Abstract. The hallmark of chronic myeloid leukemia (CML) is the abnormal activity of p210Bcr-Abl kinase. Selective kinase inhibitors such as imatinib or nilotinib have been established successfully for the treatment of CML. Despite high rates of clinical response, CML patients can develop resistance to these kinase inhibitors mainly due to point mutations within the Abl kinase domain of the fusion protein. Previously, we reported that a crude extract of the mushroom Daedalea gibbosa inhibited kinase activity of Bcr-Abl kinase. Here we report on the identification of the active component of Daedalea gibbosa, oleic acid, which inhibited Bcr-Abl kinase autophosphorylation in Ba/F3 cells and exhibited anti-CML activity in a BCR/ABL-positive mouse model. Medicinal mushrooms have emerged as an important and potentially novel source of therapeutic substances for the treatment of various human diseases (1, 2). Traditionally, antitumor activity of mushrooms has been associated with the presence of high molecular weight polysaccharides with immune-modulation function. We and others focused on the ability of low molecular weight moieties that can penetrate the cell membrane easily and exhibit anti-tumor activity with a defined mechanism of action (2-4).

CML is a clonal myeloproliferative disorder of hematopoietic stem cells that accounts for 15% of leukemias in adults (5). The translocation-related (9, 22) protein product is the Bcr-Abl kinase fusion protein, which displays constitutive tyrosine kinase activity leading to the induction of aberrant proliferation and neoplastic transformation by constitutive activation of target signaling pathways (6). The Bcr-Abl kinase-induced phenotype in hematopoietic cells is characterized by increased proliferation, as well as reduced susceptibility to a variety of pro-apoptotic stimuli, including growth factor deprivation (6). The inoculation of hematopoietic stem cells retrovirally transduced with Bcr-Abl kinase induces a CML-like disease in sublethally irradiated recipient mice within 3-4 weeks, which proves that Bcr-Abl kinase is responsible for the pathogenesis of Philadelphia-chromosome-positive (Ph+) leukemias (7).

Imatinib (Gleevec), a selective inhibitor of Abl kinase, is approved for the treatment of chronic phase CML (CP) and is included in most clinical trials for the treatment of Ph+ acute lymphatic leukemia (ALL) (8-10). Unfortunately, the clinical efficacy of treatment with imatinib decreases continuously with the advancement of the disease. Blast crisis CML or Ph+ ALL patients benefit from treatment with tyrosine kinase inhibitors only temporarily or not at all (11). Acquired resistance is mostly due to the acquisition of point mutations in the Bcr-Abl kinase by either reducing the affinity of the kinase domain to the ATP-competitor or stabilizing the active conformation of the Abl kinase domain.

Second-generation inhibitors of Abl kinase activity consists of compounds that can overcome imatinib resistance. Nilotinib (Tasigna) is a novel ATP-competitive effective against a variety of cell lines expressing Imatinib-resistant Abl kinase mutants. Dasatinib (Sprycel) is a dual Src/Abl kinase inhibitor that inhibits kinase activity of 14 out of 15 imatinib-resistant Abl kinase mutants (12).

The major therapeutic challenge in Ph+ leukemia remains the ‘gatekeeper’ mutation T315I, which confers global resistance against nearly all molecular therapy approaches targeting Bcr-Abl kinase, such as ATP competition,
oligomerization inhibition, and allosteric inhibition (13, 14). Recently, a number of kinase inhibitors such as VX-680, PHA739358 and AT9283 were reported to exhibit good activity in inhibiting the activity of T315I-mutated Bcr-Abl kinase (15-17). Furthermore, treatment of imatinib-resistant patients carrying T315I with VX-680 resulted in a promising outcome (15, 18), while clinical trials with PHA739358 and AT9283 are ongoing.

Recently, we identified a mycelium organic extract from the medicinal mushroom *Daedalea gibbosa* (*D. gibbosa*) exhibiting selective antiproliferative and apoptosis-inducing activities against CML-derived K562 cells. In addition, we isolated an active fraction capable of inhibiting directly the kinase activity of Abl kinase, as well as the auto-phosphorylation activity of native Bcr-Abl kinase and its mutated forms, including the T315I mutation. The active fraction of *D. gibbosa* also inhibited the clonigenicity of native Bcr-Abl kinase and its mutants when expressed in Ba/F3 cells (4).

In this study, we further characterized the active fraction of *D. gibbosa* in order to isolate the component responsible for the inhibitory effect against Bcr-Abl kinase.

**Materials and Methods**

*Mushroom growth conditions and extractions.* The *D. gibbosa* (#514) strain used in the present research is from a culture collection of the Institute of Evolution, University of Haifa, Israel. Mushroom growth medium and cultivation of *D. gibbosa* were as previously described (4).

*Crude fractionation and isolation of active components.* Dry ground mycelia of *D. gibbosa* mushroom (1.5 kg) were extracted with ethyl acetate, filtered and evaporated to dryness under vacuum. The crude extract (10 g) was loaded on a silica gel column (200 g, 0.063-0.200 mesh) suspended in hexane, and the mixture was eluted with a gradient of hexane-ethyl acetate and finally with methanol to obtain eight fractions. Fraction 2 (SiF2), which eluted with 85% hexane and 15% ethyl acetate, was the most active fraction and was selected for further fractionation. Two grams of fraction SiF2 were purified further on an atmospheric C18 reverse phase column (d=1 cm, L=25 cm), particle size 15-35 micron, and three fractions were eluted with a gradient of acetonitrile and water. Biological analysis revealed that fraction SiF2C18A (2) is the active fraction and it was separated further by a preparative HPLC column kromasil C18 250×20 mm; flow of 20 ml/min, detection at wavelength 220 nm) using a mobile phase of 95% acetonitrile and 5% water, and 14 fractions were collected. Fraction SiF2C18A(2)-6 was the most active fraction containing two peaks with retention times of 10 and 13.3 minutes. A semi-preparative C18 column (kromasil C18 250×10 mm, flow of 4.7 ml/min, detection at wavelength 220 nm, mobile phase of 95% acetonitrile and 5% water) was used to separate the two peaks; the peaks of 10 and 13.3 minutes were designated as SiF2C18A(2)-6.1 and SiF2C18A(2)-6.2, respectively.

*Chemical analysis of the active fractions.* A Hewlett-Packard (HP) 1090 HPLC, equipped with a manual injector C18 column (4.6×250 mm, analytical column and 10×250 mm, semi-preparative column) pump and a UV-Vis detector, was used. Twenty microliters were injected into the analytical column and 0.5 ml was injected into the semi-preparative column. The mobile phase for all of the samples analyzed was a gradient of water and acetonitrile, beginning with 95% water to 5% water over 30 minutes. The flow of the mobile phase using the analytical column was 1 ml/min and 4.7 ml/min for the semi-preparative column. All fractions were detected in the diode array UV detector at three wavelengths: 220, 254 and 280 nm.

A Hewlett-Packard (HP) 6890 gas chromatograph equipped with an auto-sampler was interfaced with a mass spectrometer. Samples were injected into an HP-1 100% methyl silicon capillary fused silica column (30 m × 0.2 mm i.d.; 0.33 μm film thickness). Helium was the carrier gas at a column pressure of 160 kPa and a column flow rate of 1 ml/min. The injector temperature was 250°C, and the split ratio of inlet to column gas flow was 15:1. All fractions were dissolved in a glass well with dichloromethane at a concentration of 1-2 ppm, and 1 μl was injected into the GC. The fractions were
separated by a GC temperature program: initial temperature at 60˚C increased at 10˚C/min to 300˚C, with a solvent delay of 1 min. The GC analysis was completed in 30 min. MS was carried out in an electron ionization mode, and both total and selected ions were monitored.

Cell lines and cell cultures. Ba/F3 cells expressing Bcr-Abl kinase, as previously described (4), were grown in RPMI-1640 with 2 mM L-glutamine supplemented with 10% fetal bovine serum. Penicillin at 100 U/ml and streptomycin at 100 μg/ml were added to the culture media. All cell lines were grown at 37˚C in a humidified atmosphere with 5% CO2.

Cellular autophosphorylation of Bcr-Abl kinase. Ba/F3 cells expressing the native Bcr-Abl kinase protein (4×10^5 cells/ml) were treated with D. gibbosa mycelia crude extract, active fractions of the crude extract or DMSO for 1 h. Cells were collected, washed once with cold PBS and lysed with 100 μl of lysis buffer (10 mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM NaPO₄, 2 mM Na₂VO₄, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 0.1% SDS, and 0.5% sodium deoxycholate, supplemented with protease and a phosphatase inhibitors cocktail). Cell lysate supernatants (40 μg protein) were resolved on 8% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and analyzed by immune-blotting with anti-phospho-c-Abl(Tyr245) polyclonal antibody (Cell Signaling Technology, USA). The phosphorylation levels of Bcr-Abl kinase protein were compared to total Abl kinase, which was detected using anti-c-Abl monoclonal antibody (Santa Cruz Biotechnology, USA).

Mouse tumor model. Six- to eight-week-old female athymic mice (Harlan Laboratories Ltd., Israel) weighing ~20 g±20% were housed in polyethylene cages in a climate-controlled environment. Ba/F3 Bcr-Abl kinase cells (2×10^6 cells) were implanted s.c. by an injection of 100 μl/mouse into the animal’s lower flank. Mice were then treated by daily i.p. injections for 16 days with either the material-free formula (vehicle) or the formula containing D. gibbosa mycelia-enriched extract (450 mg/kg; 10 ml/kg mouse). Tumor diameters were measured with digital calipers, and tumor volumes in mm³ were calculated and recorded three times a week.

Results

D. gibbosa active fraction inhibits Ba/F3 p185 Bcr-Abl kinase tumor. Previously, we showed that organic fraction F6 prepared from D. gibbosa inhibited kinase and auto-phosphorylation activity of native and T315I-mutated Abl kinase (4). Thus, we tested whether the enriched D. gibbosa fraction is able to suppress tumor growth in vivo. Large-scale fermentation was carried out and the active fraction was isolated. The ability of the crude extract, as well as the active fraction, to inhibit
autophosphorylation of Bcr-Abl kinase in Ba/F3 Bcr-Abl cells was measured utilizing the autophosphorylation assay (data not shown). The active fraction was used to treat 6 to 8-week-old female athymic mice previously inoculated with Ba/F3 cells expressing Bcr-Abl kinase. Mice were treated daily with i.p. injections of 10 mg/kg imatinib (Figure 1A), 450 mg/kg of D. gibbosa active fraction (Figure 1B), or vehicle for 16 days. Results shown in Figure 1 illustrate that treatment with D. gibbosa active fraction resulted in a significant reduction in the volume of Ba/F3 Bcr-Abl tumors compared to vehicle-treated animals (Figure 1B) and this treatment performed better than imatinib (Figure 1A).

Active component from D. gibbosa isolated by activity-guided purification was able to inhibit Bcr-Abl kinase autophosphorylation. Given the in vivo efficacy of the silica-purified active fraction, we attempted to purify further the active fraction aimed at isolating the active component. The active fraction was purified further with a bioactive-guided process using atmospheric reverse phase C18 column followed by preparative HPLC column, as shown in Figure 2. The fractions obtained were examined for activity using our autophosphorylation assay.

The effects of the different fractions obtained from the semi-preparative HPLC on the autophosphorylation of Bcr-
Abl kinase were investigated. As shown in Figure 3A, the mother fraction (SiF2) showed moderate activity in inhibiting the autophosphorylation of Bcr-Abl kinase at increasing concentrations. In contrast, the daughter fraction SiF2C18(2)-6 showed significant inhibition of activity at both concentrations used. The other fractions, such as the daughter fractions SiF2C18(2)-7 and SiF2C18(2)-8, showed minimal activity even at higher concentrations (Figure 3A). These data clearly indicate that the active component is concentrated mainly in the daughter fraction SiF2C18(2)-6 (Figure 3A), which was subjected to further analysis. Interestingly, the HPLC profile revealed the presence of two major peaks at retention times of 10 and 13.3 minutes (Figure 3B). The two peaks were separated using semi-preparative HPLC (Figure 2).

Oleic acid is the component in D. gibbosa inhibiting Bcr-Abl kinase autophosphorylation. Based on the HPLC profile (Figure 3B), we separated the two peaks and generated SiF2C18(2)-6.1 and SiF2C18(2)-6.2 fractions. The ability of these fractions to affect Bcr-Abl kinase autophosphorylation activity was examined, and data presented in Figure 4A illustrate that a significant portion of the auto-phosphorylation inhibitory activity resides in the daughter fraction SiF2C18(2)-6.1. In addition, SiF2C18(2)-6.2 exhibited marginal activity in Bcr-Abl kinase autophosphorylation inhibition (Figure 4A). GC-MS analysis of the two fractions SiF2C18(2)-6.1 and SiF2C18(2)-6.2 revealed that fraction SiF2C18(2)-6.1 contains palmitic as well as oleic acid, in approximately equal amounts (Figure 4B), whereas fraction SiF2C18(2)-6.2 contains mainly diisooctyl phthalate with traces of palmitic and oleic acids (Figure 4C).

To confirm whether the activity is mediated by one of the three compounds, namely palmitic, oleic and phthalic acids, we repeated the experiment with pure commercial chemicals and a mixture of the two fatty acids (palmitic and oleic acids). Data shown in Figure 4D demonstrate that the presence of oleic acid inhibited autophosphorylation of Bcr-Abl kinase with a minimal effect of palmitic acid and phthalic acid. Furthermore, when the two fatty acids (palmitic and oleic acids) were mixed, an inhibitory effect was observed only at the higher concentration, which further
supports the conclusion that the activity exhibited by \textit{D. gibbosa} extract is due to the presence of oleic acid.

**Discussion**

Previously, we showed that the active fraction F6, which was isolated from mycelial extract of \textit{D. gibbosa}, was significantly active in inhibiting the kinase and autophosphorylation activity of Bcr-Abl kinase (4). Furthermore, the active fraction was also effective in inhibiting Abl kinase harboring imatinib-resistant mutations (4).

In this study, we evaluated the in vivo antitumor activity of the active fraction isolated from \textit{D. gibbosa} in an in vivo model of Bcr/Abl-positive leukemia. Our data show that the active fraction exhibited greater activity, at the concentration used, than imatinib without any noticeable toxicity (data not shown).

The proven active fraction was subjected to a series of further activity-guided purification steps in order to obtain a highly pure active fraction. This aim was achieved by a series of liquid chromatography using silica and C18 gels followed by preparative HPLC fractionation, resulting in two fractions. Further chemical characterization of these two fractions by HPLC, GC-MS, LC-MS and NMR (data not shown) revealed that these active fractions contain a mixture of palmitic and oleic acids. To disclose which component is capable of inhibiting Bcr-Abl kinase autophosphorylation activity, we used commercial preparations of the two fatty acids and mixed them according to their concentration in the active fraction. This approach allowed us to illustrate clearly that the inhibitory activity against Bcr/Abl kinase resides within the oleic acid and not within the palmitic acid.

Our studies on isomers (\textit{cis} and \textit{trans}) of oleic and other fatty acids showed that only oleic acid in its \textit{cis} isomer was active against the aberrant kinase activity of Bcr-Abl. No significant activity was observed with \textit{trans} oleic acid (data not shown).

Fatty acids were reported previously to affect kinase activity (19). Specifically, oleic acid and elaidic acid (\textit{trans} oleic acid) were active in inhibiting cyclic AMP-dependent protein kinase (PKA) activity (19). Furthermore, oleic acid was reported to induce activity of PKC, IKK α/β, the stress kinase S6 kinase p70 (p70SK), stress-activated protein kinase (SAPK), JNK, as well as p38 MAP kinase (p38 MAPK) (20, 21). Interestingly, oleic acid-mediated PKC activation was implicated in insulin resistance and in vascular complications of diabetes (22, 23). The exact mechanism of action by which oleic acid modulates the activity of the different kinases remains to be elucidated. However, our preliminary docking data (not shown) illustrated that oleic acid might interact with the Abl kinase protein in a hydrophobic pocket in the C-lobe of the kinase domain, an area that is proposed to interact with the myristate in the Abl kinase protein. Thus, one could speculate that oleic acid might be an allosteric inhibitor targeting the myristate binding pocket leading to a change in conformation of Abl kinase and consequently to the inhibition of its kinase activity. This hypothesis is awaiting experimental evidence.

In conclusion, our data presented provide evidence for the potential use of oleic acid and probably of more selective and potent derivatives in inhibiting the transformation potential of Bcr-Abl kinase in vivo and represent the basis of a novel approach for the molecular therapy of Ph+ leukemia.

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**References**


