Laboratoire de Pharmacognosie et de Mycologie. UPRES 2234 "Synthèse et extraction de molécules à visée thérapeutique", Rennes, France

Cytotoxic activity of methanol extracts from Basidiomycete mushrooms on murine cancer cell lines

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Received July 24, 2003, accepted September 18, 2003

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Pharmazie 59: 290–293 (2004)

Crude methanol extracts of 58 mushroom species were screened for their cytotoxic activities against two murine cancer cell lines, L1210 and 3LL, using the tetrazolium assay. A majority of extracts (74%) exhibited $IC_{50} > 100$ μg/ml against both cell lines. A most marked activity against one of the cell lines was noted for nine species (14% of the tested species). While Amanitales and Russulales tested were not found active, Polyporales and Boletales gave better results. Four species exhibited a significant cytotoxic activity ($IC_{50} \leq 20$ μg/ml) against at least one of the two murine cancer cell lines (*Ganoderma lucidum*, *Meripilus giganteus*, *Suillus granulatus*, *S. luteus*). The last one had never been investigated for its cytotoxic compounds before.

1. Introduction

Mushrooms have been used all over the world as century-old traditional medicines (Chang 1996). High mushrooms (Basidiomycetes) produce various classes of secondary metabolites with a wide variety of biological activities, particularly in the antimicrobial, antitumor and antiviral fields (Jong et al. 1989; Wasser et al. 1999). Over 300 antitumor substances have been isolated from microbial origin (76% from Actinomycetes, 13% from Fungi, 11% from Bacteria). Among the 43 antitumor substances from fungi, 23 were obtained from Fungi imperfecti, 15 from Basidiomycetes, 5 from Ascomycetes (Jong et al. 1989). Searching for new antitumor fungal substances became a matter of great significance (Wasser et al. 1999). Recently we reported the cytotoxic evaluation of 22 species of the Tricholomatales order on two murine and four human cancer cell lines leading to the selection of *Lepista inversa* (Bézivin et al. 2002).

During our ongoing search of new antitumor metabolites from fungal species, we carried out an *in vitro* screening of randomely gathered mushrooms in Brittany forests. 58 species mainly belonging to the Amanitales, Boletales, Polyporales and Russulales orders were screened for their cytotoxic activities on two murine cancer cell lines.

2. Investigations and results

Using the tetrazolium test (Mosmann 1983) crude methanol extracts of Basidiomycetes fruiting bodies were tested for their ability to inhibit *in vitro* the growth of two murine cancer cell lines: L1210, a lymphocytic leukaemia, and 3LL, a Lewis lung carcinoma. The 58 species tested represent 19 families and most of them belong to four orders: Amanitales, Boletales, Polyporales and Russulales. These species were listed according to Courtecuisse classification (Courtecuisse et al. 2000) and the inhibitory concentrations 50% (IC_{50}) observed with these two cell lines are shown in the Table. IC_{50} values were compared to those obtained with a crude methanol extract of the bark of *Taxus baccata*, used as positive control. A majority of extracts (74%) exhibited $IC_{50} > 100$ μg/ml against both cell lines. According to the standards of the National Cancer Institute (NCI), a crude extract may be considered significantly cytotoxic with a $IC_{50} \leq 20$ μg/ml (Cordell et al. 1993). Four out of 58 tested species exhibited significant cytotoxic activity against at least one of the two murine cancer cell lines. The most active extracts, from *Suillus granulatus* and *Ganoderma lucidum*, exhibited a significant cytotoxic activity against both cell lines, IC_{50} L1210 = 4.7 μg/ml; IC_{50} 3LL = 6.8 μg/ml and IC_{50} L1210 = 15 μg/ml; IC_{50} 3LL = 10 μg/ml, respectively. An interesting activity against one of the cell lines was noted for nine species (14% of the tested species). The four most active species against L1210 were: *Strobilomyces strobilaceus*, IC_{50} = 30.5 μg/ml; *Suillus bovinus*, IC_{50} = 37.5 μg/ml; *Daedaleopsis confragosa*, IC_{50} = 74.5 μg/ml; *Boletus edulis*, IC_{50} = 75.8 μg/ml and the five most active against 3LL were: *Meripilus giganteus*, IC_{50} = 19.8 μg/ml; *Lactarius quietus*, IC_{50} = 45 μg/ml; *Amanita phalloides*, IC_{50} = 59 μg/ml; *Trametes versicolor*, IC_{50} = 79.5 μg/ml; *Cantharellus tubiformis*, IC_{50} = 94.5 μg/ml. Among them, only the *Meripilus giganteus* extract demonstrated a significant cytotoxicity against the 3LL cell line (IC_{50} = 19.8 μg/ml). *Tylopilus felleus* was two-fold more active against L1210 (IC_{50} = 29.5 μg/ml) than against 3LL (IC_{50} = 70.3 μg/ml) as so as *Suillus luteus* (IC_{50} L1210 = 18.1 μg/ml; IC_{50} 3LL = 40.3 μg/ml). The extracts of *Oligoporus stypticus* and *Piptoporus betulinus* showed a similar but not relevant activity on L1210 and 3LL, while *Gymnopilus spectabilis* showed activities to be consid-

Table: IC_{50} values (μg/ml) of crude methanol extract of fungi on murine cancer cell lines (L1210, 3LL)

Family Species	IC_{50}[a] (μg/ml) ± SD	
	L1210	3LL
Amanitaceae		
Amanita citrina (Sch.) Pers.	>100	>100
Amanita citrina var. *alba* (Price) Q.& Bat.	>100	>100
Amanita malleata (Piane ex Bon) Contu	>100	>100
Amanita muscaria (L.: Fr.) Pers.	>100	>100
Amanita pantherina (DeCand.: Fr.) Krombh.	>100	>100
Amanita phalloides (Vaill.: Fr.) Link	>100	59 ± 22
Amanita rubescens Pers.: Fr	>100	>100
Amanita spissa (Fr.) Kummer	>100	>100
Boletaceae		
Boletus edulis Bull.: Fr.	75.8 ± 12.8	>100
Boletus erythropus Pers.	>100	>100
Boletus luridus Sch.: Fr.	>100	>100
Suillus bovinus (L.: Fr.) Roussel	37.5 ± 11.2	>100
Suillus granulatus (L.: Fr.) Roussel	**4.7 ± 0.5**	**6.8 ± 2.5**
Suillus luteus (L.: Fr.) Roussel	**18.1 ± 4.1**	40.3 ± 3.2
Tylopilus felleus (Bull.: Fr.) Karst.	29.5 ± 9.7	70.3 ± 3.2
Xerocomus badius (Fr.: Fr.) Gilbert	>100	>100
Xerocomus chrysenteron (Bull.) Quel.	>100	>100
Xerocomus parasiticus (Bull.: Fr) Quel.	>100	>100
Bjerkanderaceae		
Oligoporus caesius (Sch.: Fr.) Gilbn & Ryv.	>100	>100
Oligoporus stypticus (Pers.: Fr.) Gilbn & Ryv.	56.8 ± 6.7	84.3 ± 14.7
Phaeolus schweinitzii (Fr.) Pat.	>100	>100
Cantharellaceae		
Cantharellus tubiformis Fr: Fr.	>100	94.5 ± 5.3
Coprinaceae		
Coprinus micaceus (Bull.: Fr.) Fr.	>100	>100
Coprinus picaceus (Bull.: Fr.) Gray	>100	>100
Psathyrella piluliformis (Bull.: Fr.) Orton	>100	>100
Coriolaceae		
Trametes gibbosa (Pers.: Fr.) Fr.	>100	>100
Trametes versicolor (L.: Fr.) Lloyd	>100	79.5 ± 17.5
Craterellaceae		
Craterellus cornucopioides (L.: Fr.) Pers.	>100	>100
Crepidotaceae		
Gymnopilus penetrans (Fr.: Fr.) Murr.	>100	>100
Gymnopilus spectabilis (Weinm.: Fr.) Smith	34.2 ± 4.8	32.8 ± 1.1
Daedaleaceae		
Daedaleopsis confragosa (Bolt: Fr.) Schroet.	74.5 ± 4.2	>100
Fistulinaceae		
Fistulina hepatica (Sch.: Fr.) With.	>100	>100
Fomitopsidaceae		
Heterobasidion annosum (Fr.: Fr.) Bref.	>100	>100
Ganodermataceae		
Ganoderma lucidum (Curt.: Fr.) Karst.	**15**	**10**
Grifolaceae		
Meripilus giganteus (Pers.: Fr.) Karst.	>100	**19.8 ± 2.6**
Hygrophoropsidaceae		
Hygrophoropsis aurantiaca (Wulf.: Fr.) Mre	>100	>100
Meruliaceae		
Merulius tremellosus Schrad.: Fr.	>100	>100
Paxillaceae		
Paxillus involutus (Batsch: Fr.) Fr.	>100	>100
Polyporaceae		
Laetiporus sulfureus (Bull.: Fr.) Murr.	>100	>100
Piptoporus betulinus (Bull.: Fr.) Karst.	77.5 ± 10.2	88.2 ± 18.5
Ramariaceae		
Ramaria stricta (Pers.: Fr.) Quel.	>100	>100
Russulaceae		
Lactarius blennius (Fr.: Fr.) Fr.	>100	>100
Lactarius chrysorrheus Fr.	>100	>100
Lactarius controversus (Pers.: Fr.) Fr.	>100	>100
Lactarius necator (Bull.: Fr.) Karst.	>100	>100
Lactarius pubescens Fr.	>100	>100
Lactarius quietus (Fr.: Fr.) Fr.	>100	45 ± 5.8
Lactarius torminosus (Sch.: Fr.) Gray	>100	>100
Lactarius vellereus (Fr.: Fr.) Fr.	>100	>100
Russula amara Kucera	>100	>100
Russula cyanoxantha (Sch.) Fr.	>100	>100
Russula drimeia Cooke	>100	>100
Russula fageticola (Meltzer) Lundell	>100	>100
Russula fellea (Fr.: Fr.) Fr.	>100	>100
Russula nigricans (Bull.) Fr.	>100	>100
Russula ochroleuca (Hall.) Pers.	>100	>100
Russula vesca Fr.	>100	>100
Strobilomycetaceae		
Strobilomyces strobilaceus (Scop.: Fr.) Berk.	30.5 ± 12.3	>100
Taxaceae		
Taxus baccata L.[b]	**14.3 ± 3.1**	**14.7 ± 4.7**

[a] Inhibitory Concentrations 50% indicated with standard deviations (SD)
Values in bold characters are considered to be relevant.
All species were evaluated at least three times, except *Ganoderma lucidum* (one assay).
[b] Methanol extract of bark of *Taxus baccata* was used as positive control.

ered against the two cell lines (IC_{50} L1210 = 34.2 μg/ml; IC_{50} 3LL = 32.8 μg/ml).

3. Discussion

Some Basidiomycetes (*Trametes versicolor*, *Ganoderma lucidum*, *Lentinus edodes*, *Grifola frondosa*) are clinically used for cancer treatment and prevention in Asian countries (Mizuno et al. 1995; Wasser et al. 1997). Activity of these mushrooms is generally related to the presence of polysaccharides which are considered as Biological Response Modifiers (BRM). Among the species tested in our study, some of them were reported to contain polysaccharides and β-glucans with *in vivo* antitumor activities e.g. *Amanita muscaria*, *Daedaleopsis confragosa*, *Fistulina hepatica*, *Piptoporus betulinus*, *Phaeolus schweinitzii*, *Tylopilus felleus*, *Trametes versicolor* (Wasser 2002). *Ganoderma lucidum* is more particularly described for its antitumor polysaccharides (Miyazaki et al. 1981; Sone et al. 1985; Nano et al. 2002). All these various polysaccharides acting as BRM showed no direct cytotoxicity on tumors but increased the ability of the host to defend itself from tumor progression (Jong et al. 1991; Wang et al. 1997; Bao et al. 2002). Thus, the activity of this type of compounds could not be revealed using our *in vitro* cytotoxic assays against cell lines.

Besides the antitumor polysaccharides, various extracts of *Ganoderma lucidum* showed significant activities either *in vitro* against HeLa human cervical cancer (Lovy et al.

1999), Human T4 leukaemic (Zhu et al. 2000), MCF-7 breast cancer cells (Hu et al. 2002) or *in vivo* (Min et al. 2002; Chung 2001). Although $IC_{50} \leq 15$ μg/ml found for *G. lucidum* in our study have been obtained with one assay only, this result is in agreement with other studies where triterpenoids (specially lucidenic and ganoderic acids) are described with IC_{50} in a range of 15 nM (Kim et al. 1999; Min et al. 2000; Wu et al. 2001; Gao et al. 2002).

Among other previously known cytotoxic species (*Suillus granulatus*, *Meripilus giganteus*, *Gymnopilus spectabilis*), the most active against the two cell lines, *Suillus granulatus*, contained cytotoxic compounds, i.e the phenolic suillin active *in vitro* against three cell lines (KB, P388, NSCLC-N6) with $0.69\ \mu g/ml \leq IC_{50} \leq 1.02\ \mu g/ml$ (Tringali et al. 1989) and the benzofuran suillisin which showed a relative *in vitro* cytotoxic activity against various cancer cell lines ($12\ \mu g/ml \leq IC_{50} \leq 30\ \mu g/ml$) (Yun et al. 2001). The hydroethanol extract of *S. granulatus* was however inactive *in vivo* on Yoshida sarcoma, Crocker sarcoma and Ehrlich ascite tumor (ECA) cells (Christov et al. 1974).

Meripilus giganteus presented an interesting activity against 3LL. A mixture of saturated and unsaturated fatty acids (i.e. palmitic, oleic and linoleic acids) and ergosterol peroxide were isolated and identified as immunosuppressive components (Narbe et al. 1991). Additionally, a cytotoxic activity was demonstrated for ergosterol peroxide on four solid ($9\ \mu g/ml \leq IC_{50} \leq 158.2\ \mu g/ml$) and two liquid ($18.7\ \mu g/ml \leq IC_{50} \leq 74.1\ \mu g/ml$) tumor models (Kahlos et al. 1989; Nam et al. 2001).

The Cortinariale *Gymnopilus spectabilis*, which exhibited valuable activities against the two cell lines, was reported to contain a cytotoxic compound, ostopanic acid (Nunez-Alarcon et al. 2001) with *in vitro* inhibition of the growth of P388 cells ($ED_{50} = 1.5\ \mu g/ml$) (Hamburger et al. 1987).

Some species inactive in our study were already known as such. They were *Coprinus micaceus* (Porte et al. 1982; Nano et al. 2002), *Hygrophoropsis aurantiaca*, *Heterobasidion annosum*, *Lactarius chrysorrheus*, *L. torminosus*, *Oligoporus caesius*, *Xerocomus badius* (Porte et al. 1982) and *Phaeolus schweinitzii* (Nano et al. 2002). Conversely, *in vitro* cytotoxic compounds were reported for some species, but corresponding extracts did not show here any activity as for *Amanita phalloides*, *Cantharellus tubiformis*, *Lactarius deliciosus*, *L. vellereus* and *Merulius tremellosus*. This could be due to a stability problem or to a compound concentration in extract below its activity threshold. Thus, the instability (Sterner et al. 1985) of the cytotoxic isovelleral and velleral sesquiterpenoids ($2\ \mu g/ml \leq IC_{50}$ ECA and $L1210 \leq 20\ \mu g/ml$) (Anke et al. 1989a; Anke et al. 1991; Jonassohn et al. 1997) could partly justify the lack of activity of the *Lactarius vellereus* extract. In addition, the cytotoxic activities of lactaroviolin and deterrol ($10\ \mu g/ml \leq IC_{50} \leq 50\ \mu g/ml$) on ECA and L1210 cells found in *L. vellereus* (Anke et al. 1989b; Sterner 1995) are not high enough to be revealed as they are diluted in the extract. The same argument can be given for a fatty acid derivative (10-hydroxy-*trans*-8-decenoic acid) (IC_{50} on L1210 and BHK cells around 50 μg/ml) found in *Cantharellus tubiformis* (Anke et al. 1996) and for merulidial (IC_{50} on L1210 and ECA cells = 20 μg/ml) (Anke et al. 1991) found in *Merulius tremellosus* (Quack et al. 1978). The well known toxic *Amanita phalloides* was found lightly active against 3LL and inactive against L1210. This species contained about 0.05% of toxic cyclopeptides (Faulstich et al. 1976) e.g. α-amanitin which inhibit L1210 cells isolated RNA polymerase II at a dose of 1 μg/ml (Maniglia et al. 1979). In the conditions used in our assay, the most concentrated extract (2.8 μg cyclopeptides/ml) was around or below the toxic threshold for cells.

Six species (*Boletus edulis*, *Oligoporus stypticus*, *Piptoporus betulinus*, *Suillus bovinus*, *Tylopilus felleus*, *Trametes versicolor*) showing $IC_{50} < 100\ \mu g/ml$ had not previously been reported for *in vitro* cytotoxic activities. However, *in vivo* studies have been carried out for some of them. Aqueous and alkaline extracts of *P. betulinus* presented antitumor activity *in vivo* (Shibata et al. 1968) and the Sarcoma 180 tumor inhibitory properties of a mucoprotein isolated from *Boletus edulis* was also reported (Lucas et al. 1957). A cultured mycelial extract of *Suillus bovinus* and *O. stypticus* were found inactive *in vivo* on ECA (Porte et al. 1982).

None antitumor or cytotoxic activity was previously reported for half of the species tested. Among them, the most interesting active species were *Lactarius quietus*, *Strobilomyces strobilaceus* and *Suillus luteus*. *L. quietus* and *S. strobilaceus* had a moderate activity against 3LL and L1210, respectively. Although less active than the previously studied *Suillus granulatus*, the methanolic extract of *S. luteus* showed good cytotoxic activities particularly against L1210 ($IC_{50} = 18.1\ \mu g/ml$). As this species has only be investigated for the inhibition of human blood platelets aggregation (Czarnecki et al. 1995) and antibiotic (Park et al. 1995) activities, it could be a good candidate for phytochemical investigation of its cytotoxic compound(s).

The number of tested species is not exhaustive within each families and orders, but some remarks can be drawn out with regard to the activity scores. Among the 8 tested species of the *Amanitaceae* family and the 16 tested species of the *Russulaceae* family, none showed a relevant cytotoxic activity on the two murine cell lines (only a moderate activity against 3LL for *A. phalloides* and *L. quietus*). From the Polyporales order grouping the *Bjerkanderaceae*, *Coriolaceae*, *Daedaleaceae*, *Fistulinaceae*, *Fomitopsidaceae*, *Ganodermataceae*, *Grifolaceae*, *Polyporaceae* and *Ramariaceae* families, only two species, *Ganoderma lucidum* and *Meripilus giganteus*, were active and four had $IC_{50} < 100\ \mu g/ml$ against at least one of the cell lines (*Daedalopsis confragosa*, *Oligoporus stypticus*, *Piptoporus betulinus*, *Trametes versicolor).*

The Boletales order including the *Boletaceae*, *Hygrophoropsidaceae*, *Paxillaceae* and *Strobilomycetaceae* families presented 6 interesting species out of 12 tested: *Suillus granulatus* > *S. luteus* > *Strobilomyces strobilaceus* > *S. bovinus* > *Tylopilus felleus* > *Boletus edulis*. Interestingly, three of them were most active against 3LL which is generally found less sensitive. These findings suggest that these species might contain some cytotoxic substances related to a similar metabolism pattern.

So, the interest to look for anticancer compounds in mushrooms is again illustrated as some species have a cytotoxic activity on cancer cells comparable to the well known *Taxus baccata* bark. Four out of 58 tested species exhibited significant cytotoxic activity. Three of these species were already studied (*Suillus granulatus*, *Ganoderma lucidum*, *Meripilus giganteus*) and studies for isolating new active low molecular weight compounds should be more particularly focused on species of the Boletales order, namely *Suillus luteus*.

4. Experimental

4.1. Preparation of methanol extracts

Different species of mushrooms were collected in autumn 1999, 2000 and 2001 from their natural habitat in mixed forest near Rennes, France. All species were identified in the laboratory by Dr. M. Amoros, Pr. J. Boustie and Dr. S. Tomasi. Fresh mushrooms were cleaned, sliced, frozen and kept

at −20 °C until their extraction. 50 g of frozen mushrooms were extracted three-times by a mechanical shaking with 300 ml of methanol for 24 h. After evaporation of methanol under reduced pressure, dry residues were dissolved in dimethylsulphoxide at a 50 mg/ml concentration and stored at −20 °C and used as mother liquor for all experiments. A crude methanolic extract from the dried bark of *Taxus baccata* L. (*Taxaceae*) was prepared following the same procedure and used as positive control.

4.2. Cells

Two murine cancer cell lines were used: L1210 – lymphocytic leukaemia (ATCC CCL 219) and 3LL – Lewis lung carcinoma (CRL-1642). The cells were grown following the procedure previously described (Bézivin et al. 2003). For the adherent 3LL cells, the plates were seeded the day before the experiment in order to obtain good adherence.

4.3. Cytotoxic assays

On the day of the experiment, fungi extracts (at a 50 mg/ml concentration) were serially diluted in RPMI 1640 media to obtain concentrations ranging from 0.1 to 100 μg/ml and then deposited into 3 wells for each cell line.
Cytotoxicity was measured using the tetrazolium assay as described (Mosmann 1983) with modifications (Bézivin et al. 2003).
All experiments were repeated at least three times except for *G. lucidum* species for which one test was performed.

Acknowledgements: We gratefully thank Nadège Bébin, Elsa Lemonnier, Sébastien Bellier du Boisière for their taking part in cytotoxic assays, Dr. M. Amoros for her help in collection and identification of mushrooms.

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