Combination therapy of benzylpenicillin IV and silybin IV vs. monotherapy of benzylpenicillin IV or silybin IV in the treatment of *A. phalloides* intoxication

University of Groningen
Mentor: S. Notenboom
Student: Anne Kamphuis, S2995727

Abstract

Amanita phalloides mushroom poisoning is a rare but serious occurrence, which seems to have the highest frequency in Europe. The *phalloides* syndrome is hard to recognize and institution of an effective treatment as soon as possible is important in order to avoid fatal complications. Since the occurrence of the poisoning is rare, there is not many known about effective treatments. The aim of this thesis is to decide whether combination therapy of benzylpenicillin IV and silybin IV is more effective against *Amanita phalloides* poisoning than intravenous monotherapy of these drugs. Comparing clinical studies and *in vitro* studies from European countries, it seems that silybin IV is the most effective therapy.

Contents

Abstract 2

1. Introduction 4

1.1. Amanita phalloides: biology and appearance in Europe 4

1.1.1. Phallotoxins: characteristics and toxicology 5

1.1.2. Virotoxins: characteristics and toxicology 5

1.1.3. Amatoxins: characteristics and toxicology 5

1.1.4. Phases of *A. phalloides* syndrome 6

1.2 Dealing with *A. phalloides* intoxication 7

2 Literature research 9

2.1 Mechanism of action benzylpenicillin 9

2.2 Mechanism of action silybin 9

2.3 Combined mechanism of action benzylpenicillin and silybin 10

2.4 Safety profile benzylpenicillin 10

2.5 Safety profile silybin 10

2.6 Benzylpenicillin therapy in Europe 11

2.7 Silybin therapy in Europe 13

2.8 Combined therapy benzylpenicillin and silybin in Europe 14

2.9 Overview combined data 15

Discussion 16

Conclusion 19

References 20

1. Introduction

Nowadays, mushrooms have become more popular for humans to be a part of their diet. Mushrooms contain lots of proteins and have an exquisite taste (Garcia, 2015). Next to that, there is a growing craze for 'organic' food and when specifically looking at Europe, there is a growing variability in cultures within the continent due to immigration. Some of these cultures are used to collecting their own food and thereby wild mushrooms (Das, 2007). Some mushroom 'hunters' believe that poisonous varieties can be easily identified, but there is a great change that misidentifications of edible and toxic mushrooms occur, due to similarities of morphological characteristics (Garcia, 2015). Many have the erroneous thought that some toxic species are harmless if they are cooked or frozen, causing fatalities even under experienced seekers (Becker, 1976).

More than 90% of the fatalities caused by mushroom poisonings are a result of ingestion of amatoxin-containing species (Giannini, 2007). The species that contain amatoxin are *Amanita (Amanita phalloides, A. virosa* and *A. verna), Lepiota* and *Galerina*. Among these, *A. phalloides* has caused the most fatalities (Enjalbert, 2002). Fatalities caused by *A. phalloides* particularly occurred in Europe (Barceloux, 2008).

1.1. Amanita phalloides: biology and appearance in Europe

Amanita phalloides are also referred to as 'death cap' or 'deadly angel' due to its fatal effects. In figure 1 an illustration of the death cap is shown. Looking at its physical properties, it has a grayish olive to greenish yellow cap which is about 5-15 cm. The stalk of the mushroom contains a membranous volva surrounding an enlarged bulb at its base. *A. phalloides* is the primary European poisonous mushroom, particularly in Central and Occidental Europe (Barceloux, 2008). They are found most common in damp woods, especially in beech and oak forests, between the months June and October. *A. phalloides* have a sweetish, not unpleasant smell (Bonnet, 2002). There are three classes of cyclic peptide toxins present in *A. phalloides*, which can be grouped into phallotoxins, virotoxins and amatoxins (Garcia, 2015).

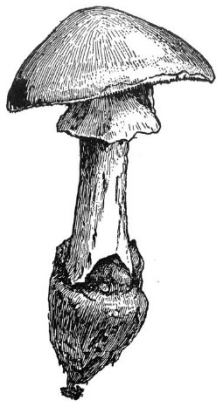

Figure 1. Illustration of the so called 'death cap' or 'deadly angel' (Allen).

1.1.1. Phallotoxins: characteristics and toxicology

Phallotoxins are bicyclic heptapeptides and are mostly present in the volva of *A. phalloides* (Wong, 2013). These toxins are not taken up by the gastrointestinal tract and are therefore not lethal, although they do cause toxic effects in the GIT resulting in clinical symptoms such as vomiting and diarrhea. Phallotoxins have a great heat stability and are not destroyed by cooking. This is shown in a case where a man cooked the mushroom before ingestion, though still developed clinical symptoms corresponding to the GIT toxic effects (Vo, 2017). When administered parenterically, the major *in vivo* toxic effects are affecting the liver (Wieland T. , 1977). In humans, phallotoxins are mostly taken up by a hepatocyte-specific organic anion-transporting octapeptide OATP1B1/SLC21A6, but OATP1B3 is also capable of transporting phallotoxins across the hepatocyte membrane (Mcqueen, 2010). When taken up in the hepatocytes, cytotoxicity through phallotoxins is caused by its ability to bind to F-actin. This stabilizes the actin filaments and thereby microfilaments depolymerization is prevented. The correct function of the cytoskeleton is disturbed (Wieland T. , 1977). Uptake of these toxins causes cholestatis (Mcqueen, 2010). These effects might be relevant for human ingestion, since the structures of phallotoxins, virotoxins and amatoxins are much alike and thus might cause similar effects. Also, if the phallotoxins do manage to enter the blood by e.g. damaged epithelia, it is relevant to know the effects.

1.1.2. Virotoxins: characteristics and toxicology

Virotoxins are found to show structural similarities to phallotoxins, although virotoxins are monocylic peptides. The biological activity and the behavior on molecular level are also comparable with phallotoxins, thus causing toxicity through binding to F-actin (Faulstich, 1980). Virotoxins also have a great heat stability and are nog destroyed by cooking (Vo, 2017). As with phallotoxins, virotoxins are not taken up by the gastrointestinal tract and thus are not considered lethal after oral intake, but they do cause toxic effects in the GIT (Garcia, 2015).The affinity of virotoxins towards F-actin is very similar to that of phallotoxins. However, the flexibility of the monocylic structure and the presence of two additional hydroxy groups in virotoxins suggest a different mode of interaction. While there is proof that the bicyclic phallotoxins possess a rigid binding site, the virotoxins may derive their biologically active conformation by an induced-fit mechanism upon contact with actin (Faulstich, 1980).

1.1.3. Amatoxins: characteristics and toxicology

Amatoxins are bicyclic octapeptides which are formed by at least nine different compounds; α-amanitin, β-amanitin, γ-amanitin, ε-amanitin, amanin, amaninamide, amanullin, proamanullin and amanullinic acid. From these compounds, α-amanitin and β-amanitin were the main focus of toxicological studies (Garcia, 2015). These toxins have a great heat stability and they are resistant to enzyme degradation. Amatoxins cannot be destroyed by cooking, drying of freezing, which makes them exceptionally toxic (Poucheret, 2010). Unlike phallotoxins and virotoxins, amatoxins are absorbed from the human gastrointestinal tract after ingestion of *A. phalloides*. Amatoxins can be detected in urine as early as 90-120 min after ingestion, which is proof of rapid absorption by the gastrointestinal tract and excretion through the kidneys (Homann, 1986). Amatoxins do not bind to albumin, which causes a rapid elimination from the blood. It is distributed to the liver and the kidneys within a period

of 48 h (Faulstich, 1985). Amatoxins are taken up in the liver by OATP's, which are present in the sinusoidal membranes of human hepatocytes. OATP1B3 is identified as the main uptake transporter (Letschert, 2006). The mechanisms of toxicity caused by amatoxins on a molecular level differ from phallotoxins and virotoxins. Multiple studies have shown that the main mechanism in which amatoxins cause toxicity is through inhibition of the DNA-dependent RNA-polymerase II. RNA-polymerases II are present in the nuclei of all eukaryotic cells, where they are responsible for the transcription of DNA to give messenger-RNA. A more detailed investigation of the individual steps of transcription showed that amanitin inhibits the elongation step. Binding of amanitin to RNA-polymerase II blocks movement of the proteins along the 'single-stranded' DNA. As a result, protein synthesis is inhibited, which causes death of the affected cells (Wieland & Faulstich, 1991); (Bushnell, 2002). It is thought that the binding site of α-amanitin is located in the interface of subunits Rpb1 and Rpb2 (Bushnell, 2002). However, the characterization of this particular mechanism needs to be further investigated. Another mechanism through which α-amanitin causes liver injury is apoptosis. Studies have shown that presence of α-amanitin in human hepatocyte cultures induce p53-dependent apoptosis (Magdalan, 2011) and that the concentration that is required for p53-dependent apoptosis was in relation to the concentration that is required for inhibition of mRNA-synthesis (Ljungman, 1999). This might indicate that there is a certain relation between the two effects. Next to that, an *in vivo* study suggest that there is a synergistic effect between α-amanitin and TNF-α. After *in vivo* administration of a high dose of α-amanitin in mice, hepatic TNF-mRNA levels were increased and the hepatocytes underwent apoptosis (Leist, 1997). Although there is no more proof of this effect and the effect is not confirmed in another study (El-Bahay, 1999).

1.1.4. Phases of *A. phalloides* syndrome

Amanita phalloides poisoning in general consists of three different phases. The first phase is the gastrointestinal phase, which is caused by virotoxins and phallotoxins. The second phase is an asymptomatic latency period and in the third phase the hepatic-kidney failure takes place, which is caused by amatoxins (Poucheret, 2010); (Becker, 1976). In more detail, the first phase occurs abruptly within 6-24 hours after ingestion and it is characterized by vomiting, abdominal pain, hypoglycemia and diarrhea that can lead to severe dehydration. It seems that phallotoxins and virotoxins are responsible for this effects (Bonnet, 2002); (Becker, 1976); (Poucheret, 2010). The second phase takes place during the next 24-48 hours. During this latency period there appears to be remission of the symptoms which gives an impression of recovery, though progressive impairment of hepatic and renal function takes place (Becker, 1976); (Bonnet, 2002). The final phase starts 48-72 hours after ingestion and it lasts for about 6-16 days. Hepatic and renal states worsen and symptoms such as jaundice, hypoglycemia, delirium, coagulation disorders and confusion develop. Symptoms such as coagulation disorders are a result of renal failure. Renal failure in the so called hepatorenal syndrome is a consequence of multiple factors caused by acute liver failure, e.g. changes in blood flow. In this phase, the symptoms will worsen and ultimately cause death (Becker, 1976); (Bonnet, 2002); (Poucheret, 2010).

1.1.4.1. Identification A. phalloides intoxication

The time between ingestion and onset of symptoms and the type of systemic involvement, for example neurologic of gastrointestinal, can be very helpful pointers for characterizing a certain type of mushroom poisoning. However in the case of an *A phalloides* intoxication, delay in onset of symptoms, individual susceptibility variation and lack of rapid and reliable identification have contributed to the significant mortality of this type of intoxication (Becker, 1976). Symptoms that occur during the first phase such as nausea, vomiting and abdominal pain are often erroneous associated with other gastrointestinal diseases. Suspicion of *A. phalloides* intoxication is most often obtained by assessment of history concerning the patient's diet (Santi, 2012) (Becker, 1976).

After ingestion of *A. phalloides,* α-amanitin can be identified by multiple analytical methods such as thin-layer chromatography, radioimmunoassay, enzyme-linked immunosorbent assay, RP-HPLC (reversed-phase high pressure liquid chromatography), LC-MS (liquid chromatography-mass spectrometry), capillary zone electrophoresis with photodiode array detection, GC (gas chromatography), GC/MS (gas chromatography, mass spectrometry) or multistage linear ion trap mass spectrometry. Vomitus, blood and urine should be collected from the patient to carry out the analysis (Barceloux, 2008) (Garcia, 2015). RP-HPLC is the method that is used most common, although analysis with LC-MS seems to provide the most reliable and sensitive results (Garcia, 2015). Another method that could be used for identification is the Wieland-Meixner test, which is a test for the presence of amatoxins that is relatively sensitive. The test is based on an acid-catalyzed reaction of amatoxins with the biopolymer lignin, which will form a blue product. The test is carried out by squeezing juice from mushroom tissue onto a piece of newsprint, which contains the lignin. If the spot turns blue, this is an indication of a positive test. This is a qualitative test. (Barceloux, 2008) (Garcia, 2015). The toxins itself could also be used as a marker for detection, since α- and β-amanitin are present in the blood and urine after intoxication. Although there are few data that show a correlation between blood amanitin concentrations to symptoms or outcome. Urine samples usually contain a higher concentration of amanitin and since amanitin disappears from the blood rapidly, the urine samples are preferred (Barceloux, 2008).

1.2 Dealing with *A. phalloides* intoxication

Treatment of an amatoxin poisoning is primarily supportive with attention directed towards the acid-base, fluid, coagulation, and the electrolycte imbalances which are caused by the gastrointestinal phase of the poisoning (Barceloux, 2008) (Becker, 1976). Patients with an altered mental status should be immediately evaluated for hypoglycemia, since altered mental status could be a result of hypoglycemia that needs rapid treatment (Banh, 2008). Patients who are suspected to be late in the course of amatoxin poisoning, should be examined for hepatorenal failure, hemorrhage and coagulopathy. This suspicion might occur if it is known that there is a large time interval between ingestion of the mushrooms and hospitalization. If coagulopathy due to hepatic damage has occurred, fresh-frozen plasma, vitamin K and packed red blood cell transfusions may be necessary to correct it (Barceloux, 2008). Next to these supportive measures, detoxification procedures are carried out. These procedures consists of two methods, namely the reduction of absorption by the intestine

and enhancement of excretion (Santi, 2012). During the oral detoxification, treatment with activated charcoal takes place to absorb remaining toxins and to interrupt the enterohepatic circulation, although there is no evidence that this improves clinical outcome (Bergis, 2012) (Santi, 2012) (Barceloux, 2008). Another detoxification procedure is treatment with Molecular Adsorbent Recirculating System (MARS). The real efficacy of this method should be analyzed in appropriate trials, but their use is a potential additional option for the treatment of patients with severe amanita poisoning. MARS is a modified dialytic method that mimics the biological function of the hepatocyte membrane by transferring toxic metabolites from the bloodstream into a dialysate compartment. This method was shown to be efficient in improving the state of the liver. However, the treatment is only useful if it is started very early, soon after the gastrointestinal symptoms have occurred (Santi, 2012). In some fatal courses, immediate liver transplantation may be required as the only curative treatment option (Bergis, 2012).

Condition of the patient and the state of internal damage are important factors that determine the procedure that is carried out for treatment. Next to these measures, drugs are also used to prevent and treat hepatocellular damage (Barceloux, 2008) (Becker, 1976) (Bergis, 2012) (Santi, 2012). Although, due to lack of case reporting and lack of knowledge about the effectiveness of various treatments, there are no worldwide widely accepted guidelines regarding the drug treatment of amatoxin-poisoning. The Portuguese poisoning information center, The clinical toxicology database of the United Kingdom (TOXBASE) and the national poisons center of New Zealand for example all have different guidelines (Garcia, 2015). Since *A. phalloides* intoxication is most prominent in European countries (Křenová, 2007), the main focus of this thesis will be regarding the drug therapies in Europe.

1.2.1. Current drug therapy in Europe

Based on clinical therapies in amatoxins poisoning, drugs that are used for treatment in Europe are β-lactam antibiotics (mostly benzylpenicillin), silybin, N-acetylcysteine and thiotic acid (Giannini, 2007) (Křenová, 2007) (Enjalbert, 2002) (Bergis, 2012). Silybin in derived from silymarin. Silymarin is isolated from the seeds of Mediterranean milk thistle and contains the three isomers silydianin, silychristin and silybin, whereas silybin is the major compound (Enjalbert, 2002). Since benzylpenicillin IV and silybin IV are the most used drugs in the clinical trials and since they are the most promising (Mengs, 2012) (Křenová, 2007), the main focus of this thesis will be on these drugs.

Some suggest that the combination therapy of silybin plus benzylpenicillin is more beneficial than other combinations and some think that both drugs act as competitive inhibitors of the amatoxin uptake transporters in the hepatic cells and thus should not be used in combination (Enjalbert, 2002). Since the mortality rates due to *A. phalloides* poisoning are still too high and there are still a lot of uncertainties when it comes to drug therapy, the research question of this thesis is: '*Is combination therapy of benzylpenicillin combined with silybin a better approach to handle hepatocellular damage and prevent mortality caused by A. phalloides poisoning compared to monotherapy of silybin or benzylpenicillin?*'

2 Literature research

2.1 Mechanism of action benzylpenicillin

There have been several hypotheses proposed towards the explanation of the mechanism of benzylpenicillin in amatoxin poisoning (Garcia, 2015). A study showed that amatoxin uptake by the hepatocellular membranes is inhibited by most of the used drugs, but not by benzylpenicillin. This study was carried out using hepatic cell particles from rat liver (Kröncke, 1986). Although, a more recent study using human hepatocytes, suggests that benzylpenicillin does block the cellular uptake of α-amanitin. It is shown to be a potent inhibitor of the OATP1B3 transporter (Gundala, 2004) (Letschert, 2006), although there is no data on the kinetics of the interaction that takes place between α-amanitin and OATP1B3 (Roth, 2012). Floersheim has a hypothesis that the drug could displace α-amanitin from binding on serum protein, but this hypothesis is challenged by evidence that both α-amanitin and benzylpenicillin do not bind serum albumin (Enjalbert, 2002) (Garcia, 2015). Other reports presented evidence of an anti-proliferative effect of benzylpenicillin on cultured eukaryotic cells concerning the DNA replication systems. The intracellular target of benzylpenicillin appears to be the enzyme polymerase I. RNA polymerase I is located in the nucleoli. It is responsible for transcription of the tandem array, a serie of copies of a gene, for certain ribosomal RNA. As already mentioned, amatoxins are blockers of DNA-dependent RNA polymerase II. Polymerase II is located in the nucleoplasm and is resposible for synthesizing both the precursors of messenger RNA and several small RNA molecules, such as the ones of the splicing appartus. There is a possibilty that benzylpenicillins have a protective effect through their effects on eukaryotic DNA replication mentioned above (Do, 1987) (Neftel, 1987) (Berg, 2002). Although the mechanism how it actually prevents damge caused by amatoxin needs further investigation.

2.2 Mechanism of action silybin

As with benzylpenicillin, there have been several hypotheses proposed towards the explanation of the mechanism of silybin in amatoxin poisoning. Data in the literature suggests that silybin acts in different ways. One of the protective mechanisms of silybin is due to its strong antioxidant activity. This could explain its action against hepatotoxic agents that cause damage through oxidative stress, for example lipid peroxidation or cellular necrosis caused by paracetamol. The drug does not only act as an antioxidant because it is a scavenger of free radicals that induce lipid peroxidation, but it also influences enzyme systems associated with glutathione and superoxide dismutase (Fraschini, 2002) (Campos, 1989). Another protective mechanism through which silybin acts is by stimulating protein synthesis. Silybin is able to enter the cell nucleus and act on RNA polymerase I enzymes, which causes an increase of ribosomal formation. This stimulates protein and DNA synthesis, thus working as a balancing effect with respect to the damage that is caused by amanitin through RNA polymerase II. This mechanism has important therapeutic implications in repairing damaged hepatocytes and to restore the normal function of the liver. The increase of protein synthesis induced by silybin was only seen in injured livers, and not in healthy ones (Fraschini, 2002) (Pradhan, 2006). This is interesting, because it might suggest that the efficacy of therapy with silybin increases in livers who are more damaged. Next to these mechanisms, silybin acts against damage through anti-inflammatory and anti-fibrotic effects

on hepatic stellate cells. Silybin is able to inhibit growth factor-induced pro-fibrogenic actions of activated human hepatic stellate cells, including proliferation, motility, and synthesis of extracellular matrix components. The anti-inflammatory activity was shown, since silybin inhibited the synthesis of the inflammatory cytokines MCP-1 and IL-8 (Trappoliere, 2009). Another important mechanism of action of silybin is the inhibition of OATP1B3 activity, which may be a crucial part of preventing the uptake of amanitin by hepatic cells (Wleck, 2013). Just as with benzylpenicillin, there is no data on the kinetics concerning the interaction between silybin and OATP1B3.

2.3 Combined mechanism of action benzylpenicillin and silybin

As with the mechanisms of the individual drugs, there are different thoughts about the combined mechanism and efficacy of benzylpenicillin and silybin together. Some suggest that the combination is more efficient (Tong, 2007), but the mechanism behind this observed effect needs to be further investigated. Others think that the drugs should not be used in combination, since the drugs both act as competitive inhibitors of the amatoxin-transporting system (Rumack, 1994). Although setting this hypothesis, disparate effects of benzylpenicllin and silybin on the α-amatin uptake were not considered (Enjalbert, 2002) (Kröncke, 1986). This weakens the hypothesis.

2.4 Safety profile benzylpenicillin

Benzylpenicillin is reported to be efficient, although it does have safety issues (Garcia, 2015). Benzylpenicillin causes allergic drug reactions with an incidence of 1-10%. Also the electrolyte balance in the body may be disrupted when a large amount of sodium ions is administered with this antibiotic agent to amatoxin intoxicated patients. High doses of benzylpenicillin are also observed to cause severe granulocytopenia. Degradation products that are formed *in vitro* are often the cause of such adverse effects rather than the parent antibiotic. Use of freshly prepared single doses of benzylpenicillin prevents the most adverse effects. Although, given the bone narrow toxicity of β-lactams, benzylpenicillins are able to affect all the hematopoietic cell lines. Next to these effects, massive benzylpenicillin therapy may also cause neurotoxic symptoms in patients with nervous system disease and renal insufficiency and induce convulsions (Enjalbert, 2002). It is already known for many years that penicillin is neurotoxic, but in patients with impaired renal tubular function the risk of neurotoxic symptoms is even greater, since 60-90% of penicillin in the body is excreted by the tubules. If this excretion cannot take place, prolonged exposure of penicillin to the body causes a greater change of (neuro)toxic effects (Borman, 1968).

2.5 Safety profile silybin

No serious side effects have been observed with the treatment of silybin. Although, nausea, epigastric discomfort, arthralgia, headaches, severe itching of the skin and rashes have been reported. Due to the lack of clinical investigations, silybin is not used to treat children under the age of 12, unless the benefits of the treatment are greater than the risks (Enjalbert, 2002).

2.6 Benzylpenicillin therapy in Europe

Clinical data

A study (Křenová, 2007) concerning the clinical findings and follow-up evaluation of *A. phalloides* poisoning included 34 patients, whereof 18 patients were treated with benzylpenicillin. The method used for the study was analyzing data concerning the clinical course of the poisoning. The data that was collected originates from reports to the Czech Toxicological Information Centre and from toxicological laboratories and were collected between 2000 and 2004. Of the 18 patients, 1 patient died (5,6%).

A different study (Giannini, 2007) concerning clinical evaluation and follow-up evaluation of *A. phalloides* poisoning included 111 patients, who were all treated with the same therapeutic protocol that consisted of intensive supportive therapy and continuous intravenous administration of benzylpenicillin. The study is a retrospective chart review, so the method used for the study was reviewing the charts of the treated patients concerning the clinical course of the poisoning. The data collected originates from patients treated between 1988 to 2002 at the Toxicological Unit of Careggi General Hospital (University of Florence). Of the 111 identified patients, 2 patients died (1,8%). These patients were both hospitalized more than 60 hours after mushroom ingestion. Early hospitalization (<36 hours after ingestion) was significantly correlated with lower severity of the poisoning and earlier discharge, which suggests that earlier treatment is associated with better outcome.

The overall data of the patients of both studies are shown in table 1.

Table 1. Data of 129 patients intoxicated with *A. phalloides* treated with benzylpenicillin

Number of patients	Delay (hours) MEAN	Length (days) MEAN	Number of LT's	Outcome	Reference
18	24,8	4,4	-	17 recovery's, 1 deceased	(Křenová, 2007)
111	38,3	5,9	-	109 recovery's, 2 deceased	(Giannini, 2007)

Delay, time interval between ingestion *A. phalloides* and hospitalization in hours; Length; length of hospitalization in days; LT, liver transplantation; -, unknown

In vitro data

In an experimental study (Magdalan, 2010) with human hepatocyte cultures, it is shown that administration of benzylpenicillin resulted in a strong protective effect against cell damage in α-amanitin toxicity. The protective effect was not dose-related. Administration of the drug to the cultures was not associated with any cytotoxic effects in the hepatocytes. Another study (Magdalan, 2011) confirms that treatment with benzylpenicillin did not reduce cell viability nor did it induce apoptosis. It was also shown that human hepatocyte cultures that are exposed to α-amanitin and benzylpenicillin showed significantly lower apoptosis compared to the cultures who were only exposed to α-amanitin.

In another study (Letschert, 2006) concerning the inhibition of the amatoxin transporter OATP1B3 by several antidotes it is shown that the concentration of benzylpenicillin

necessary for inhibition of amanitin-induced cell damage is 25 µM. At this dosage, 50% of the amanitin-uptake by OATP1B3 was inhibited.

Contradictory, an *in vitro* study (Tong, 2007) using a murine model showed that benzylpenicillin had no antitoxic effect whatsoever concerning an α-amanitin poisoning.

2.7 Silybin therapy in Europe

Clinical data

A study (Hruby, 1983) where the effect of silybin therapy on *A. phalloides* poisoning was evaluated included a total of 18 cases who were treated during 1980 and 1981. All of the treatments consisted of conventional therapeutical measures and intravenous administration of silybin. This study was a retrospective review, so the method used for the study was reviewing the followed-up case records of the cases. The data collected originates from the Austrian Poison Information Centre in Vienna. The length of the 'delay' intervals from ingestion to the start of silybin therapy was correlated with the severity of the liver damage. Except for one case with a suicidal intake of *A. phalloides,* all patients recovered showing healthy liver functions.

A different study (Kieslichova, 2017) analyzed the clinical course and outcome of treatment of patients with an *A. phalloides* intoxication. Twenty-three patients with acute liver failure due to *A. phalloides* poisoning were admitted to the intensive care unit in Czech Republic between July 2007 and April 2016. The courses of the patients were studied retrospectively. Since there are almost no cases reported where *A. phalloides* poisoning was treated with monotherapy of silybin, the data of this study where bi-therapy of silybin and acetylcysteine is used as a treatment is presented. Six of the treated patients underwent a liver transplantation. One transplant recipient died two months after liver transplantation. The other 5 transplant recipients and all other patients treated are alive and prospering.

The overall data of the patients of both studies are shown in table 2.

Table 2. Data of 41 patients intoxicated with *A. phalloides* treated with silybin

Number of patients	Delay (hours) MEAN	Length (days) MEAN	Number of LT's	Outcome	Reference
18	29,0	3,3	-	17 recovery's, 1 deceased	(Hruby, 1983)
23	58,3	23,7	6	22 recovery's, 1 deceased	(Kieslichova, 2017)

Delay, time interval between ingestion *A. phalloides* and hospitalization in hours; Length, length of hospitalization in days; LT, liver transplantation; -, unknown

In vitro data

An experimental study (Magdalan, 2010) with human hepatocyte cultures showed that also administration of silybin resulted in a strong protective effect against cell damage caused by amanitin. Also the dose of silybin was found to be not dose-related and administration of the drugs was nog associated with any cytotoxic effects.

In another *in vitro* study (Letschert, 2006) where the inhibition of the amatoxin transporter OATP1B3 was examined, it was shown that the dosage of silybin needed for inhibition of amanitin-induced cell damage is 0,4 µM. At this dosage, 50% of the amanitin-uptake by OATP1B3 was inhibited.

Just as with benzylpenicillin, the same study (Tong, 2007) using a murine model showed that silybin had no antitoxic effects on hepatocytes concerning an α-amanitin poisoning.

2.8 Combined therapy benzylpenicillin and silybin in Europe

Clinical data

In a report (Alves, 2001) the clinical courses of 4 patients intoxicated with *A. phalloides* are evaluated. The patients were all treated in 2000 in the Garcia de Orta Hospital in Portugal, and the treatments all included drug therapy with intravenous benzylpenicillin and silybin. All the 4 patients survived, whereof two of the patients received a liver transplantation.

In a different report (Serné, 1996) the clinical course of 2 cases of *A. phalloides* intoxication are presented. The patients were both treated in 1994, first in the Medical Center Alkmaar and later in the University Hospital Groningen. Both treatments included intravenous benzylpenicillin and silybin.

The overall data of the patients of both reports are shown in table 3.

Table 3. Data of 6 patients intoxicated with *A. phalloides* treated with benzylpenicillin and silybin

Number of patients	Delay (hours) MEAN	Length (days) MEAN	Number of LT's	Outcome	Reference
4	23,75	5,6	2	4 recovery's, 0 deceased	(Alves, 2001)
2	18	10,5	0	2 recovery's, 0 deceased	(Serné, 1996)

Delay, time interval between ingestion of *A. phalloides* and hospitalization in hours; Length, length of hospitalization in days; LT, liver transplantation

In vitro data

There is little *in vitro* data on therapies where benzylpenicillin and silybin are combined. However, a study (Floersheim, 1978) were dogs were given a sub lethal dose of *A. phalloides* showed that treatment with both benzylpenicillin and silybin prevented the rise of liver enzymes in the blood and the decrease of clotting factors.

2.9 Overview combined data

Table 4 shows an overview of the clinical data of all the presented studies/reports. Results of the studies concerning the same therapy (monotherapy benzylpenicillin, monotherapy silybin or combination therapy of benzylpenicillin and silybin) are combined. All the studies included have focused on multiple parameters during the trial such as blood values, differences in age, gender, etc. Although for the literature research of this thesis only certain results are selected that are most relevant for answering the research question. Mortality rates and time needed to restore normal liver function are hard endpoints that are relevant for determination of the efficacy of a therapy.

Table 4. Average data of the trials of monotherapy with benzylpenicillin IV, monotherapy with silybin IV and bi-therapy with IV benzylpenicillin and silybin

Therapy	Total number of patients	Delay (hours) MEAN	Length (days) MEAN	Number of LT's	Outcome	Overall mortality (%)
BZP	129	26,8	9,6	1	126 recovery's, 3 deceased	2,3
SB	41	35,5	23,7	6	39 recovery's, 2 deceased	4,9
BZP + SB	6	21,8	4,4	2	6 recovery's, 0 deceased	0,0

BZP, benzylpenicillin; SB, silybin; Delay, time interval between ingestion of *A. phalloides* and hospitalization in hours; Length, length of hospitalization in days; LT, liver transplantation

Discussion

When comparing the overall results presented in table 8, it is observed that combination therapy of benzylpenicillin and silybin shows the lowest mortality rate. None of the cases which included this therapy showed a fatal ending. After this, the monotherapy of benzylpenicillin showed the lowest mortality rate of 2,3%. The therapy of silybin shows the highest mortality rate of 4,9%. This rates include the liver transplants. However, based on these rates, it is not justified to say that the combination therapy has the highest efficacy. There are a few aspects that need to be taken in account when comparing the mortality rates. For one thing, the total number of patients is clearly different in the different trials. Since there are only 6 patients in the combination therapy trials, the change is rather high that there are no fatal endings. Although when there would be a fatal ending by change, the mortality rate would rise with 16,7%. This means that the combination therapy would seem way worse than the mono-therapies, though the fatal outcome might be even caused by a different factor than the sort of therapy, such as poor liver function or the presence of a different liver disease such as hepatitis. This could be possible, since these factors were no exclusion criteria in the studies. The more patients included in the study, the more reliable it gets. Another aspect that needs to be taken in account comparing the clinical are the different time intervals between ingestion of *A. phalloides* and hospitalization. The more time passes, the more time there is for the toxins to cause hepatocellular damage. The therapy with silybin might have the highest mortality rate, but the mean delay is also clearly higher compared to the delay of the other therapies. The mean delay of the combination therapy is the smallest. It seems that this corresponds to the low mortality rate, although there were still two liver transplantations necessary. Next to this, the number of liver transplantations also needs to be taken in account. This number says something about the state that the livers were in during the treatments, since liver transplantations are only carried out when liver functions are irreversible deteriorated. Just as with the delay, the total number of liver transplantations is the highest in the trials were silybin is used as a therapy. Compared to the liver transplantations and so the state of the livers in the mono-therapy with benzylpenicillin, the mortality rate is relatively higher in the trials with benzylpenicillin. The reason that the total number of transplantations in the silybin therapy trials is much higher is that one trial concerning silybin therapy only included patients who suffered from acute liver failure. This makes comparing the data harder, since the mortality rate might underestimated. In other words, if the transplantations would not have taken place, there were probably more patients who were deceased. There is no clear evidence now that the recovery's are due to the treatment with silybin. But since there is little data on mono-therapy of silybin, the data is yet included in the literature research.

The *in vitro* data showed that both benzylpenicillin and silybin showed an antitoxic effect in human hepatocyte cultures poisoned with amatoxin. Although, the concentration needed to inhibit 50% of the α-amanitin uptake by OATP1B3 for benzylpenicillin was 62,5 times higher than the concentration needed for silybin. The safety profiles of benzylpenicillin and silybin show that treatment with benzylpenicillin comes with much more risks than treatment with silybin. Since treatment of *A. phalloides* poisoning with benzylpenicillin requires a high dose

anyhow, there is a great risk at adverse effects. This data together shows that the risk-benefits profile of silybin is way more beneficial than the risk-benefits profile of benzylpenicillin.

Taking all these aspects of both the clinical and *in vitro* data in account, mono-therapy with silybin seems to be the most efficient therapy towards liver function and reducing mortality.

When looking at the mechanisms of action of benzylpenicllin and silybin, this finding seems to be supported. Benzylpenicillin and silybin both seem to limit toxicity caused by amanitin through inhibiting the OATP1B3 transporter (Letschert, 2006) (Wleck, 2013). They both also seem to have an intracellular effect. Benzylpenicillin is able to bind to RNA-polymerase enzymes, which inhibits the toxic effects caused by amantin, but it also inhibits cell proliferation (Berg, 2002). Silybin also binds to RNA-polymerase enzymes, though it stimulates protein synthesis instead of inhibiting it , which might help regenerating cells in a damaged liver. Next to that, silybin seems to cause more antitoxic effects than benzylpenillin does, as example it also has an anti-oxidant mechanism (Fraschini, 2002).

The preference of therapy with silybin also seems to be supported by other clinical data. A recent review (Mengs, 2012) showed that in nearly 1500 documented cases, the overall mortality in patients who were treated silybin is less than 10%. In patients who were treated with benzylpenicillin or a combination of silybin and penicillin, the mortality rate seemed to be more than 20%. Another 20-year retrospective analysis (Enjalbert, 2002) were clinical data from 2108 hospitalized amatoxin poisoning cases collected, seems to support the idea that monotherapy of silybin IV is the most efficient. Treatments of benzylpenicillin alone or in combination were most frequently utilized, but showed little efficacy. Treatment with silybin however, as mono-therapy or in combination with other drugs, seemed to be the most beneficial.

Looking at the different methods by which the clinical data is obtained, there is little change the data is biased. The information all originates from case reports, so the patients are not ‘selected’ from a bigger population.

limitations

The efficacy of individual drugs in the treatment of *A. phalloides* poisoning is hard to compare, since therapies almost always consist of multiple drugs and every therapy consists of supportive measures. Because of this, it is hard to say that it is actually the drug that is responsible for recovery that took place.

There is a great possibility that differences in personal characteristics such as age and gender might also influence the outcome of the trails, but those are not taken into account when comparing the data. This is a limitation that can’t be corrected for, since these personal differences also exist in practice.

For further investigation, it is relevant to look at the farmacokinetics of the binding of benzylpenicillin and silybin to the amatoxin-transporter OATP1B3. The affinity of the drugs could possibly reveal a lot about the efficacy, but such kinetic parameters could also have an effect on the therapy. For example, the concentration of benzylpenicillin that needs to be

administered is much more higher than the concentration of silybin, but if the affinity of benzylpenicillin for OATP1B3 is also much higher, the frequency of administering drugs is also less, which would be beneficial for the patient.

Conclusion

In conclusion, the results of the literature research confirm that poisoning with *A. phalloides* is a rare but serious occurrence. Since there are not many case reports, data on the efficacy of different treatments are limited. Based on the cases that are reported and the *in vitro* data, it seems that monotherapy with intravenous silybin is the most efficient.

References

Allen, D. *Death Cap (Amanita Phalloides).* chicago.

Alves, A. (2001). *Mushroom poisoning with Amanita phalloides - a report of four cases.* Almada: European Journal of Internal Medicine.

Banh, K. (2008). *Hypoglycemia*. Retrieved from CDEM curriculum: https://cdemcurriculum.com/hypoglycemia/

Barceloux, D. (2008). *Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals.* Los Angeles, California: John Wiley & Sons.

Becker, C. (1976). Diagnosis and Treatment of Amanita Phalloides-Type Mushroom Poisoning. *Western Journal of Medicine*, 100-109.

Berg, J. M. (2002). *Biochemistry.* New York: W.H. Freeman.

Bergis, D. (2012). Treatment of Amanita Phalloides intoxication by fractionated plasma separation and adsorption. *Journal of Gastrointestinal and Liver Diseases*, 171-176.

Bonnet, M. (2002). The toxicology of Amanita phalloides. *Elsevier*, 249-254.

Borman, J. (1968). Neurotoxic Effects of Large Doses of Penicillin Administered Intravenously . *Arch Surg.* , 662-665.

Bushnell, D. (2002). Structural basis of transcription: alfa-amanitin-RNA polymerase II cocrystal at 2.8 A resolution. *PNAS*, 1218-1222.

Campos, R. (1989). Silybin dihemisuccinate protects against glutathione depletion and lipid peroxidation induced by acetaminophen on rat liver. *Planta Medica*, 417-419.

Cooper, J. (1987). Effects of cytochalasin and phalloidin on actin. *Journal of Cell Biology*.

Das, R. (2007). “Last supper with mushroom soup”: a case report of amatoxin poisoning. *McGill Journal of Medicine*, 93-95.

Do, U. (1987). Betalactam antibiotics interfere with eukaryotic DNA-replication by inhibiting DNA polymerase alpha. *Nucleic Acids Research*, 10495-10506.

El-Bahay, C. (1999). Influence of Tumor Necrosis Factor-a and Silibin on the Cytotoxic Action of a-Amanitin in Rat Hepatocyte Culture. *Toxicology and Applied Pharmacology* , 253-260.

Enjalbert, F. (2002). Treatment of Amatoxin Poisoning: 20-Year Retrospective Analysis. *Journal of Toxicology: Clinical Toxicoloy*, 715-757.

Faulstich, H. (1979). New aspects of Amanita poisoning. *Journal of Molecular Medicine*, 1143-1152.

Faulstich, H. (1980). Virotoxins; Actin-Binding Cyclic Peptides of Amanita virosa Mushrooms. *Biochemistry*, 3334-3343.

Faulstich, H. (1985). Toxicokinetics of labeled amatoxins in the dog. *Archives of Toxicology* , 190-194.

Floersheim, G. (1978). Effects of penicillin and silymarin on liver enzymes and blood clotting factors in dogs given a boiled preparation of Amanita phalloides. *Toxicology and Applied Pharmacology*, 455-462.

Fraschini, F. (2002). Pharmacology of Silymarin. *Clinical Drug Investigation*, 51-65.

Garcia, J. (2015). Amanita phalloides poisoning: Mechanims of toxicity and treatment. *Elsevier*, 41-55.

Giannini, L. (2007). Amatoxin poisoning: A 15-year retrospective analysis and follow-up evaluation of 105 patients. *Clinical Toxicology*, 539-542.

Gundala, S. (2004). The hepatocellular bile acid transporter Ntcp facilitates uptake of the lethal mushroom toxin α-amanitin. *Archives of Toxicology*, 68-73.

Homann, J. (1986). Early detection of amatoxins in human mushroom poisoning. *Archives of Toxicology*, 190-191.

Hruby, K. (1983). Chemotherapy of Amanita phalloides Poisoning with Intravenous Silibinin. *Hum Toxicology*, 183-195.

Kieslichova, E. (2017). Acute Liver Failure due to Amanita Phalloides Poisoning: Therapeutic Approach and Outcome . *Elsevier*, 192-197.

Křenová, M. (2007). Survey of Amanita phalloides poisoning: clinical findings and follow-up evaluation. *Human and Experimental Toxicology*, 955-961.

Kröncke, K. (1986). α-Amanitin uptake into hepatocytes. Identification of hepatic membrane transport systems used by amatoxins. *The Journal of Biological Chemistry*, 12562-12567.

Leist, M. (1997). Tumor Necrosis Factor–Induced Apoptosis During the Poisoning of Mice With Hepatotoxins. *gastroenterology*, 923-934.

Letschert, K. (2006). Molecular Characterization and Inhibition of Amanitin Uptake into Human Hepatocytes . *Toxicological sciences*, 140-149.

Ljungman, M. (1999). Inhibition of RNA polymerase II as a trigger for the p53 response. *Oncogene*, 583-592.

Magdalan, J. (2009). Comparative antidotal efficacy of benzylpenicillin, ceftazidime and rifamycin in cultured human hepatocytes intoxicated with α-amanitin. *Archives of Toxicology*, 1091-1096.

Magdalan, J. (2010). Benzylpenicillin, acetylcysteine and silibinin as antidotes in human hepatocytes intoxicated with α-amanitin. *Elsevier*, 367-373.

Magdalan, J. (2011). Benzylpenicyllin and acetylcysteine protection from α-amanitin-induced apoptosis in human hepatocyte cultures. *Elsevier*, 311-315.

Mcqueen, C. (2010). *Comprehensive Toxicology.* Auburn: Academic Press.

Mengs, U. (2012). Legalon SIL: The Antidote of Choice in Patients with Acute Hepatotoxicity from Amatoxin Poisoning. *Current Pharmaceutical Biotechnology*, 1964-1970.

Neftel, K. (1987). Effects of bèta-Lactam Antibiotics on Proliferating Eucaryotic Cells. *Antimicrobal agent and chemotherapy*, 1657-1661.

Poucheret, P. (2010). Amatoxin poisoning treatment decision-making: Pharmaco-therapeutic clinical strategy assessment using multidimensional multivariate statistic analysis. *Elsevier*, 1338-1345.

Pradhan, S. (2006). Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine. *Indian Journal of Medicine*, 491-504.

Roth, M. (2012). OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *British Journal of Pharmacology*, 1260-1287.

Rumack, B. (1994). *Handbook of mushroom poisoning: diagnosis and treatment .* Denver: CRC.

Santi, L. (2012). Acute Liver Failure Caused by Amanita phalloides Poisoning. *International Journal of Hepatology*, 1-6.

Serné, E. (1996). *Amanita phalloides, a potentially lethal mushroom: its clinical presentation and therapeutic options.* Alkmaar: Netherlands Journal of Medicine .

Tong, T. C. (2007). Comparative Treatment of α-Amanitin Poisoning With N-Acetylcysteine, Benzylpenicillin, Cimetidine, Thioctic Acid, and Silybin in a Murine Model. *Annals of Emergency Medicine*, 282-288.

Trappoliere, M. (2009). Silybin, a component of sylimarin, exerts anti-inflammatory and anti-fibrogenic effects on human hepatic stellate cells. *Journal of Hepatology*, 1102-1111.

Vo, K. (2017). Amanita phalloides Mushroom Poisonings . *Morbidity and Mortality Weekly Report*, 549-553.

Wieland, T. (1977). Interactions of phallotoxins with actin. *Elsevier*, 285-299.

Wieland, T., & Faulstich, H. (1991). Fifty years of amanitine. *Experientia*, 1186-1193.

Wleck, K. (2013). Hepatocellular Organic Anion–Transporting Polypeptides (OATPs) and Multidrug Resistance–Associated Protein 2 (MRP2) Are Inhibited by Silibinin. *Drug Metabolism and Disposition* , 1522-1528.

Wong, J. H. (2013). *Handbook of Biologically Active Peptides.* London: Academic Press.