Toxic Mechanisms of α-Amanitin and Its Potential to Fight Cancer

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Abstract: In fighting one of the major health problems in the world, the common approach to cancer treatment is the combination of chemotherapy and radiation therapy yet from which the cytotoxic effects on normal tissues and the drug tolerance gained through remain a huge obstacle ahead; thus, new approaches are in immediate demand. In the past decades, toxins such as α-Amanitin have been studied in treating colorectal cancer, breast cancer and pancreatic tumor as a therapeutic agent mostly by conjugating to moieties with targeting property and reduced toxicity. The Amanitin toxin blocks RNA polymerase II activity and is the most specific and most potent inhibitor of the eukaryotic transcription, hepatotoxicity being the main syndrome. For the selective inhibition of RNAPII and induced RNPII activity in cancer cells, using this transcriptional arrest to fight cancer cells appears to be a novel approach with broad applications. Considering the liver toxicity of α-Amanitin, conjugation of α-Amanitin to antibodies or small molecules minimizes its toxicity and increases the efficacy of treatment with relatively accurate targeting properties. In addition, further enhancements such as photocaged α-Amanitin analog, conjugation with pH low insert peptide (pHLIP) and Fc Domain provide access to more controlled drug release and ideal pharmacokinetics.

1 INTRODUCTION

Cancer is a major health problem across the world; from the statistics of the National Health Center, 28.4 million of new cancer cases are expected by 2040 Sung. Cancer is a disease caused by certain genes changes; TP53 is such a well-known tumor suppressor gene and is frequently inactivated by a deletion in a majority of human tumors. Cancer cells grow uncontrollably and invade other parts of the body, instead of dying through a process known as programmed cell death as normal cells do. Additionally, cancer relapse after chemotherapy is a frequent biological phenomenon, along with survival of the subpopulations drug-tolerant colonies (DTCs). While common cytotoxic therapies are detrimental to normal tissues, cancer cells can also develop resistance to chemotherapy. Consequently, new approaches are urgently required for successful administration in humans where the inhibitory effect is strongly selective thus reducing adverse effects on normal tissues. Recent studies have found the enhanced antitumor activity of monoclonal antibodies by conjugation to cytotoxic agents (Shuptrine, 2012). Combining the highly-selective property of antibody and the cytotoxic molecules (payloads) with a linker providing covalent binding, Antibody-Drug Conjugates (ADC) make it possible for this specific route where the toxin is delivered to kill target cells avoiding normal tissues; several of these them are currently being evaluated in clinical trials against cancer (Strassz, 2020). Except for ADCs, Small Molecule-Drug Conjugates (SMDC) have also been designed to overcome limitations in ADCs. Following specific approaches to targets, the conjugates readily enter tumor cells and release active payloads for further inhibition. So far ADCs are based on only a few toxic compounds, which are

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largely limited to microtubule- or DNA-targeting toxins that mainly impact proliferating cells and have limited efficacy in diseases with a low proliferative fraction such as indolent lymphomas or multiple myeloma (Strassz, 2020). This limitation further urges the studies of new compounds with alternative toxic mechanisms and the ability to selectively inhibit cancer cells.

Amatoxins can be found in several species of the mushroom genus Amanita, one being the famous death cap (Amanita Phalloides), and also in the mushrooms Galerina marginate and Conocybe filaris. Among the amatoxin family, α-Amanitin is possibly the most fatal one. The toxin is notorious for its specific and non-covalent binding to RNA polymerase II, thereby decreasing mRNA levels and protein synthesis, which is the primary toxic mechanism for liver necrosis or apoptosis (Arima, 2005, Leu and George, 2007, Ljungman, 1999).

Among the twelve subunits in the human RNA polymerase II complex, POLR2A encodes the largest subunit that is indispensable for the polymerase activity in mRNA synthesis. In addition, genomic deletion of the tumor suppressor gene TP53 frequently comes with encompassment of neighboring essential gene POLR2A, rendering cancer cells with hemizygous TP53 deletion vulnerable to further suppression of POLR2A expression, which can be inhibited by α-Amanitin (Liu, 2015). Molecular events that cause tumor formation involve a number of Homeobox (Hox) genes, proteins of which can bind to regulatory DNA at the level of transcription of target gene DNA to messenger RNA by RNA polymerase II (Boube, 2014). Comparing with other toxins employed in ADCs development such as microtubule inhibitors and DNA cross-linkers, α-Amanitin ADCs have an effect on slowly dividing tumor cells (Hechler, 2014). Additionally, considering the number of intracellular targets of ADCs, RNAPII are fewer than targets of other developments, which leads to a lower concentration of α-Amanitin having complete inhibition (Rudd and Luse, 1996). Thus, the selective inhibition of RNAPII plays a crucial role in the inhibitory effect against cancer.

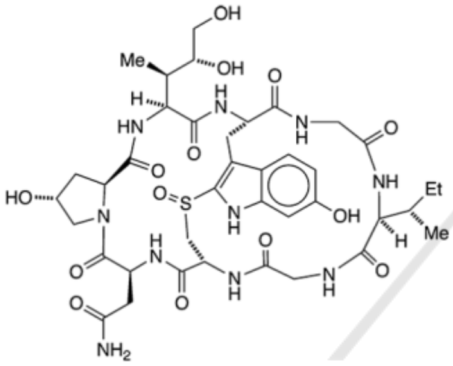
This review discusses the recent research on α-Amanitin as an RNAPII inhibitor with a focus on its high selectivity and controlled drug pathway that allows new innovative and effective therapeutics against cancer. The chemical properties of α-Amanitin and its activity in cancer cells are also discussed in this review. As the non-stop mRNA synthesis and gene expression are essential in the endless growth of cancer cells, and studies have demonstrated the mechanism underlying relapse is based on transcriptional regulation, α-Amanitin presents itself as a novel therapeutic agent against human cancer (Kume, 2016). Although applications of free α-Amanitin are limited in use due to its liver toxicity, α-Amanitin-based conjugates were evaluated with reduced toxicity for its potential to suppress DTC formation and reduce cancer cells resistance. The conjugates appeared optimistic in vivo. While the large size of ADCs impairs the penetration and SMDCs have better tumor-penetrating properties, the pharmacokinetics of SMDCs limit their anti-tumor activity. Consequently, more enhancements of these approaches are discussed to overcome the limitations; photocaged Amanitin analogs are introduced as a novel method to control drug release (Matinkhoo, 2021); conjugation with an Fc Domain enhances the pharmacokinetics and anti-tumor activity of α-Amanitin-based-SMDCs (Gallo, 2021).

2 PROPERTIES OF AMANITINS

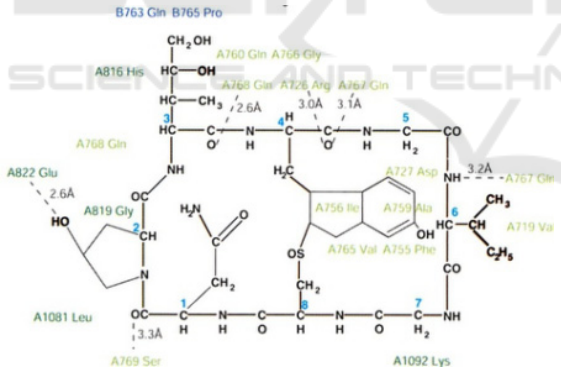
α-Amanitin is a highly toxic bicyclic octapeptide with eight amino acids and is produced by the hepatotoxic mushroom genus Amanita with its relatives β-, γ-, and ε-Amanitin. The Amanitins are special peptides with the amino acid chains branched and the branches giving rise to an inner loop. The structural derivatives of α-Amanitin show the importance of bridge helix interaction for inhibitory activity (Fig.1a) (Wang, 2011, ZANOTTI, 1987). α-Amanitin is possibly considered to be the deadliest among all the amatoxins by far. The mushroom species which called Amanita Phalloides ("death cap") or Amanita verna (“destroying angel”) is famous for its red cap and lethal toxin Amanitin. It has been estimated that Amanita Phalloides is responsible for 90% of the mushroom-ingested fatalities worldwide with hepatocellular failure being the main syndrome (α-Amanitin poisoning may require liver transplantation when the toxicity is severe). Chemical and physical properties of α-Amanitin were shown in Table 1. Amatoxins can be absorbed rapidly after ingestion. The ingested amounts as low as 0.1 mg/kg are sufficient to be lethal (Lewis and Seeff 2020). The oral LD_{50} of Amanitin is 0.4-0.8 mg/kg in mice and it takes nearly 2–8 days to cause death (Saravanapriya and Devi 2021).

Table 1: Chemical and physical properties of α-Amanitin.

Property Name	Property Value
Empirical Formula	$C_{39}H_{54}N_{10}O_{14}S$
Average Molar Mass	918.97 g/mol
Hydrogen Bond Donor Count	13
Hydrogen Bond Acceptor Count	15
Rotatable Bond Count	7
Melting Point	254-255 ºC
Water Solubility	≈1-10 g/L



(a) The bicyclic octapeptide amatoxins



(b) Interaction of α-amanitin with RNAPII

Figure 1: Chemical structure of α-Amanitin with residues of with RNAPII (Bushnell, 2002).

3 MOLECULAR TOXICITY MECHANISMS OF ALPHA-AMANITIN

Amatoxins can block nuclear RNA polymerases via the target organs (intestinal mucosa, liver and kidneys) to inhibit synthesis of proteins. Liver is the privileged target of α-Amanitin (Amatoxins can be absorbed by the gastrointestinal tract and then travel within the enterohepatic circulation and reach the hepatocytes, where the inhibition of mRNA production and protein synthesis occurred, causing cell necrosis, inducing apoptosis and glutathione depletion), internalizing the toxin through the organic-anion transporter 1B3 (OATP1B3) (Letschert, 2006), and thus receiving a massive amount of Amanitin after rapid gastrointestinal absorption.

3.1 Pro-apoptosis Activity

Inside the cell, α-Amanitin induces the stress signals (ex. RNA polymerase inhibition) which lead to an induction of the p53 protein, allowing the formation of complexes with the two anti-apoptotic proteins which called B-cell lymphoma-extra-large (Bcl-XL) and B-cell lymphoma-2 (Bcl-2) and triggering apoptosis by the mitochondrial release of cytochrome c in the cytosol (Arima 2005, Leu and George 2007, Ljungman 1999). F.A. Derheimer et al. presented two possible mechanisms of tumor suppressor gene p53 induction by α-Amanitin (Derheimer 2007). The study suggested that the export of p53 from the nucleus is dependent on the export of mRNA so that when the synthesis or export of mRNA is blocked, p53 accumulates in the nucleus by default. Secondly, inhibition of transcription elongation leads to the phosphorylation of the Ser-15 site of p53. Blockage of transcription is sufficient for the nuclear accumulation of p53 even though it's unclear that which mechanism takes place in the cells. A report pointed out that α-Amanitin induced significant changes in the mitochondrial proteome, associated with the destruction of membrane potential (Wang 2018). Hence, Amanitin-induced apoptosis has been considered to play a vital role in the pathophysiology of these intoxications (Figure 2) (Magdalan, 2010).

3.2 Enhancement of Oxidate Stress

Some mechanisms have been suggested the formation of reactive oxygen species (ROS) leading to oxidative stress-related damages. The production of oxidative stress has been seen as an essential factor in the development of that severe hepatotoxicity. In fact, some studies have shown that the accumulation of α-Amanitin leads to the increase of superoxide dismutase (SOD) and glutathione (GSH) peroxidase activities, malondialdehyde products, and lipid peroxidation, which is related to the inhibition of catalase activity (Dündar, 2017, Rodrigues, 2020,

Steurer, 2018, Zheleva, 2013, Zheleva, 2007) (Figure 2). Recent researches have indicated that α-Amanitin induces the production of GSH and tGSH, confirming the hypothesis of the involvement of oxidative stress in the pathophysiology (Rodrigues 2020, Steurer 2018). A scientist found that α-Amanitin could form phenoxyl-free radicals that might be involved in the increased production of reactive oxygen species (Figure 2) (Zheleva, 2013). With the case of Amanitin intoxication, induction of the NF-κB (nuclear factor-kappa B) pathway has been observed to have a certain protective effect without establishing a link with the levels of production of SOD, GSH, or catalase (Garcia, 2015, Morgan and Liu, 2011).

3.3 Inhibition of Protein Synthesis

Furthermore, the cytotoxicity α-Amanitin will result in the inhibition of RNA polymerase, especially RNA polymerase II (it is more sensitive to this mushroom toxin than other polymerases in eukaryotic cells). Since RNA polymerase II is responsible for mRNA synthesis in the cell, α-Amanitin is a potent and selective inhibitor of mRNA synthesis (Kume, 2016). The main toxicity mechanism of Amanitin is attributed to non-covalent nuclear inhibition of RNA polymerase type II (RNAP II), which reduces mRNA levels and protein synthesis (WIELAND 1983). By complexing with the intracellular RNA polymerase II enzyme, amatoxins inhibit the formation of mRNA and then restrain the protein synthesis, leading to cell necrosis rapidly. This enzyme inhibition has been proved to be the cause of RNAP II ubiquitination and its degradation by proteasomes, which is related to the increased intracellular ATP concentration (Rodrigues, 2020, Steurer, 2018) (Figure 2). Up to now, there are still many unknown areas about the toxic effects of Amanitin at the cellular level that need further exploration. The treatment mainly remains symptomatic.

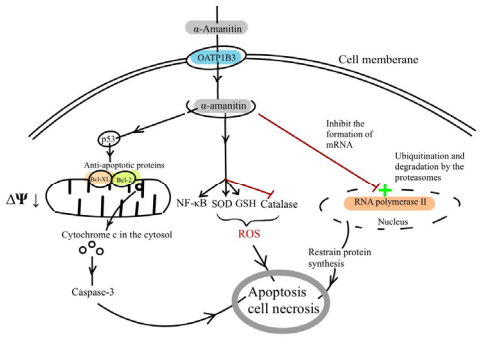


Figure 2: Main toxicity mechanisms of Amanitin within hepatocytes (modified from (Le Daré, 2021)

4 INTRACELLULAR ACTIVITIES OF ALPHA-AMANITIN

4.1 Inhibition of Tumor Growth through Suppression of POLR2A

While there is no denying that most cancers come along with the deletion of the tumor suppressor gene p53, no effective p53-based therapy has been successfully applied in clinical treatment due to its complexity in signalling. However, 104 (53%) out of 195 colorectal cancer cells (CRC) cases bear the hemizygous loss in the 17p13 region, resulting in concomitant deletion of TP53 and POLR2A(Liu, 2015). The gene POLR2A encodes the largest subunit of RNA polymerase II and is indispensable in cell proliferation. No homozygous deletion was observed in cancer cells, in accordance with the fact that POLR2A is essential for cancer cell survival. Studies demonstrated that POLR2A expression tightly correlates with its gene copy numbers, resulting in significantly lower levels of POLR2A loss (hemizygous deletion) cells(Liu, 2015). However, by comparing POLR2Aneutral cells and POLR2Aloss cells, the similar proliferation rates indicate this hemizygous loss is sufficient to main proliferation in HCT116 cells. The half-maximum inhibitory concentration (IC50) for the POLR2Aneutral cells was 10-fold greater than the POLR2Aloss cells; when examined for drug sensitivity to different chemotherapy drugs of these two cell types, POLR2A inhibited by α-Amanitin notably increased the cell-killing effects yet no significant enhancements were observed in normal cells. POLR2Aloss cells were more sensitive to POLR2A inhibition by α-Amanitin while re-expression of POLR2A rescued resistance to α-Amanitin, where this loss happens to be concurrent with p53 deletion in major cancer cases. High concentrations of α-Amanitin caused complete deaths while with low doses, the inhibition had significantly higher levels of cell-killing effect on the POLR2A loss cells than the neutral ones(Liu 2015). Taken together, this research implied using α-Amanitin as a potential therapeutic method against CRC.

4.2 Inhibition of RNAPII by Amanitin via TAF15 Mrna

Kume et al. investigated the impact of RNA polymerase II inhibition on DTC survival(Kume, 2016). Among four compounds that affect different steps including chromatin formation, transcription, or protein synthesis, α-Amanitin showed remarkable suppression of colony formation in the shortest exposure period. Moreover, colony formation was also clearly suppressed by the other less-specific RNAP inhibitor AMD, suggesting the selective inhibition of RNAPII plays a crucial role in suppressing colony formation. Their study also demonstrated that TAF15 is especially responsible for DTC formation as TAF15 gene products are upregulated in DTC-forming cells. Recent studies had suggested TAF15 binds the C-terminal domain of RNAPII more avidly than other RNA-binding proteins in the TET family and acts as a coactivator of RNAPII(Kwon, 2013). Both TAF15 mRNA and protein levels were decreased after treatment, suggesting RNAPII activity towards TAF15 mRNA was inhibited by α-Amanitin. Furthermore, the subsequent colony formation assay showed that TAF15 knockdown suppressed the emergence of both DTCs with or without α-Amanitin treatment, meaning TAF15 depletion inhibited DTC formation. Additionally, TAF15-knockdown and α-Amanitin-treated cells induced similar morphological changes, suggesting a TAF15 depletion by α-Amanitin treatment. Both TAF15 mRNA and protein levels were decreased in response to α-Amanitin, consistent with similar morphological changes. These results suggested TAF15 is a mediator of RNA polymerase II-dependent DTC formation and a crucial target of α-Amanitin. The RNAPII-dependent inhibition of early-phase mRNA synthesis is sufficient to produce a nearly complete suppression of colony formation, and the practicality of α-Amanitin treatment for disease caused by DTCs, such as Peritonitis Carcinomatosa by chemotherapy, via inhibition of TAF15.

5 THERAPEUTIC USE OF ALPHA-AMANITIN

α--Amanitin has a low cellular uptake due to its polar structure and low permeation except in liver cells where OATP1B3 internalizes the toxin(Bodero 2018). Free Amanitin permeation mediated by transporter protein results in apoptosis and necrosis of hepatocytes(Bodero, 2018, Letschert, 2006). The strong inhibition effect caused by Amanitin aroused the interest of scientists to target cancer cells while preventing liver toxicity. Numerous studies have shown promising cell-killing effects after direct α-Amanitin treatment in tumor cells. In order to enhance the selectivity of Amanitin treatment, scientists have developed conjugates targeting tumor cells and novel drug release methods.

5.1 Antibody-drug Conjugates Approach

Amanitin antibody-drug conjugates (ADC) were found to successfully increase α-Amanitin activity in target cells while not being a substrate of OATP1B3 to avoid systematic toxicity. The antibody is responsible for targeting specific antigens and bringing the toxin α-Amanitin into the cells. Moreover, epithelial cell adhesion molecule (EpCAM) is a target antigen highly and frequently expressed on carcinoma cells and its metastasis (Gires 2020). α-Amanitin that was conjugated with chiHEA125, a chimerized anti-EpCAM antibody, reduced cell proliferation in human pancreatic (BxPc-3 and Capan-1), colorectal (Colo205), breast (MCF-7), and bile duct (OZ) cancer cell lines (IC50 = 2.5×10−10 to 5.4×10−12 M)(Moldenhauer 2012). The antiproliferative effect of chiHEA125-ama was up to 10,000 -fold higher than α-Amanitin alone. Anti-tumor effects of chiHEA125-Amanitin conjugates were tested in an experimental human BxPc-3 pancreatic cancer model, induced by injecting BxPc-3 cancer cells subcutaneously into the right flank of female mice. A single dose (50 μg/kg with respect to α-Amanitin) suppressed BxPc-3 xenograft tumor growth, while two higher doses (100 μg/kg with respect to α-Amanitin), administered one week apart, inhibited tumor recurrence for 3-4 weeks. Besides, the treatment was well-tolerated in tumor-bearing mice and no substantial difference was observed compared with the control mice treated with unconjugated chiHEA125 (Moldenhauer, 2012).

Drug resistance is one of the major concerns of existing chemo- and targeted therapies of colorectal cancer cells (CRC)(Van der Jeught, 2018). Escaping mechanisms including enhanced DNA repair and drug metabolism result in worse clinical outcomes. Research showed that α-Amanitin-HEA125 selectively killed human CRCs with a hemizygous deletion of POLR2A(Liu 2015). Those cells were more sensitized to chemotherapy drugs owing to the strong inhibition of POLR2A by α-Amanitin. It was observed that a very low dose of α-Amanitin-

HEA125 (10μg/kg) was sufficient to inhibit tumor growth in mice bearing POLR2Aloss HCT116 tumors, reducing the effective doses of α-Amanitin by at least 10,000-fold (IC50 = 0.01 ng/ml). Increasing specificity and effects on chemo-resistant cancer cells by conjugating with antibody HEA125 implied a potential therapeutic way to EpCAM expressed human cancers and other Amanitin ADCs might be synthesized in future studies to target other cancers. Although α-Amanitin ADC has shown positive outcomes in various animal studies, this approach is relatively high cost and may result in unfavorable pharmacokinetics and immune response (Bodero, 2018).

Antibody-targeting Amanitin conjugates (ATACs) have a broader application than other toxin compounds as payloads because of their unique interaction with RNAPII. Comparing with other intracellular targets like microtubule(Nasiri 2018), RNAPII maintains a much lower number of 100 to 1000, rendering low concentration able to achieve optimistic cell-killing effects. Slow growth of tumor is common in prostate cancers, and another advantage of α-Amanitin as a payload is the active inhibition on dormant cells. Despite the fact that Prostate-specific membrane antigen (PSMA) are expressed in normal tissues as well, the expression levels are much higher in prostate carcinomas, indicating a potential high specificity for antibody therapy(Osborne 2013). Scientists conjugated α-Amanitin to the anti-PSMA mAb 3F11(Hechler, 2014). To test the active inhibition of ATACs on non-proliferating cells, growth arrest of LNCaP cells is made by addition of Interleukin 6 (IL-6). Inhibition effects were observed both on rapidly dividing and growth arrested LNCaP cells. The viability curve of dormant cells is comparable to dividing ones under increasing concentration of Amanitin ADC, indicating the proliferation-independent inhibition on tumor by ATACs (Hechler, 2014).

However, the large size and high affinity to the target of ADCs restrict their ability of penetration, especially in solid tumors. Furthermore, their long circulatory half-life might lead to immunogenicity and unspecific toxicities. Thus, alternative approaches may still catch scientists' attention in the future.

5.2 Small Molecule-drug Conjugates Approach

Small molecule-drug conjugates (SMDCs) are an alternative approach where a specific cell-membrane-receptor ligand assists drug delivery to the target site and internalization by the receptor. Tripeptide arginine-glycine-aspartate (RGD) and the related sequence isoaspartate-glycine-arginine (isoDGR) were two ligands that have been conjugated to α-Amanitin targeting the αVβ integrin receptor family (Bodero 2018). αVβ integrin receptors are strongly expressed on blood vessels in human cancers such as breast cancer, glioblastoma, pancreatic tumor, prostate carcinoma (Desgrosellier and Cheresh 2010). The conjugates demonstrated great binding affinity to the receptor like the free ligands. However, the toxicity and inhibitory effect of the integrin ligand-α-Amanitin conjugates were either worse or slightly better than free α-Amanitin. Similar results were achieved in other studies involving small molecule-amanitin conjugates (Moshnikova, 2013, Zhao, 2015). In addition, SMDCs have a shorter half-life compared to ADCs due to their smaller size, limiting their distribution to tumor cells and therapeutic effect. In order to prolong circulatory half-life, the immunoglobulin Fc domain was conjugated to α-Amanitin-based SMDCs (Gallo, 2021). Apparently, the interaction between the Fc domain and neonatal Fc receptors (FcRN) results in prolonged exposure to drugs that contain Fc peptides (Wang, 2011, Wu, 2012). Both SMDCs (IC50 = 0.863 nM)) and Fc-SMDCs (IC50 = 15.2 nM) had higher cytotoxicity in vitro compared to unconjugated α-Amanitin (IC50 = 476 nM). In vivo pharmacokinetics and biodistribution study were conducted to evaluate half-live, tumor, and organ accumulation of SMDCs and Fc-SMDCs. Fc-SMDCs showed a dramatic decrease in their clearance, thus extending their half-lives from 44 min to approximately 7.2 days (Gallo, 2021).

Table.2: Evaluation of inhibitory effect of α-Amanitin and α-Amanitin conjugates in various cell lines.

Compound (name)	IC_{50} (cell lines)	References
ChiHEA125-Ama	2×10^{-12} M (Colorectal, Colo205), 8.7×10^{-11} M (Bile duct, OZ), 2.1×10^{-11} M (Pancreatic, Capan-1), 2.5×10^{-10} M (Pancreatic, BxPC-3), 5.4×10^{-12} M (Breast, MCF-7)	(Moldenhauer 2012)

Ama-HEA125	0.1 μg/ml (POLR2A^{loss} HCT116)	(Liu 2015)
HDP 30.2284	8.63×10^{-10} M (LNCaP)	(Gallo 2021)
HDP 30.2972	1.52×10^{-8} M (LNCaP)	(Gallo 2021)
α-Amanitin	~4.76×10^{-8} M	(Gallo 2021)

To increase the cytotoxicity effect, α-Amanitin was combined with another cytotoxic drug displaying an independent mode of action. Dual conjugation of α-Amanitin and monomethyl auristatin E (MMAE) with fibroblast growth factor 2 (FGF2) tend to increase the toxicity of both drugs and maintain the selectivity (Świderska, 2018). Fluorescence microscopy was performed to track the internalization process and found that FGF2 dual conjugate internalization was strongly correlated with FGF receptors level on the cell surface. None of the tested conjugates displayed severe toxicity towards receptor-negative cells, suggesting its selectivity to FGF receptor expressed cells. After binding to the high affinity FGFRs on the cancer cell surface, dual FGF2 conjugate is internalizing by endocytosis. Processing through the endosome–lysosome pathway leads to release of MMAE and α-Amanitin inside the cell and next respectively inhibit tubulin polymerization and DNA transcription. α-Amanitin inhibited DNA transcription and MMAE stopped microtubule polymerization simultaneously, leading to cell apoptosis. The dual conjugate had a greater cytotoxic effect than any of single-drug FGF2 conjugates, which can be explained as a result of the combined cytotoxic action of α-Amanitin and MMAE. With the highest concentration, α-Amanitin/MMAE-FGF2 conjugate reduced 95% of cell viability. No or minimal immune response was triggered since the ligand sequence was fully from Homo sapiens (Świderska, 2018). The positive result of combining two cytotoxic agents allows more possibilities in the future against different cancer cells with different intracellular impair.

5.3 Drug Delivery and Release

On the basis of cell targeting models including ADCs and SMDCs, controlled drug release further increases selectivity. Stimuli-responsive prodrugs and carriers allowed on-demand drug delivery and release (Dunkel and Ilaš, 2021). Photocaged α-Amanitin analog, Ama-Flash has been synthesized by Matinkhoo and coworkers (Matinkhoo 2021). Nitroveratryl (Nv) ether was attached to modified α-Amanitin as a photo-masking group which is extensively used in photo-pharmacology studies. Nv-protected α-Amanitin was inactive against RNAP II and didn't result in cell death even at a high dose of 100 μM. α-Amanitin analog was released through a 25 min irradiation with λ = 366 nm and CHO (Chinese hamster ovary) cell viability was measured after 48 and 72 hours, with α-Amanitin as a control. The IC50 value was not significantly different from free α-Amanitin, suggesting that irradiation may not affect toxicity. Furthermore, irradiated wavelengths are minimal cytotoxic and won't be absorbed by tryptathionine in α-Amanitin. The toxicity of byproduct 4,5-dimethoxy-2-nitrobenzyl (DMNB) was not tested. With light-activated α-Amanitin analog, it creates the possibility to preload the drug into cells and trigger RNAP II inhibition and ubiquitination when needed. Amanitin conjugated with pH low insert peptide (pHLIP) is another drug delivery method based on pH-dependent conformational change of pHLIP which leads to its insertion into the membrane (Moshnikova, 2013). Acidic environment protonates Asp/Glu residues and increases hydrophobicity of pHLIP. Studies showed that the antiproliferative effect was 4-5 times higher at pH 6 compared to pH 7.4, creating the selectivity due to the negative transmembrane pH gradient of cancer cells. Cytotoxic effect was achieved on four different human cancer cell lines at concentration of 0.25–1 μM. Taken together, the new techniques of improving drug delivery and release such as ADCs, SMDCs, as well as photo-pharmacology methods, give more flexibility to cancer chemotherapy.

6 CONCLUSIONS

In this review, the toxic mechanisms of α-Amanitin, a fetal chemical present in the mushroom species Amanita Phalloides were discussed. Its ability to inhibit mRNA polymerase II and induce p53 protein makes it a new possibility against cancer. We reviewed multiple animal studies in which α-Amanitin conjugates were formed with either antibodies or small molecules and showed strong selectivity and toxic effect. Novel drug delivery methods provide new insight into future cancer chemotherapy. More in vivo and in vitro animal studies with larger sample sizes are necessary in order

to confirm the cytotoxicity effect as some cytotoxic mechanisms are still unclear. There are risks associated with animal-to-human extrapolation due to differences in metabolism and size. A first-in-human phase 1/2a study started enrolling participates in early 2021 (Strassz, 2020). and this study aims to determine the maximum tolerated dose and assess the anti-tumor activity of HDP101, an ADC targeting BCMA (B cell maturation antigen) carrying a synthetic version of Amanitin as a payload. Future research focusing on the mechanisms of α-Amanitin anti-cancer effects and related clinical trials may be required to promote the understanding of α-Amanitin as a potential therapeutic way for cancer treatment.

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