyces cerevisiae* inhibited the growth of lung cancer cells *in vitro* and *in vivo via* p53-dependent G_1 arrest, which probably resulted from a rFIP-glu1 (rLZ-8)-induced disruption of ribosome biogenesis [44]. rFIP-glu1 (rLZ-8) treatment resulted in redistribution of RPS7 and improved its interaction with MDM2, a p53-specific E3 ubiquitin ligase, which interacted directly with the N region of p53 and inhibited its stability *via* polyubiquitination and proteasomal degradation [46]. The MDM2–p53 interaction could then be suppressed. p53 was decreased in the RPS7–MDM2–p53 complex and was activated. This then activated p53-dependent cell cycle arrest [47].

Inhibition of migration, invasion and metastasis

Cell migration is an essential and complex process involving the reorganization of the actin cytoskeleton. In A549 cells, rFIP-gmi significantly inhibited the epidermal growth factor (EGF)-induced activation of EGF receptor (EGFR) and PKB (Akt), with little effect on the signal transducing and activating transcript 3 (STAT3) receptor, reduced EGF-promoted Cdc42 activity with little reduction in Rac1 activity and abrogated lamellipodia formation [48]. This suggested that rFIP-gmi triggers EGF-induced migration and invasion by blocking the EGF/phosphoEGFR-phosphatidylinositol-3-kinase (PI3K)/PKB (Akt) pathway and through the Stat3 pathway. FIP-fve repressed A549 cell migration and invasion by reducing the expression of RacGTPase-activating protein 1 (RacGAP1) by regulating the RacGAP1 promoter [45].

Tumor invasion and metastasis require extensive degradation of the interstitial extracellular matrix (ECM) [49]. Matrix metalloproteinases (MMPs), a family of structurally related zinc-dependent endopeptidases capable of degrading the ECM and basement membrane, play an important part in tumor invasion and metastasis [50,51]. MMP-9, also called gelatinase (gelatinase B), is one of the most important MMPs [52]. FIPs mediated antitumor invasion *via* the nuclear factor kappa B (NF-κB)/MMP-9 pathway. In A549 cells, rFIP-gmi inhibited MMP-9 transcriptional activity, which was through suppressing phosphorylation and nuclear translocation of NF-κB p65 by blocking the phosphorylation, ubiquitination and degradation of tumor necrosis factor (TNF)-α-induced IκBα and then leading to suppressing the binding of NF-κB to the MMP-9 promoter [53]. MMP-2 (gelatinase A), the other major

MMP, is the most abundantly expressed MMP in normal tissues and also plays an important part in transmigration and invasion [50,54]. Although rFIP-gts reduced the migration of SiHa and Caski cells and the invasion of Caski cells, it failed to reduce the MMP-2 and MMP-9 activities of SiHa and Caski cells [55]. It is believed that this suppression is mediated by the other signaling pathways, such as PI3K/PKB(Akt) or the Stat3 signaling pathway.

Concluding remarks and future perspectives

Immunotherapeutic approaches to the treatment of tumors have become one of the most popular methods in human tumor therapy [56]. FIPs, potential candidates that can be used as adjuvants for cancer immunotherapy [57], have been extensively studied regarding their biochemical and pharmacological properties. They have molecular weights of ~13 kDa (110–114 amino acids), which makes them easy to express in a heterologous expression system. Because most FIPs are originally derived from fungi, it is possible to produce recombinant protein *in vitro* using a yeast expression system. These systems have many advantages, such as the correct modification of the expressed proteins, with a high performance and extracellular protein harvesting. More importantly, rFIPs have also been shown to have good heat- and freeze-resistance, acid tolerance and dehydration stability, and can overcome the challenges of acidity and pepsin digestion [58]. In addition, FIPs remain effective when administered orally to mice [32,59]. It has also been suggested that the further enhancement of antitumor DNA vaccine safety through further research into more-efficient FIPs as adjunctive drugs for antitumor vaccines will be of immense help in the development and usage of antitumor vaccines [60]. Therefore, FIPs can be developed as potential novel chemoprevention and treatment agents for cancer.

With the advancement and development of biotechnology, the gene structure and function of FIPs from different fungi are very clear. In addition, small molecular weight FIPs are suitable for expression *in vitro* and structural modification. Today, FIPs have been successfully expressed in various host cells such as *Escherichia coli*, *Pichia pastoris*, insect cells, among others [2]. In addition, gene recombination of FIPs has been successfully realized by DNA shuffling technology. It is therefore possible to produce these proteins *via* an industrial fermentation process. However, several problems must be addressed including elucidation of the mechanisms of action of the pharmacological activities of proteins, the improvement of pharmacological abilities and the enhancement of expression yields in a heterologous expression system. Fortunately, these problems will soon be solved with the development of new biotechnologies such as omics technologies, gene editing techniques and HTS technologies. Therefore, we believe that the pharmacological mechanisms of mushroom proteins (such as immune regulation, antiviral and antibacterial mechanisms) will soon be revealed, and the exploration of the relationship between the structure and activity of a fungal protein will promote the design and development of new therapeutic agents for human disease. This review highlights the integrated information on the current state of structural and functional characteristics of FIP research, which could provide a useful reference for studying the pharmacological functions of proteins, as well as the use of biotechnological methods to develop these proteins as a biological or functional food ingredient.

Acknowledgments

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.drudis.2018.09.014.

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