Substances from the medicinal mushroom *Daedalea gibbosa* inhibit kinase activity of native and T315I mutated Bcr-Abl

MAJED YASSIN[1,2], SOLOMON P. WASSER[2] and JAMAL MAHAJNA[1]

[1]Cancer Drug Discovery Program, Migal, Galilee Technology Center, P.O. Box 831,
Kiryat Shmona; [2]Institute of Evolution, University of Haifa, Mount Carmel, Haifa 31905, Israel

Abstract. Human chronic myelogenous leukemia (CML) is a malignancy of pluripotent hematopoietic cells characterized by a distinctive cytogenetic abnormality resulting in the creation of a p210 [Bcr-Abl] fusion protein with abnormal tyrosine kinase activity. Recently, a selective Abl kinase inhibitor, Imatinib mesylate, was introduced as a first line therapy for CML. Despite the initial response, CML patients develop a resistantance to Imatinib, which is mediated mainly by point mutations within the Abl protein. Herein, we describe the identification of mycelium organic extracts of *Daedalea gibbosa* with selective anti-proliferating and apoptosis-inducing activities against K562 cells and other laboratory model of CML. Using activity-guided purification, we isolated an active fraction, F6, which inhibits *in vitro* kinase activity of recombinant Abl. The active fraction significantly inhibits the autophosphorylation of native and mutated Bcr-Abl, which are resistant to Imatinib treatment including the T315I mutation. Using a colony-forming assay, we demonstrated that the active fraction is effective in inhibiting the colony formation of the Ba/F3 cell line harboring either native Bcr-Abl or its mutations, including the T315I mutation. Our data illustrated the potential of natural products in cancer therapeutics.

Introduction

Medicinal mushrooms have been an important source of therapeutic substances for the treatment of various human illnesses (1). Higher basidiomycetes mushrooms (HBM) represent a major and, as yet, largely untapped source of potent new pharmaceutical products. Of the 15,000 known

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Correspondence to: Dr Jamal Mahajna, Cancer Drug Discovery Program, Migal, P.O. Box 831, Kiryat Shmona 11016, Israel
E-mail: jamalm@migal.org.il

species, 2,000 are safe for human health and ~650 of them possess medicinal properties (2). Of the 650 mushroom species with known medicinal properties, only ~20 species are already in use (3), arguing the need to explore the medicinal potential of the rest.

Several medicinal mushroom substances having anti-tumor activity were isolated including lentinan, krestin and schizophyllan. Most bioactive substances isolated from mushrooms were high-molecular-weight (HMW) polysaccharides having a molecular weight of 200-400,000 Dalton. Anti-tumor activity of HMW polysaccharides was attributed to immune-modulation function or enhancement properties of the immune system, not as a result of a direct influence on the tumor cells. Our interest is in low-molecular-weight (LMW) moieties that can easily penetrate the cell membrane and exhibit anti-tumor activity with a defined mechanism of action (4-8).

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder of hematopoietic stem cells and accounts for 15% of leukemias in adults (9). Blasts of patients suffering from CML in >95% of cases the Philadelphia chromosome, the morphological substrate of t(9;22). Patients (25-30%) from adult lymphoblastic leukemia (ALL) are Ph+ ALL and the presence of t(9;22) defines a high-risk subgroup of ALLs (9).

The t(9;22)-related translocation products are the Bcr-Abl fusion proteins. The fusion of Bcr to Abl leads to the constitutive activation of the kinase activity of Abl, whose physiological counterpart is finely regulated by a variety of stimuli. Constitutively activated Abl induces aberrant proliferation and neoplastic transformation by the constitutive activation of RAS, PI3 kinase, as well as JAK/STAT (9). The constitutively activated Abl kinase activity is indispensable for the Bcr-Abl-induced transformation of cells. The Bcr-Abl-induced phenotype of hematopoietic cells is characterized by increased proliferation as well as by reduced susceptibility to a variety of pro-apoptotic stimuli, including growth factor deprivation (9). The inoculation of stem cells retrovirally transduced with Bcr-Abl induces a CML-like disease in sublethally irradiated recipient mice within 3-4 weeks, which proves that Bcr-Abl is responsible for the pathogenesis of Ph+ leukemias (10).

The inhibition of Bcr-Abl kinase activity is a valid concept of causal therapy of Ph+ leukemia and the specific Abl kinase

inhibitor Imatinib mesylate (Imatinib, Gleevec, and before STI571) is now a part of standard therapy of chronic phase CML (CP) and is included in most clinical trials for the treatment of Ph+ ALL (11-13). Unfortunately, the clinical efficacy of the treatment with Imatinib continuously decreases with the advancement of the disease. Blast crisis, CML or relapsed Ph+ ALL benefit from the treatment of Imatinib only temporarily or not at all (14). Patients relapsing upon treatment with Imatinib are resistant to further treatment with the drug.

Secondary resistance is mostly due to the acquisition of point mutations in Bcr-Abl, which change the affinity of the protein for Imatinib. More than 25 different substitutions of amino acid residues of Bcr-Abl have been detected in CML and Ph+ ALL patients (15-17). Such mutations can pre-exist at the onset of treatment in a small subset of cells, which are selected by the treatment with Imatinib (18). Even mutations outside the kinase domain contribute to Imatinib resistance by stabilizing the active conformation, which is not accessible by Imatinib.

Several new Abl inhibitors were reported; AMN107, BMS-354825 and VX-680. AMN107 is a novel ATP-competitive inhibitor of Abl with increased potency compared with Imatinib in inhibiting cellular proliferation and was also more effective than Imatinib in inhibiting phosphorylation of p185 Bcr-Abl tyrosine kinase in cell lines and in primary ALL cells. AMN107 is effective against a variety of cell lines expressing Imatinib-resistant Bcr-Abl mutants, except for the T315I mutation (29).

BMS-354825 is another novel compound, which is a dual Src/Abl kinase inhibitor having a greater potency than Imatinib and is active against 14 of the 15 Imatinib-resistant Bcr-Abl mutants (16). The T315I mutation in the kinase domain of Abl was resistant to inhibition by Imatinib, AMN107 and BMS-354825 (19). VX-680 compound was developed as Aurora A kinase inhibitor and later turned out to inhibit Abl kinase and its mutants including T315I mutation by a novel mechanism (20). Treatment of Imatinib-resistant patients carrying the T315I with VX-680 resulted in a promising outcome (20,21).

Herein, we report the identification of a mycelium organic extract from *Daedalea gibbosa* having selective anti-proliferating and apoptosis-inducing activities against K562 cells and other laboratory model of CML. In addition, we identified an active fraction, F6, capable of directly inhibiting the kinase activity of recombinant Abl, as well as its autophosphorylation activity of native Bcr-Abl and its mutated forms including the T315I mutation. The active fraction of *D. gibbosa* also inhibited the colonigenicity of native Bcr-Abl and its mutants when expressed in Ba/F3 cell lines.

Materials and methods

Mushroom material. The strain *D. gibbosa* used in the present study is from the culture collection of HBM (HAI) of the Institute of Evolution, University of Haifa, Israel (22). Mushroom growth medium and the cultivation of *D. gibbosa* as previously described (4).

Mushroom growth conditions and extractions. Pre-inocula for cultures were prepared by transferring to 5-mm diameter discs to flasks containing 100 ml of YE medium (yeast extract 0.1%, peptone 0.1%, glucose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, supplemented with 10 ml/l of trace solution (18 mM $FeSO_4$, 3.7 mM $MnSO_4$, 1.5 mM $ZnSO_4$ and 0.8 mM $CuSO_4$) incubated for 14 days. The contents of the pre-inocula flasks were homogenized and transferred to flasks containing 1 litre of YE medium. Inoculated flasks were shaken at 160 r/min at 27˚C for 12 days, then filtered and washed to remove culture broth. Wet mycelia were dried at 37˚C. In the first phase of the study; dry mycelium were extracted as previously described (4). At a later stage, extraction was carried out using 100% dichloromethane (DCM) or 100% ethyl acetate (EA).

Fractionation of mycelium crude extract. *Daedalea gibbosa* crude extract (1.5 g) was prepared from 85 g of *D. gibbosa* mycelium, which was extracted with 5 litres of dichloromethane on a rotary shaker at 160 r/min at room temperature. Dichloromethane extract (1.3 g) was separated in a silica gel column (Silica gel 60, Merck, USA). Different fractions were eluted by increasing methanol concentrations (from 0, 0.25, 0.5, 1, 2, 5, 10, 20, 40, 60 and 100%). During the fractionation process, the eluted substances were traced by thin layer chromatography (TLC). The separated substances were visualized by staining with 2% phosphomolybdic acid.

Cell lines and cell cultures. Ba/F3 cells (a murine pro-B cell line) transfected with native Bcr-Abl, Bcr-Abl/T315I, or Bcr-Abl/E255K constructs were donated by Dr J. Duyster, Germany. Transfected Ba/F3 and Human K562 (ATCC, Rockville, MD) cell lines were grown in RPMI-1640 with 2 mM L-glutamine and supplemented with 10% fetal bovine serum. Penicillin at 100 U/ml and streptomycin at 100 μg/ml were added to the culture media. Each cell line was grown at 37˚C in a humidified atmosphere with 5% CO_2.

Bcr-Abl autophosphorylation assay by ELISA. The capture anti-Abl antibody (100 ng/well, Santa Cruz, USA) was first diluted in 0.1 M carbonate buffer, pH 9.4, then 200 μl was added to each well of a 96-well plate and the plates were kept at 37˚C overnight. Cell lysates were prepared from treated and untreated K562 or transfected Ba/F3 cell lines. After treatment for 1 h, cells were washed twice with cold PBS and lysed with lysis buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride] for 30 min on ice. After blocking the plates for 1 h, the cell lysates (100 μg/well) were diluted in a blocking buffer (PBS, 1% BSA) and then added to the plates in a volume of 200 μl per well, and the plates were incubated at 4˚C overnight. Subsequently, the plates were washed with wash buffer (PBS, 0.05% Tween-20) and a reporter antibody PY20 conjugated to HRP was added at 50 ng/well and incubated at room temperature for 1 h. The ELISA was developed using TMB as a substrate and OD was measured at 630 nm.

Cellular autophosphorylation of Bcr-Abl. Ba/F3 cell lines that express the native Bcr-Abl protein and its mutated forms ($4x10^5$ cells/ml) were treated with mycelium crude extract, fraction 6 of the *D. gibbosa* extract and DMSO for 1 h. Cells

Table I. Growth inhibition and induction of erythroid differentiation by *D. gibbosa* crude extracts.

Extracts		Growth inhibition (%)				Erythroid differentiation
Ex	Extract symbol	K562	DU145	MH3924A	HT-29	Relative hemoglobin levels
Meth451	Eth	51.3	-6.2	44	0.2	10.8
MH56	H	56.3	26.2	28	23.9	1.6
ME424	E	45.4	11.9	54	24.50	2.3
MC57	C	52.7	16.2	14	7.6	1.7

Percentage of growth inhibition caused to a variety of cell lines. Relative hemoglobin levels expressed in treated cells.

were collected, washed once with cold PBS and lysed as previously indicated. Cell lysate supernatants (40 μg protein) were resolved on 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and analyzed by immunoblotting with phospho-c-Abl (Tyr245) polyclonal antibody (Cell Signaling Technology, USA). Phosphorylated levels of Bcr-Abl proteins were compared to total Abl, which was detected using c-Abl monoclonal antibody (Santa Cruz Biotechnology, USA).

Colony forming assay. Ba/F3 cells ($2x10^3$) that express Bcr-Abl or its mutated forms (Bcr-Abl/T315I and Bcr-Abl/E255K) were re-suspended in RPMI-1640 with L-glutamine, supplemented with 10% fetal bovine serum and containing 0.3% Noble agar (Sigma, USA). Cells were seeded in 6-well plates above a layer of 0.6% Noble agar. The cells were fed every two days by overlaying the agar with 3 ml of a medium containing mycelium crude extract, fraction 6 of *D. gibbosa* extract, Imatinib or DMSO. The presence or absence of colonies was scored after 14 days (23).

Abl in vitro kinase assay. The activity of Abl protein kinase using recombinant Abl enzyme (expressed in Sf21 cells from Upstate Cell Signaling Solutions, USA) was assayed in the presence or absence of inhibitors by measuring the incorporation of ^{33}P from [γ-^{33}P]-ATP (PerkinElmir, USA) into Abltide (EAIYAAPFAKKK peptide from Upstate). Imatinib (Novartis, Basel), AG957 (Sigma) and fraction 6 of *D. gibbosa* (#514) mycelium crude extract were dissolved and stored at -70˚C. The Abl protein kinase assay was carried out at 30˚C in a total volume of 25 μl: 20 ng recombinant Abl kinase, 50 mM Hepes buffer pH 7.4, 20 mM $MgCl_2$, 0.1 mM $MnCl_2$, 0.2 mM Na_3VO_4, 1% DMSO, 25 μM ATP (1 μCi/assay [γ-^{33}P]-ATP) and 200 μg/ml Abltide and was carried out for 10 min. The assay was terminated with the addition of 5 μl of 72 mM EDTA. Subsequently, 25 μl of the reaction mixture were transferred onto the center of a 2x2 cm P81 paper (Upstate Cell Signaling Solutions), washed three times for 5 min with 0.75% phosphoric acid, then washed once for 5 min with acetone and counted using a liquid scintillation analyzer (TRI-CARB 2100TR, Packard Bio Science Company, USA).

Results

The medicinal mushroom *D. gibbosa* was grown as a submerged culture, and four different solvent mixtures

including Eth (70% ethanol), E (33% ethyl acetate, 50% methanol), H (22% ethyl acetate, 11% methanol) and C (33% chloroform, 50% methanol) were used to prepare crude extracts. Initially, growth inhibition activity of the various extracts was monitored utilizing an XTT assay (4,24). The anti-proliferation activity was evaluated using a number of cell lines including HT29 (human colon adenocarcinoma cells), MH3924A (rat Morris hepatoma) and DU145 (human prostate carcinoma).

Results summarized in Table I show that each organic extract of *D. gibbosa* was significantly active in inhibiting the growth of K562 cells with varying potency and selectivity. Crude extracts H (MH56) and C (MC57) were selective in inhibiting the growth of K562 cells. In contrast, organic crude extract Eth (Meth451) and E (ME424) show significant growth inhibition activity against K562 and MH3924A (rat Morris hepatoma) cell lines, though exhibit minimal activity against HT29 (human colon adenocarcinoma cells) and DU145 (human prostate carcinoma).

Induction of erythroid differentiation in K562 by crude extracts of Daedalea gibbosa. Overexpression of p210 [Bcr-Abl] is implicated in mediating arrested differentiation and resistance to apoptosis (25). K562 are patient-derived cell lines (26) and serve as the *in vitro* CML model. K562 cell lines are pluripotent cells that are able to undergo differentiation along megakaryocytic and erythroid lineages (4,27). Induction of differentiation in CML patients might serve as a potential route for CML therapy. Extracts of *D. gibbosa* were evaluated for their ability in inducing erythroid differentiation in K562 cell lines. The ability to induce erythroid differentiation was monitored by measuring hemoglobin production in treated cells. Results shown in Table I demonstrate that crude extracts of *D. gibbosa* show a varying potency in inducing hemoglobin expression in treated cells. Extracts Meth451 and ME424 induce hemoglobin expression by 10.8- and 2.3-fold, respectively, above the solvent-treated cells. Notably, extract Eth shows the highest activity of hemoglobin production and was more potent than that of butyric acid (4), a well established differentiation agent.

Growth inhibition of K562 by D. gibbosa extracts is mediated by the induction of the apoptosis pathway. Induction of apoptosis is the principle mechanism by which the majority of chemotherapeutic agents exert their function. The execution of apoptosis requires specific caspases including
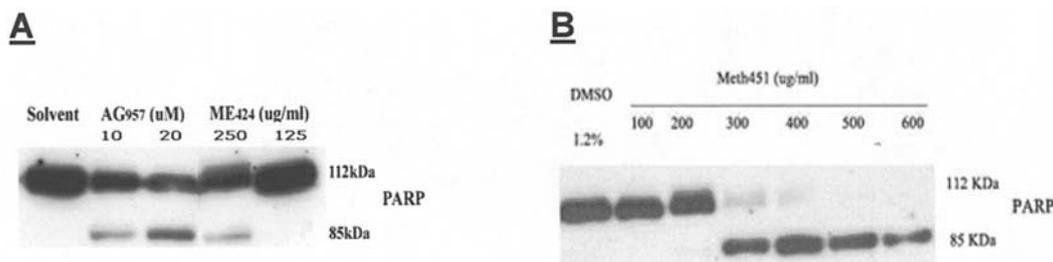
Figure 1. Induction of apoptosis by *D. gibbosa* crude extracts. (A) and (B) induction of PARP cleavage in treated cells by ME424 (A) and Meth451 (B).
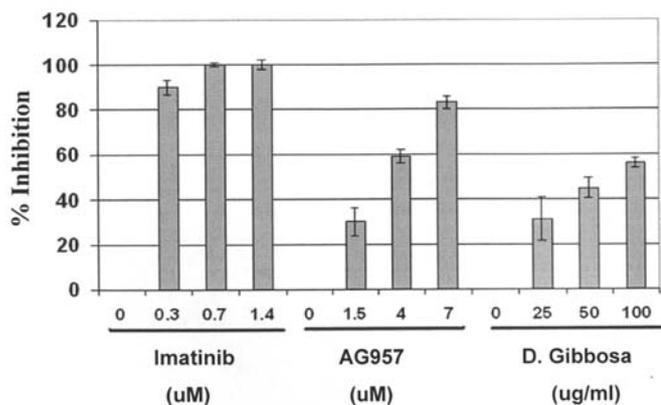
Figure 2. Inhibition of Bcr-Abl phosphorylation using ELISA assay. Cells were treated with the indicated concentration for 1 h and 100 μg of proteins were used to determine levels of phopshorylated Bcr-Abl.

caspase-3, which cleaves death substrates such as the nuclear protein poly (ADP-ribose) polymerase (PARP). In this study, we followed PARP cleavage as an endpoint for apoptosis assessment (28).

Consistent with published data, treatment with AG957, a known substrate inhibitor of Abl, causes a significant cleavage of PARP. Crude extract E and Eth were active in inducing apoptosis in treated K562 cell lines as determined by the PARP cleavage assay (Fig. 1).

Effect of Daedalea gibbosa crude extract on the auto-phosphorylation of Bcr-Abl. The creation of Bcr-Abl fusion protein resulted in enhanced and uncontrolled tyrosine kinase activity of the p210 [Bcr-Abl]. It is well documented that tyrosine kinase activity is responsible for the oncogenic potential of Bcr-Abl. A number of anti-CML compounds, including Imatinib (Gleevec) and the tyrphostin AG957, are known to inhibit tyrosine phosphorylation of Bcr-Abl and consequently exert anti-CML effects. In order to investigate the mechanism of action by which the organic extract of *D. gibbosa* exerted its function, we followed levels of phosphorylated Bcr-Abl protein. For this purpose, we developed an ELISA assay in which a monoclonal anti-c-Abl antibody (Santa Cruz) is used to capture the Bcr-Abl and c-Abl protein, while the anti-phosphorylated tyrosine antibody, PY20, is used as a reporter antibody. Initially, we monitored phosphorylated c-Abl/Bcr-Abl in a variety of cell lines such as K562, LNCaP, Cos-1 and Ba/F3 p185 Bcr-Abl. The signal obtained was very minimal in LNCaP and Cos-1 cells. Furthermore,

signals from K562 and Ba/F3 p185 Bcr-Abl cells were significantly higher than the background signal. However, signal-to-noise ratio from Ba/F3 p185 Bcr-Abl was reproducibly and significantly better than that obtained with the K562 cells. Thus, we proceeded to investigate the ability of the *D. gibbosa* extract in affecting phosphorylation of Bcr-Abl using Ba/F3 p185 Bcr-Abl. Ba/F3 p185 Bcr-Abl cells were treated with various concentrations of test compounds for 1 h and levels of phosphorylated Bcr-Abl were measured as described in Material and methods. Results shown in Fig. 2 demonstrate that treatment with Imatinib at 0.35-1.5 μM caused a significant reduction in the phosphorylation status of Bcr-Abl. In addition, treatment with AG957 caused an inhibition of the phosphorylation levels of Bcr-Abl in a concentration-dependent manner. Treatment with 1.75 μM of AG957 cause a reduction of ~30% of in phosphorylation of Bcr-Abl. Increasing the drug concentration to 7 μM caused >80% inhibition of the phosphorylation levels of Bcr-Abl. Similarly, treatment with 70% ethanol extract of *D. gibbosa* (Meth 451) causes a decrease in the phosphorylation levels of Bcr-Abl. The effect of *D. gibbosa* extract exhibited a concentration-dependent phenomenon. Treatment with 100 μg/ml of *D. gibbosa* extract caused an almost 50% inhibition in phosphorylation levels of Bcr-Abl. These data argue that the anti-CML activity of *D. gibbosa* extracts might be mediated by the inhibition of autophosphorylation of Bcr-Abl.

Identification of the active fraction that inhibits Abl phosphorylation. In an attempt to identify the active moieties that mediate the activity of the mushroom extracts *D. gibbosa* against K562, we used an activity-guided approach to isolate the active fraction using liquid chromatography. Crude organic extract was separated on a Silica gel using dichloromethane and the concentration of methanol was increased. Fractions were tested for their ability to inhibit autophosphorylation of Bcr-Abl using our ELISA assays that measure tyrosine phosphorylation of Abl proteins. As shown in Fig. 3, fractions F5-F6 contained potent activity that inhibited the autophosphorylation of Bcr-Abl, while fraction F7-F8 showed a moderate inhibitory activity against Bcr-Abl autophopshorylation. In contrast, fractions F2-F4 showed minimal autophosphorylation inhibitory activity. We focused on the fraction F6, which was the most active fraction for the upcoming analysis.

Fraction 6 of the D. gibbosa extract inhibits the auto-phosphorylation of Bcr-Abl harboring mutations responsible for Imatinib resistance. To investigate whether fraction 514 F6 is active against mutated Bcr-Abl found in patients' refractory
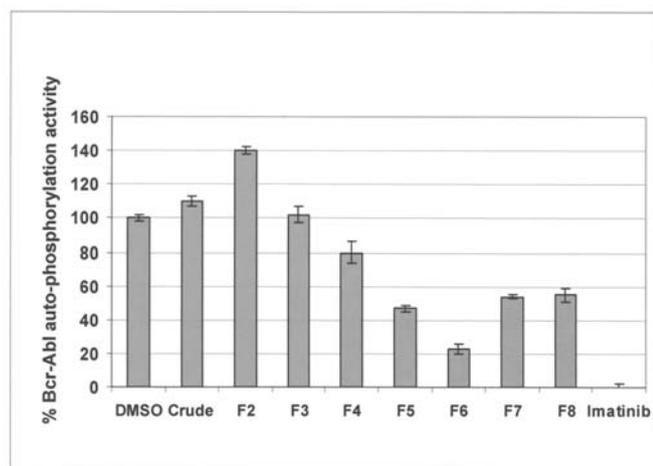
Figure 3. Inhibition of autophosphorylation of Bcr-Abl by *D. gibbosa* fractions. *D. gibbosa* fractions were isolated as described in Materials and methods and were used to evaluate the ability to inhibit Bcr-Abl auto-phosphorylation. K562 cells were treated with appropriate concentrations of the fractions for 1 h and cell lysates were used to measure levels of Bcr-Abl tyrosine phosphorylation as described in Materials and methods.

Figure 4. Inhibition of Abl autophosphorylation of native Abl and its mutants by Imatinib (A), crude extract (CR) and fraction 6 (F6) (B) compared to solvent-treated samples (U). BaF/3 cells carrying either native p185 Bcr-Abl or its mutated forms E255K or T315I were treated for 1 h with Imatinib (1 μM), crude extract (CR) or active fraction F6 as described in Materials and methods.

to Imatinib treatment, the effect of fraction F6 on the auto-phosphorylation of Bcr-Abl stably expressed in Ba/F3 cells was monitored. Bcr-Abl constructs were used harboring either the E255K, one of the most frequent mutations in Ph[+] ALL patients, or the T315I mutation, which confers resistance not only against Imatinib, but also against most other Abl kinase inhibitors (16,29,30). Levels of phosphorylated Bcr-Abl in relation to levels of total Bcr-Abl were determined using Western blotting. As shown in Fig. 4, crude extract as well as fraction F6 were able to inhibit the autophosphorylation of not only the native Bcr-Abl but also of the two mutations Bcr-Abl; E255K and T315I.

Fraction 6 of the D. gibbosa extract inhibits the colonigenicity of Ba/F3 cells expressing Bcr-Abl harboring mutations responsible for Imatinib resistance. In order to evaluate the biological relevance of the inhibition of the autophosphory-lation of Bcr-Abl and its kinase mutants, the effect of fraction F6 on the colonigenicity of Ba/F3 cells expressing native Bcr-Abl and its mutants was studied. Ba/F3 cells expressing native Bcr-Abl, Bcr-Abl/T315I or Bcr-Abl/E255K were plated in the absence of IL-3 in a semi-solid medium and in the presence and absence of appropriate treatments. Exposure to fraction F6 as well as to the *D. gibbosa* crude extract abolished like Imatinib, the colonigenicity of Bcr-Abl-positive Ba/F3 cells. While in Ba/F3 cells expressing the Bcr-Abl/ T315I or Bcr-Abl/E255K, no inhibition of colonigenicity was seen upon exposure to Imatinib; fraction F6 as well as *D. gibbosa* organic crude extract completely abrogated the colony formation of these cells (Fig. 5).

Fraction 6 of D. gibbosa inhibits Abl kinase activity. Previously, we showed that fraction F6 inhibits the auto-phosphorylation of Bcr-Abl in Ba/F3 cells trasfected with Bcr-Abl constructs. Since the experiment was carried out in cells, one might argue that the observed effect against Bcr-Abl is indirect and not due to the direct inhibition of the kinase

activity of Bcr-Abl. Thus, we tested the activity of fraction F6 on the kinase activity of recombinant Abl kinase using Abltide (EAIYAAPFAKKK) as a substrate.

Results shown in Fig. 6 indicate that fraction F6 inhibited the *in vitro* kinase activity of recombinant Abl protein, arguing that fraction F6 contains substances that directly interfere with the Abl kinase activity. The experiment was repeated three times with a comparable outcome. Furthermore, Imatinib at 1 μM was able to inhibit Abl kinase activity by almost 50%. Similarly, AG957 was active in inhibiting Abl kinase by 50% at a concentration of 5 μM.

Discussion

CML is a malignancy of pluripotent hematopoietic cells characterized by the presence of the Philadelphia (Ph) chromosome, which resulted from reciprocal translocation between the long arms of chromosomes 9 and 22 {(t(9;22) q34;q11)} resulting in the creation of the fusion gene p210 [Bcr-Abl] exhibiting abnormal tyrosine kinase activity. The presence of active p210 [Bcr-Abl] renders CML cells resistant to apoptosis and delays differentiation.

Medicinal mushrooms have been an important source of therapeutic substances for the treatment of various human diseases. Anti-tumor activities from mushrooms were described in many reports (1,2,4-7).

In many cases, activity was due to HMW polysaccharides having a molecular weight of 200-400,000 Dalton. Anti-tumor activity of HMW polysaccharides was attributed to the immune-modulation function or enhancement properties of the immune system, not as a result of a direct influence on the tumor cells.

In this study, we identified mycelium crude extracts of *D. gibbosa,* which showed anti-tumor effects against the CML
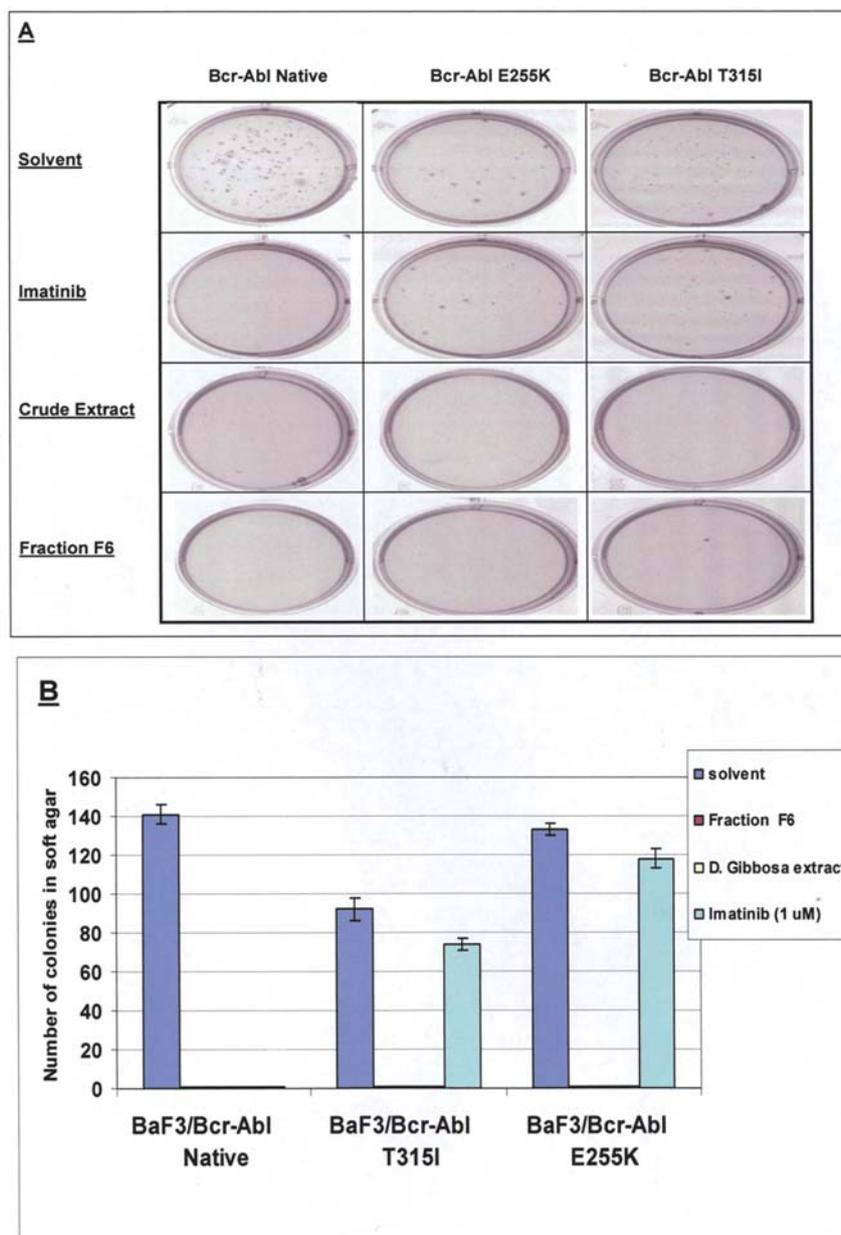
Figure 5. Effect of active fraction F6 and crude *D. gibbosa* extract compared to Imatinib on the clonogenicity of Ba/F3 cells expressing either native or mutated Bcr-Abl (A). (B) A number of stained colonies found in each treatment.
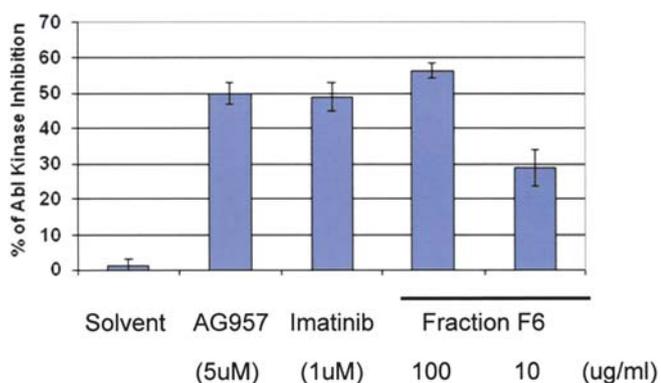
Figure 6. Inhibition of Abl *in vitro* kinase activity by *D. gibbosa* active fraction. The Abl *in vitro* kinase assay was performed according to Materials and methods in the presence of AG957 (5 μM), Imatinib (1 μM) and *D. gibbosa* active fraction F6 (10 and 100 μg/ml).

cancer cell line. The anti-CML activity of *D. gibbosa* was observed with each organic extraction; however, selectivity against other tested cancer cell lines was dependent on the composition of solvents used during the extraction. For example, extracts prepared using mixtures H (22% ethyl acetate, 11% methanol) and C (50% methanol and 33% chloroform) were CML-selective showing a significant anti-proliferating activity against the K562 cell line (CML) with minimal activity against other tested cell lines. However, extractions with mixtures Eth (70% ethanol) and E (50% methanol, 33% ethyl acetate) produced crude organic extracts that are less selective, causing a significant growth inhibition of MH3924A (rat Morris hepatoma) cell line, but minimal anti-proliferative activity against other tested cell lines. It is worth investigating the activities found in Eth and E extracts missing in extracts H and C, which cause significant

inhibition of the MH3924A (rat Morris hepatoma) cell line. Our data argue that the two observed anti-proliferation activities against the K562 (CML) and MH3924A (rat Morris hepatoma) cell lines might be separated.

Based on the extraction method employed and the ability to exert biological effects on an *in vitro* system, we argued that the active moieties in the organic extraction are not HMW substances, rather LMW substances.

K562 cells are pluripotent cells that are able to differentiate along a megakaryocytic, erythroid, or, to a lesser extent, monocytic lineage. Erythroid differentiation can be induced by a number of compounds including hemin and butyric acid (31). The different organic extracts of *D. gibbosa* were evaluated for their ability to induce erythroid differentiation in K562 cells. Each extract was able to induce hemoglobin expression above the control levels, however, the Eth extract was the most potent one and induced hemoglobin expression by >9-fold-treated cells. Our data show that there is no correlation between anti-proliferation activity against CML cells and the ability to induce erythroid differentiation. Eth and C extracts show comparable anti-proliferation activity against CML, however, they induced hemoglobin expression by 10.8 and 1.7, respectively.

It is worth noting that the hemoglobin produced in K562 is a fetal hemoglobin, mainly γ-globin. Increased expression of the endogenous γ-globin gene is a realistic approach to therapy of ß-globin disorders such as sickle cell anemia and ß-thalassemia (32,33). Thus, our mycelium extract has the potential to serve as a therapy for ß-globin disorders.

The observed anti-proliferation activity of the organic extracts of *D. gibbosa* against K562 was attributed to the induction of apoptosis. PARP cleavage assay showed that E and Eth extracts were active at a concentration above 250-300 μg/ml to induce apoptosis in treated cells (Fig. 1). Similarly, other organic extracts induce apoptosis in K562 with a comparable potency (data not shown).

Bcr-Abl exhibited uncontrolled tyrosine kinase activity, which accounted for its tumorigenesis. Consequently, inhibitors of the tyrosine kinase activity of Bcr-Abl were developed as an effective molecular therapy for the chronic phase of CML such as Imatinib (STI571; Gleevec). In an attempt to verify the mechanism of action of our *D. gibbosa* organic extracts, we investigated the effect of *D. gibbosa* extracts on the kinase activity of Bcr-Abl. Treatment with the *D. gibbosa* extract for 1 h caused a significant reduction in the amount of phosphorylated Bcr-Abl, implying that the anti-proliferation effect exhibited by *D. gibbosa* might be mediated by substances of the extract that interfere with the phosphorylation process of Bcr-Abl.

Although there are no published reports showing biological activity or substances isolated from *D. gibbosa*, anti-tumor activity from a related species has been reported. *D. dickinsii* (Berk.) Bond *et al* (34) and *D. biennis* (Bull.) Fr. (35) showed anti-tumor activity. Active molecules, such as lanostane triterpenoids and lanostane triterpene glucosides, were isolated from fruit bodies of *D. dickinsii* by 70% ethanol and inhibited the growth of the HL-60 and HCT-15 cell lines. Furthermore, lanostane triterpene glucosides promoted apoptosis in the HL-60 cell line (36).

Upon activation, Bcr-Abl becomes autophosphorylated on multiple tyrosines, making Bcr-Abl tyrosin-phosphorylation a useful indicator of Bcr-Abl kinase activity. The inhibitory effects of *D. gibbosa* fractions on the autophosphorylation of Bcr-Abl showed that fraction F6 was the most potent in decreasing the autophosphorylation activity of Bcr-Abl. In addition, contrary to Imatinib, the *D. gibbosa* crude extract and fraction F6 dramatically inhibited the phosphorylation of the native Bcr-Abl and the two mutated forms including T315I.

Furthermore, Ba/F3 cells harboring Bcr-Abl are able to form colonies in soft agar. These studies showed that Imatinib was able to inhibit colony formation by the Ba/F3 p185 Bcr-Abl cell line. However, it failed to interfere with the growth of Ba/F3 p185 Bcr-Abl/T315I and Ba/F3 p185 Bcr-Abl/E255K in soft agar. Conversely, treatment of Ba/F3 p185 Bcr-Abl by either *D. gibbosa* crude extract or the active fraction F6 caused an inhibition of colony formation by all Ba/F3 p185 Bcr-Abl cell lines.

In order to focus on the study of the mechanism of action, it was necessary to determine whether the active fraction F6 directly inhibits the Bcr-Abl kinase activity. Fraction F6 inhibited 56% of recombinant Abl tyrosine kinase activity in an *in vitro* kinase assay at a concentration of 100 μg/ml. The effect of AG957 on Abl kinase activity is consistent with other reported data since its activity does not depend on the special conformation of the enzyme Abl. Contrary to AG957, the effect of Imatinib in an *in vitro* Abl kinase assay needed a 5-fold higher concentration than its effect in previous cell culture assays. The effect of Imatinib is more potent in the cell culture assay, since it usually binds to the inactive conformation of the Bcr-Abl protein.

Our study showed the ability of natural product-derived substances to regulate the activity of Abl kinase with yet an undefined mode of action. Several Abl kinase inhibitors are available and were reported to inhibit the native form of Bcr-Abl and also most of its mutations that confer a resistance to Imatinib. However, almost all Abl kinase inhibitors, except VX-680, failed to inhibit the kinase activity of the Abl T315I mutation. Our study illustrated that substances found in the *D. gibbosa* F6 fraction were able to inhibit the kinase activity and autophosphorylation function of the native Abl and its mutants, including T315I. The nature and the chemical structure of the active moiety remain to be elucidated.

Acknowledgements

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