Available online at www.sciencedirect.com
ScienceDirect
Archives of Biochemistry and Biophysics 476 (2008) 113–123
ABB
www.elsevier.com/locate/yabbi

Insight into the apoptosis-inducing action of α-bisabolol towards malignant tumor cells: Involvement of lipid rafts and Bid

Elena Darra [a], Safwat Abdel-Azeim [b], Anna Manara [c], Kazuo Shoji [d], Jean-Didier Maréchal [e], Sofia Mariotto [a], Elisabetta Cavalieri [a], Luigi Perbellini [f], Cosimo Pizza [g], David Perahia [b], Massimo Crimi [c], Hisanori Suzuki [a,*]

[a] Dipartimento di Scienze Morfologico-Biomediche, Università di Verona, Strada Le Grazie, 8, 37134 Verona, Italy
[b] Laboratoire de Modélisation et Ingéniérie des Protéines, Université Paris-Sud, Bât 430, 91405 Orsay, France
[c] Dipartimento Scientifico Tecnologico, Università di Verona, 37134 Verona, Italy
[d] University of Human Arts and Sciences, Saitama 339-8539, Japan
[e] Unitat de Química Física, Departament de Química, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain
[f] Dipartimento di Medicina e Sanità Pubblica, Università di Verona, 37134 Verona, Italy
[g] Dipartimento di Scienze Farmaceutiche, Università di Salerno, 84084 Fisciano (Salerno), Italy

Received 16 November 2007, and in revised form 31 January 2008
Available online 9 February 2008

Abstract

In a precedent report we showed that α-bisabolol, a sesquiterpene present widely in the plant kingdom, exerts a rapid and efficient apoptosis-inducing action selectively towards human and murine malignant glioblastoma cell lines through mitochondrial damage. The present study extends these data demonstrating the apoptosis-inducing action of α-bisabolol towards highly malignant human pancreatic carcinoma cell lines without affecting human fibroblast viability. The present study further shows the preferential incorporation of α-bisabolol to transformed cells through lipid rafts on plasma membranes and, thereafter, direct interaction between α-bisabolol and Bid protein, one of pro-apoptotic Bcl-2 family proteins, analyzed either by Surface Plasmon Resonance method or by intrinsic fluorescence measurement. Notions that lipid rafts are rich in plasma membranes of transformed cells and that Bid, richly present in lipid rafts, is deeply involved in lipid transport make highly credible the hypothesis that the molecular mechanism of α-bisabolol action may include its capacity to interact with Bid protein.

Keywords: α-Bisabolol; Apoptosis; Pancreatic carcinoma cell lines; Lipid rafts; Bid; Surface Plasmon Resonance; Mitochondria

α-(−)Bisabolol, also known as levomenol, is a natural oily monocyclic sesquiterpene alcohol present in the essential oil of different plants, including chamomille flowers (Fig. 1). It has been used for hundreds of years in cosmetics because of its perceived skin healing properties and is also reported to have anti-irritant, anti-inflammatory and anti-microbial properties. Sesquiterpenes have commonly been identified as the active constituent of several medicinal plants used in traditional medicine, with a wide spectrum of biological activity including anti-inflammatory, anti-tumor and fungicidal properties. In particular, sesquiterpenes with the hydroxyl group are generally more active in anti-tumor activity than the corresponding terpene hydrocarbons [1]. For example, farnesol, a sesquiterpene alcohol, has been demonstrated to inhibit proliferation and induce apoptosis in a number of neoplastic cell lines from different origins [2] with preferential action in transformed cells versus untransformed cells [3,4]. Nevertheless, the exact molecular target(s) or the molecular mechanism of its action have not been fully elucidated yet.

* Corresponding author. Fax: +39 045 8027170.
E-mail address: hisanori.suzuki@univr.it (H. Suzuki).

0003-9861/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2008.02.004

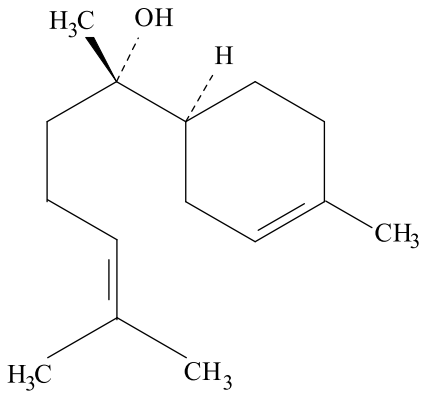

Fig. 1. Structure of α-bisabolol.

We recently reported that α-bisabolol exerts an efficient apoptosis-inducing action, via a mitochondrial pathway, towards human and murine glioblastoma cell lines [5]. Interestingly, it is further noted that α-bisabolol is far less cytotoxic towards normal cells, in line with the LD50 value reported to be around 15 ml/kg animal (mice, rats) body weight [6]. Furthermore, despite the similarity in the chemical feature of α-bisabolol and farnesol, together with their similar selective apoptosis-inducing action towards tumor cell lines, we noted that α-bisabolol is distinguished by its rapid and strong apoptosis-inducing activity [5]. This notion has led us to envisage the molecular mechanism of the anti-tumor activity of α-bisabolol.

In order to give a hint on this matter, we first hypothesized that α-bisabolol, upon contact with a plasma membrane, may be incorporated rapidly into lipid rafts. Lipid rafts are small, heterogeneous, highly dynamic, sterol- and sphingolipid-enriched membrane domains that compartmentalize cellular processes, playing an important role in intracellular protein transport and membrane fusion [7]. In this regard, it has been reported that tumor cells, compared to normal ones, contain far more lipid rafts on plasma membranes [7].

Lipid rafts contain many proteins playing a critical role in the transport of external signals inside cells. Hsu and Youle introduced an intriguing line of evidence suggesting that Bcl-2 family proteins may interact with membrane lipids and other hydrophobic molecules. They reported that hetero-dimerization between different proteins of the family was induced by non-ionic detergents like Triton X-100 [8,9]. Because other detergents did not produce the same effects, it was suggested that specific physico-chemical properties could govern the oligomerization and reciprocal binding of Bcl-2 proteins [9,10–12]. Bid, a pro-apoptotic protein, is reported to be recruited in the lipid rafts by some apoptosis-inducing agents, playing a critical role in the formation of a death-inducing signaling complex. Moreover, this BH3-only protein is part of the functional link between the intrinsic and extrinsic apoptotic pathway both ending in mitochondria activation [13]. Furthermore, recent reports describe its involvement in lipid trafficking from plasma membranes to cytoplasmic ones including mitochondrial membranes [14–17]. All these observations suggest that Bid could be involved in the intracellular pathway of α-bisabolol induced cell death.

In the present study, we first ascertained that α-bisabolol is able to induce apoptosis in highly malignant human pancreatic carcinoma cell lines and that these cells indeed incorporate more α-bisabolol than normal cells through cellular fractions rich in lipid rafts. We therefore analyzed the interaction between α-bisabolol and recombinant Bid (the wild type protein and two different mutants), a possible protein target for the binding of this compound and representative of BH3-only proteins, either by Surface Plasmon Resonance (SPR)[1] analysis or by intrinsic fluorescence measurement.

Materials and methods

Reagents

All chemicals used throughout the present study were of the highest analytical grade, purchased from Sigma Chemical Company, Milan, Italy, unless otherwise specified.

Cell culture

Human pancreatic carcinoma-derived cell lines IO and IM kindly supplied by Dr. Sorio, University of Verona, Italy and human fibroblast cells, a gentle gift from Prof. Armato, University of Verona, Italy. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Bio-Whittaker, Cambrex BioScience, Belgium), supplemented with 10 fetal bovine serum (FBS; BioWhittaker, Cambrex BioScience, Belgium), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 40 μg/ml gentamycin, in a 5% CO_2 atmosphere at 37 °C, with saturating humidity.

α-Bisabolol treatment

Cells and isolated rat liver mitochondria preparations were treated with α-bisabolol (Fluka and Riedel-de Haën, Sigma Chemical Company, Milan, Italy) from a freshly prepared stock solution in absolute ethanol (1:8 v/v). Initial α-bisabolol concentration was 250 μM. However, effective concentrations were dependent on time of incubation as described [5].

Measurement of cell viability

Cells (2×10^5 cells/wells) were plated onto 6-well plates in 1 ml culture medium. After 24 h, the cells were treated with α-bisabolol and with ethanol alone (control). Cell viability was determined with trypan blue exclusion assay. Counts were performed in duplicate wells.

Western blot analysis

Cells were homogenized at 4 °C in 50 mM Tris–HCl, pH 8, containing 0.1% Nonidet-P40 (NP-40), 200 mM KCl, 2 mM $MgCl_2$, 50 μM $ZnCl_2$, 2 mM DTT, and protease inhibitors (1 mM PMSF, 1 mg/ml leupeptin, and 1 mg/ml antipain). Aliquots of the homogenates (40 μg total protein/lane) were loaded on 7.5% polyacrylamide gels. Electrophoresis was performed at 100 V with a running buffer containing 0.25 M Tris–HCl, pH

[1] *Abbreviations used:* SPR, surface plasmon resonance; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NP-40, Nonidet-P40; PARP, poly(ADP-ribose) polymerase; DEVD-AMC, DEVD-7-amino-4-coumarin; GC–MS, gas chromatography–mass spectroscopy; EI, electron impact.

8.3, 1.92 M glycine, and 1% SDS. The resolved proteins were electroblotted onto a PVDF membrane (Immobilon P, Millipore, Bedford MA, USA). Membranes were then incubated with a monoclonal IgG antibody to poly(ADP-ribose) polymerase (PARP) (Zymed, South San Francisco, CA, USA) and, after washing, incubated with an anti-mouse IgG-peroxidase conjugate (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The blots were washed again, then successively incubated with enhanced chemiluminescence detection reagents (ECL, Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's instructions, and proteins were detected by ChemiDoc XRS Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of caspase 3 activity

For detection of caspase 3 activity, 2×10^6 cells were first lysed and caspase 3-like activity was detected after 10 min of incubation at 30 °C by measuring the proteolytic cleavage of the fluorogenic substrate DEVD-7-amino-4-coumarin (DEVD-AMC, Biomol). The fluorescence was measured at 460 nm using an excitation wavelength of 380 nm.

Measurement of DNA ladder formation

For the internucleosomal DNA laddering, 3×10^6 cells, resuspended in 0.3 ml of culture medium containing 10% FBS, were incubated for 45 min at 65 °C, and then overnight at 37 °C in the presence of 0.4 M NaCl, 5 mM Tris–HCl, pH 8, 2 mM EDTA, 4% SDS, and 2 mg/ml proteinase K. The lysates, brought to a final concentration of 1.58 M NaCl, were centrifuged twice for 10 min at 6000*g* to separate the DNA fragments from intact DNA. The supernatants were recovered and DNA was precipitated by the addition of three volumes of absolute ethanol at 80 °C for 1 h. The DNA pellets were recovered by centrifugation (10 min, 12,000*g*), and resuspended in a minimal volume of 40 µl of 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1 mg/ml DNase-free ribonuclease A. Aliquots of 5 µg of DNA were then loaded onto a 1% agarose gel containing 0.25 µg/ml ethidium bromide. After electrophoresis, the DNA was visualized by a ChemiDoc XRS imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of cytochrome c release

IO and IM cells were washed twice with ice-cold PBS and scraped off the plates. Cells were collected by centrifugation at 500*g* for 5 min. Cell pellets were suspended in 1 ml of a solution containing 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5, 1 mM sodium orthovanadate and a complete EDTA-free protease inhibitor cocktail (prepared as recommended by Boehringer–Mannheim GmbH). Cells were then chilled on ice for 10 min and gently lysed by adding 0.3% (v/v) NP-40. In order to restore an isotonic environment, a solution containing 525 mM mannitol, 175 mM sucrose, 12.5 mM Tris–HCl, pH 7.5, 2.5 mM EDTA, and the protease inhibitor cocktail was added. Lysates were centrifuged at 17,000*g* for 30 min at 4 °C. Supernatants containing cytosols were separated on a 15% SDS–PAGE and probed using an anti cytochrome c polyclonal antibody as described above.

JC-1 estimation of inner transmembrane potential ($\Delta\Psi m$) in living cells

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazol carbocyanine iodide (JC-1) is a lipophilic cationic dye that enters into mitochondria and forms red fluorescent J-aggregates (~590 nm) at high membrane potential. However at a lower potential JC-1 exists as a green fluorescent monomer (~527 nm). IO and IM cells were grown on cover slips and treated with α-bisabolol for 15 min. After this incubation time, the medium was removed, and a modified medium containing 2 mg/ml JC-1 in pre-warmed DMEM was added. Cells were placed back into the incubator (37 °C, 5% CO_2, and 100% humidity) for 30 min, and then washed twice with PBS in order to remove the unbound dye. Fluorescence was analyzed using a confocal laser scanning microscope (Axioplan 2, LSM 510, Carl Zeiss, Göttingen, Germany) equipped with argon (488 nm) and helium/neon (543 nm) excitation beams.

Preparation of lipid rafts

Cells (3×10^7) were washed twice with ice-cold phosphate-buffered saline and then scraped into 0.5 M sodium carbonate, pH 11.0. To disrupt cellular membranes, homogenization was carried out sequentially with a loose fitting Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10-s bursts), and a tip sonicator (three 20-s bursts). An equal volume of 90% sucrose in Mes-buffered saline (25 mM Mes, pH 6.5, 0.15 M NaCl) was added to the homogenate and overlaid with a 5–35% discontinuous sucrose gradient (4 ml of 5% sucrose, 4 ml of 35% sucrose; both in Mes-buffered saline containing 250 mM sodium carbonate). After centrifugation at 38,000 rpm for 16–18 h in an SW40 rotor (Beckman) 1 ml gradient fractions were concentrated by precipitation with trichloroacetic acid. The pellet was dissolved in the SDS–PAGE sample buffer. After electrophoresis proteins were blotted to a PVDF membrane. Membranes were incubated with an anti-flottillin-1 monoclonal antibody and, after washing, with an anti-mouse IgG peroxidase conjugate.

Gas chromatography–mass spectroscopy (GC–MS) analysis

All experiments were performed with a model 7694E head space autosampler (Hewlett-Packard, Palo Alto, CA, USA) connected to a model 6890 gas chromatograph (Hewlett-Packard), interfaced with a model 5973 mass detector operating in the electron impact (EI) mode. The gas chromatograph was equipped with a SPB1 column (30 m × 0.32 mm i.d., 1 µm film thickness, Supelco, Bellefonte, PA, USA). Rubber septa lined with PTFE (150 °C proof) were obtained from Analytical Technology (Milan, Italy). Samples were analyzed according to Perbellini et al. [18].

Generation of wild type and mutant Bid recombinant proteins

The Bid recombinant proteins (wild type and mutant proteins) with a tag of six histidine at the N-terminus were expressed in the pET-12 b plasmid of *Escherichia coli* and purified by affinity chromatography (HiTrap chelating column, Healthcare). The proteins fractions were concentrated, aliquotated and stored at −80 °C. Mutations were performed by a Quick Change Site-Directed Mutagenesis Kit II (Stratagene). Mutation M194Q was generated using the mutagenic oligonucleotide 5′-CTTGGTTAGAAACGAGCAGGACTGAGGATCCGGCTGC-3′ containing an AT to CA bases change that was used to replace methionine 194 to glutamine. Mutation G94E-D95E was generated using the mutagenic oligonucleotide 5′-GCCAGACATCTCGCCCAAATAGAGGAGGAG ATGGACCACAACATCC-3′ containing a GC to AG and T to G bases changes that was used to replace glycine 94 to glutamic acid and aspartic acid 95 to glutamic acid. All mutations were confirmed by DNA sequencing.

Surface Plasmon Resonance analysis

Interaction of Bid recombinant proteins (wild type Bid or mutant Bid proteins) and α-bisabolol was examined by the SPR method using Biacore 2000 biosensor system (Biacore Inc., Uppsala, Sweden). Bid recombinant proteins, dissolved in a sodium acetate buffer, pH 4.5, were immobilized on the carboxymethylated sensor chip CM5 by an amine coupling reaction kit (Biacore Inc., Uppsala, Sweden) according to the manufacture's suggestion. Ethanolamine was injected to deactivate the remaining activated carboxymethylated residue on the sensor chip. Bid wild type, Bid mutant M194Q and Bid double mutant G94E-D95E were immobilized on the sensor chip. Two milliliters of α-bisabolol was mixed well with 10 ml of HBS-EP buffer (Biacore Inc., Uppsala, Sweden). After 45 h, the mixture was centrifuged at 10,000*g* for 20 min. The bottom phase was used as saturated solution of the α-bisabolol. The concentration of α-bisabolol

was calculated to be 600 μM by gas chromatography analysis. α-Bisabolol of different concentrations was prepared by addition of HBS-EP buffer to saturated solution of the α-bisabolol. Solutions of α-bisabolol (6–540 μM) were injected to flow cells containing immobilized Bid proteins. The flow cell without proteins was used as a control. Appropriate volume ranges of analyte were determined from anticipated preliminary experiments. The K_d values from SPR sensorgram were evaluated by BIA evaluation software (Biacore Inc., Uppsala, Sweden) and Marquardt method [19].

Computer modeling analysis

Because the sites of interaction of Bid with small chemical compounds like α-bisabolol are unknown, the identification of the possible binding sites was performed using two different computational approaches. Firstly, the Q-sitefinder program (http://www.bioinformatics.leeds.ac.uk/qsitefinder/) was used to identify hydrophobic pockets within the structure of Bid. Secondly, extensive simulations for docking the bisabolol to the Bid structure was performed by dividing the three-dimensional space occupied by Bid into four parts, and using a precise grid box that was displaced on those parts. The distance between the grid points was equal to 2 Å. The Autogrid v.4 [20] program was used to calculate the atomic affinity potentials for each atom type of the α-bisabolol at each grid point for each of the four positions of the box. Autodock v.4 [21] was used to carry out docking calculations on these grid boxes. Moreover, the 10 first models of the NMR structure of Bid (PDB code 2 Bid [21]) were used in order to partially take into account the molecular flexibility. During the docking simulations a particularly accurate procedure based on the Lamarckian algorithm was used. A population size of 100, a number of energy evaluations of 2.0×10^6, a generation number of 27,000, a mutation and crossover rates of 0.02 and 0.8, respectively, and 1.5 Å tolerance were considered for the cluster analysis. The number of runs was set to 100 to explore extensively the possible poses of the highest affinity, and the Solis and Wets algorithm was used to relax the best 10 of the obtained conformations. α-Bisabolol ligand was constructed by the Gaussview v.3.09 [22] program and minimized by the B3LYP method using the program Gaussian 03 [23]. The ADTOOLS program was used for the preparation of the structure of the Bid protein previous to Autodock calculations.

Intrinsic fluorescence analysis

Purified wild type and mutant Bid proteins were dissolved in 20 mM K-Hepes, 0.12 M mannitol, 0.08 M KCl, 1 mM EDTA, pH 7.4 at a final concentration of 1 μM. Changes in intrinsic fluorescence intensity were measured at 25 °C with a spectrofluorimeter using an excitation wavelength at 275 nm and a recording emission between 290 and 400 nm. α-Bisabolol dissolved in ethanol was added in a stepwise manner and fluorescence changes were measured after 2 min equilibration. Emissions were corrected for the effect of dilution and of lipids (in the absence of the protein).

Results

α-Bisabolol rapidly kills by apoptosis a number of human malignant pancreatic tumor cell lines

To ascertain the precedent results on malignant glioblastoma cell lines [1], we first examined the effect of α-bisabolol on the viability of other types of transformed cells. IO and IM cell lines were cultivated in the presence of α-bisabolol for 5 h and successively the number of live cells was counted. As shown in Fig. 2, 50% of IO and IM cell lines were killed in 2 h by this treatment (effective concentration of α-bisabolol was estimated to be 2 μM [1]). The same treatment, however, was not successful in inducing any

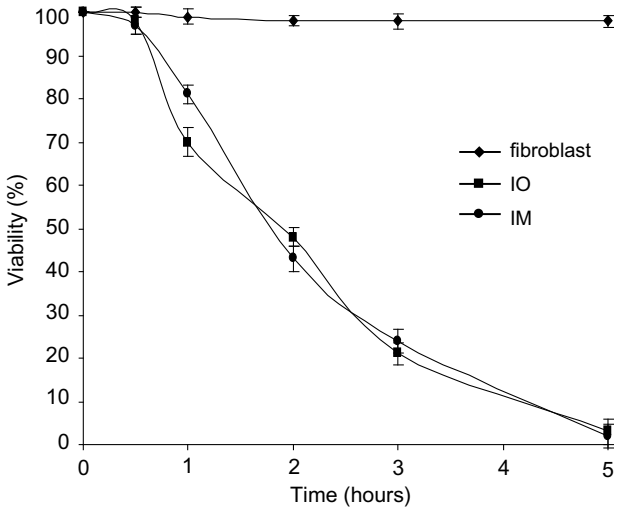

Fig. 2. Cytotoxic activity of α-bisabolol towards human malignant pancreatic carcinoma cell lines. Number of living cells after treatment was counted in duplicate wells by trypan blue assay as described under "Materials and methods". Results are the mean value (±SD) of six experiments.

change in the viability of human fibroblasts. These data, in line with our precedent results obtained in glioblastoma cell lines, confirm the rapid, efficient and highly selective action of α-bisabolol towards malignant transformed cell lines.

Induction of a specific cleavage of poly(ADP-ribose) polymerase (PARP), caspase 3 activation and DNA ladder formation observed in a short time after α-bisabolol treatment of transformed cells (Fig. 3) indicate that the death of two cell lines was mainly caused by apoptosis.

We demonstrated that α-bisabolol induces a rapid dissipation of mitochondrial membrane potential and the release of cytochrome c from mitochondria to cytoplasm (Fig. 4A and B). Furthermore, α-bisabolol fails to induce DNA ladder formation in IO and IM cell lines in the presence of 10 μM cyclosporin A, a specific inhibitor of mitochondrial permeability transition pore (mPTP) (Fig. 4C). These results indicate that the intracellular target of α-bisabolol may probably be situated on the mitochondrial membrane.

α-Bisabolol is preferentially incorporated into transformed cell lines through lipid rafts

Next, in order to explain the selective apoptosis-inducing action towards tumor cells, we first wanted to examine if α-bisabolol may preferentially be incorporated into transformed cells. As shown in Fig. 5, IO and IM cells, indeed, incorporated α-bisabolol far more than human fibroblasts did, not only in membrane fractions but also in other fractions such as cytoplasm and nuclei.

An increasing body of evidence indicates the marked difference in the plasma membrane structure and function between normal and transformed cells [7]. The presence of the so-called "lipid rafts" on plasma membranes of

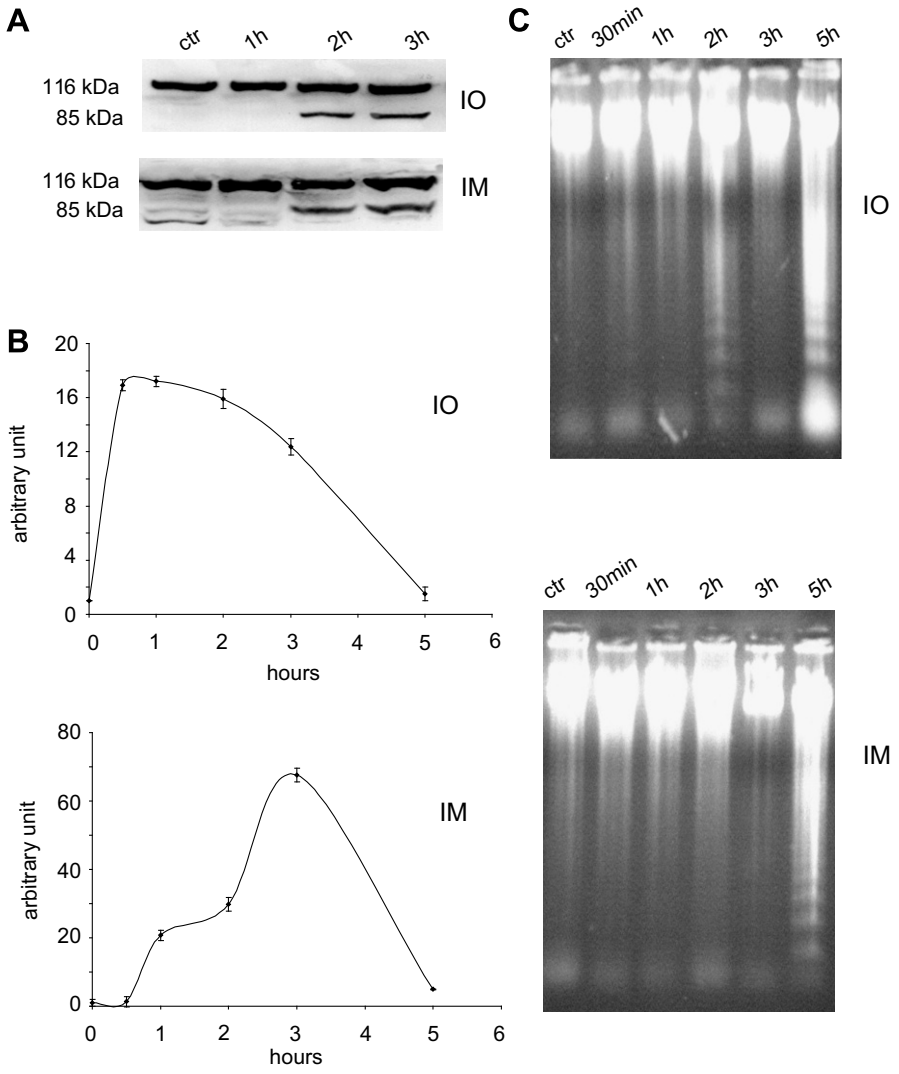

Fig. 3. Induction of apoptosis by α-bisabolol in IO/IM cell lines. (A) Western blot analysis of PARP after treatment with α-bisabolol. Ctr, cells without treatment with α-bisabolol. One hundred and sixteen and eighty-five kilo Dalton indicate, respectively, the position of native PARP and PARP fragment obtained after specific cleavage by caspase 3. (B) Caspase 3 activity after treatment with α-bisabolol. Enzymatic assay was performed as described under "Materials and methods". Results are the mean values (±SD) of six experiments. (C) Agarose gel electrophoresis of DNA after treatment with α-bisabolol. Ctr, cells without treatment with α-bisabolol.

transformed cells highlights this difference. To examine if α-bisabolol may indeed be adsorbed to transformed cells through lipid rafts regions, IO and IM cell lines were treated at different times up to 30 min with α-bisabolol and its distribution was analyzed after sucrose gradient centrifugation of cell extracts by measuring the amounts of α-bisabolol with the GC–MS method in various fractions. As shown in Fig. 6, in IO cells α-bisabolol was present only in those fractions in which flottillin, one of the specific molecular markers of the lipid rafts, is present indicating that α-bisabolol is preferentially and rapidly adsorbed into the plasma membranes of the transformed cells through lipid rafts or membrane regions rich in these structures. The same results were obtained in IM cells (data not shown). Scarce presence of lipid rafts on normal cell membranes [7] and scarce incorporation of α-bisabolol by fibroblasts (Fig. 5) are compatible with its selective presence in lipid rafts-rich fractions of IO/IM cells.

α-Bisabolol directly interacts with Bid protein

We then analyzed Bid as a possible molecular target of α-bisabolol. This member of the pro-apoptotic Bcl-2 family proteins acts at the mitochondrial level in the pro-apoptotic pathway. Recent reports described the capacity of Bid protein to transport and exchange lipids between membranes (in particular, lysolipids and cardiolipin) and to interact either directly with mitochondria or with other pro-apoptotic Bcl-2 proteins (i.e. Bak and Bax) [24]. Furthermore, upon treatment with edelfosine, a synthetic alkyl-lysophospholipid with selective apoptotic action towards tumor cells, Bid is recruited into lipid rafts,

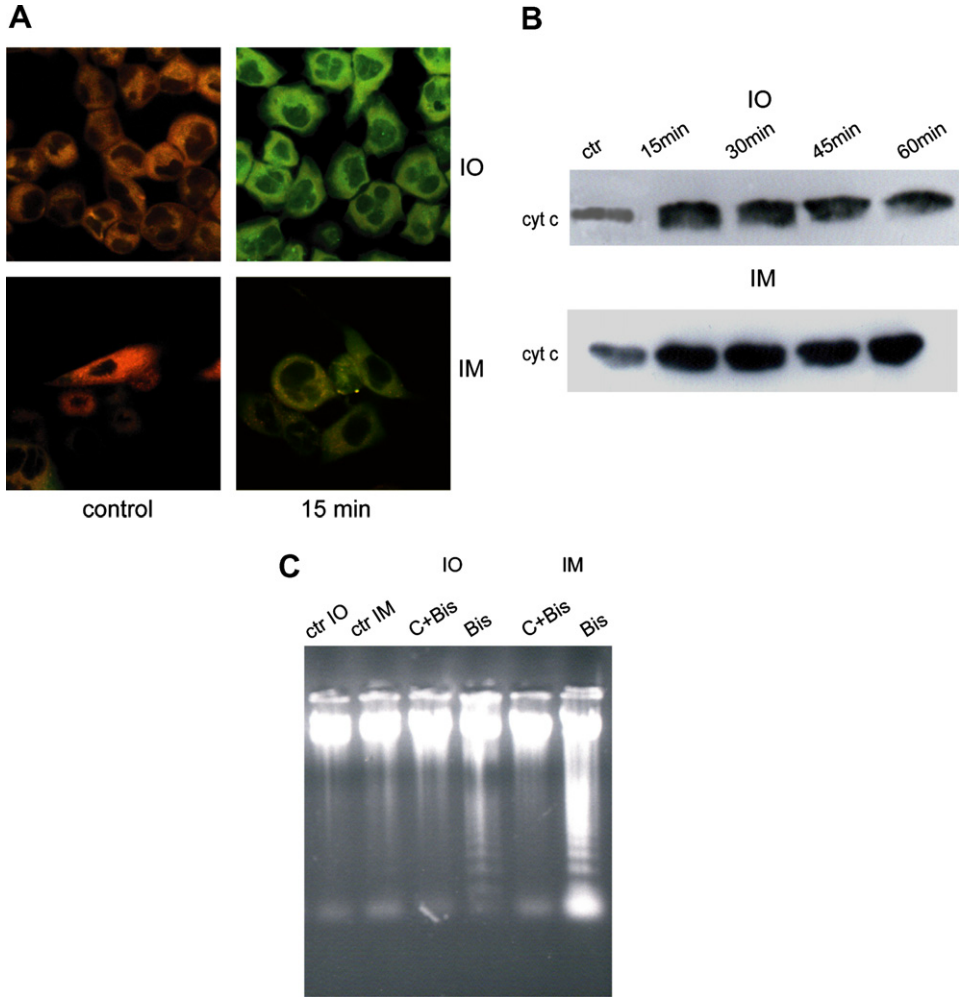

Fig. 4. Change in mitochondrial function in IO/IM cells induced by α-bisabolol. (A) Mitochondrial membrane potential before (control) and 15 min after treatment with α-bisabolol. Cells were evidenced by JC-1 coloration, performed as described under "Materials and methods". (B) Western blot analysis of cytochrome c in cytoplasmic fractions after treatment with α-bisabolol. Ctr, cells without treatment with α-bisabolol. (C) Agarose gel electrophoresis of DNA after treatment with α-bisabolol in the presence of cyclosporin A. Ctr, cells without treatment with α-bisabolol. Bis, treatment with α-bisabolol for 3 h. C + Bis, treatment with α-bisabolol for 3 h in the presence of 10 μM cyclosporin A. All images are the representative ones of at least six independent analysis.

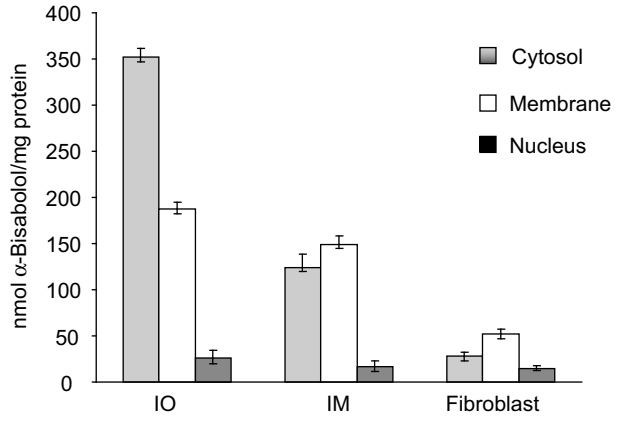

Fig. 5. Concentration of α-bisabolol in human fibroblasts and IO/IM cell lines after 30 min treatment. α-Bisabolol was measured in cytoplasmic, membrane and nuclear fractions with GC–MS method as described under "Materials and methods". Data are presented with ±SD of six independent measurements.

playing a critical role in the formation of the death-inducing signaling complex [25]. On the basis of these notions, we hypothesized that Bid protein may be a direct molecular target of α-bisabolol incorporated to lipid rafts.

To get a hint on the validity of this hypothesis, we first performed Western blot analysis of Bid protein and flottillin in the cell fractions collected after sucrose gradient centrifugation before and after treatment with α-bisabolol. As shown in supplementary figure, in non-treated IO cell Bid is widely present in low density fractions. After treatment with α-bisabolol Bid is present only in the fraction corresponding to flottillin-enriched fraction, indicating that α-bisabolol treatment induced a movement of Bid to lipid rafts-rich membrane regions. Thereafter, we performed an SPR analysis to examine if α-bisabolol directly interacts with wild type Bid protein immobilized on a chip. As shown in Fig. 7, sensorgrams clearly indicated that α-bisabolol interact directly with Bid. Estimated K_d value

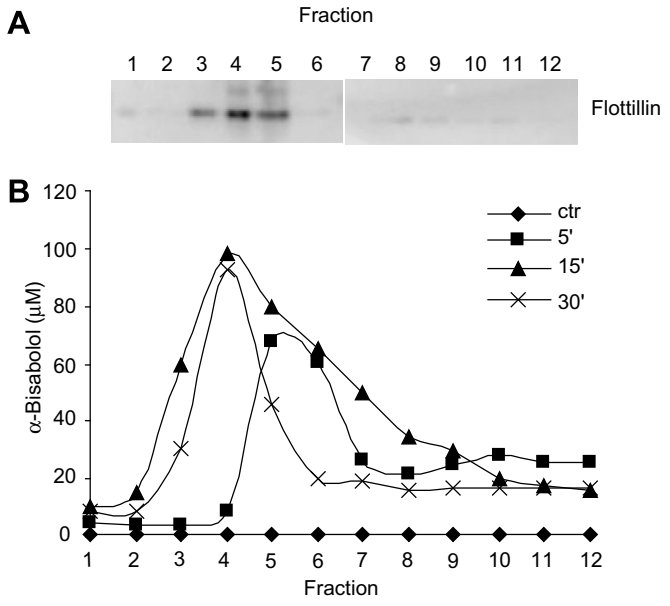

Fig. 6. Absorption of α-bisabolol on lipid rafts. (A) Western blot analysis of fractions obtained after sucrose gradient centrifugation of IO cell extract. Sucrose gradient centrifugation and Western blot analysis with anti-flottillin antibody were performed as described under "Materials and methods". The same pattern was also observed in α-bisabolol-treated cells. (B) Concentration of α-bisabolol in fractioned extract obtained from IO cells after treatment with α-bisabolol (5, 15 and 30 min) was estimated as described under "Materials and methods". Ctr, cells without treatment with α-bisabolol.

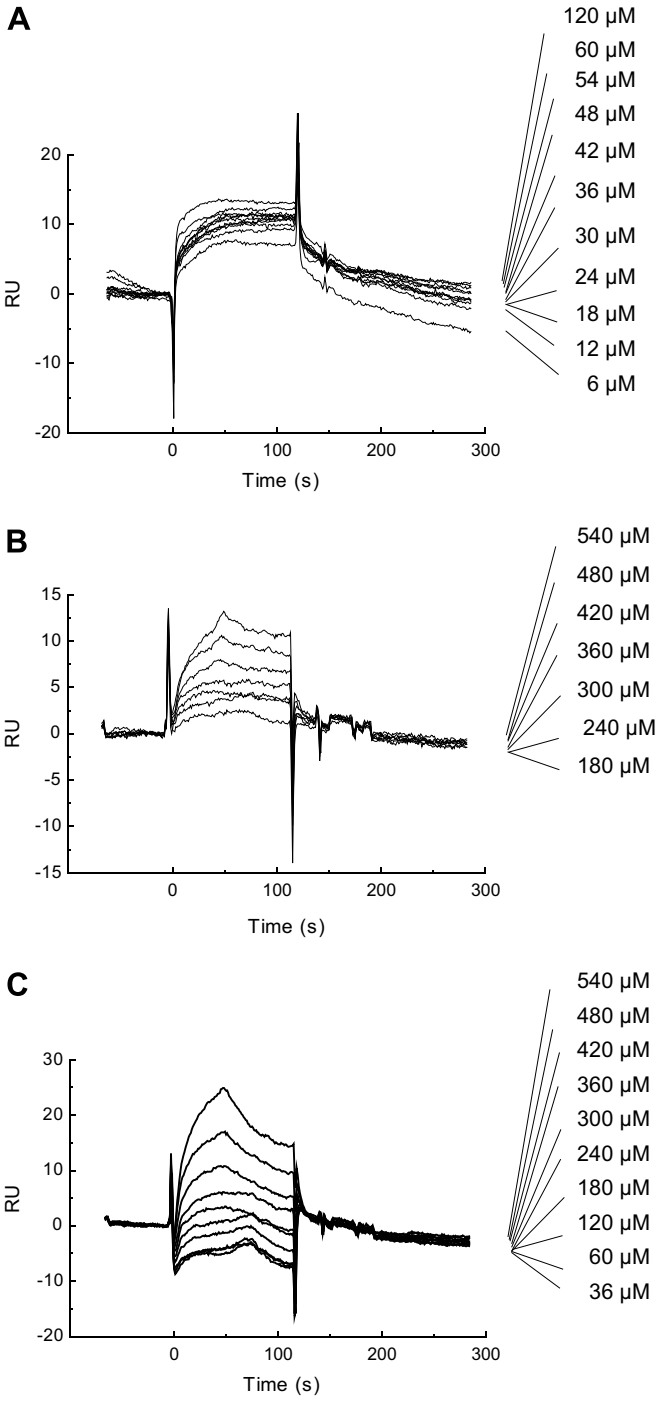

Fig. 7. Interaction between α-bisabolol and Bid wild type and mutants analyzed with SPR. Concentrations of α-bisabolol used in the experiments were 6–120 μM for wild type Bid, 180–540 μM for Bid M194Q mutant and 36–540 μM for Bid double mutant. SPR analysis was performed as described under "Materials and methods". Solutions of α-bisabolol were injected to sensor chip with immobilized wild type Bid (A), Bid M194Q mutant (B) and Bid double mutant G94E-D95E (C). The flow rate and injection volume of each experiment were 30 μl/min and 60 μl, respectively.

(2.24 ± 0.1 μM) further indicates that interaction between these molecules should be specific. Considering the preferential absorption of α-bisabolol into lipid rafts, these data suggest the possibility that Bid could indeed interact with α-bisabolol which should be present at relatively high concentrations in lipid rafts.

To shed light on the α-bisabolol action mechanism, the direct interaction between the α-bisabolol and Bid was analyzed by computational means. Using two different methods to detect the binding sites on proteins, four major putative sites were identified. Interestingly, both Autodock and Qsitefinder predict the same binding site (binding site 1) as the most favorable one. Based on the Autodock scoring function the binding sites were ranked as 1–4, and the results are presented in Table 1.

Based on the volume and the shape of the α-bisabolol most of the other binding sites found by Qsitefinder were discarded. However, with regard to the results obtained by blind dockings, Qsitefinder ranked the binding sites from most probable to least probable as being 1, 4, and 3 (according to the ranking order of Autodock), and did not identify site 2. An inhibition constant as low as 2 μM indicates that α-bisabolol should interact directly with Bid which is consistent with the SPR experiments. It may also be noted that the mutant M194Q, in which the mutation is located within binding site 1, is not able to interact with α-bisabolol. Binding site 1 seems therefore the most reasonable candidate for α-bisabolol–Bid interaction. This binding site is located quite deep within the protein core formed by the helices (Fig. 8A) and the ligand in this crevice is totally

Table 1
Ranking the putative binding sites of α-bisabolol on the molecular surface of Bid

Site	ΔG (kcal/mol)	K_i (μM)	Cluster population
1	−7.81	1.89	40
2	−6.5	16	3
3	−6.04	37	45e
4	−5.52	90	61

ΔG (kcal/mol) is the binding free energy as determined by the Autodock v.4 scoring function, K_i is the inhibition constant calculated from ΔG provided also by Autodock v.4; the last column represents the cluster population (the number of ligand conformations in this cluster with the RMS tolerance of 2 Å).

protected from the surrounding environment (Fig. 8B). On the contrary in the three other probable sites the ligand remains partially exposed to the solvent (not shown). The residues that constitutes the binding site 1 are two leucines (Leu 189 and Leu 154), one phenylalanine (Phe 24), two sulfur containing residues (Cys 28 and Met 194), two lysines (Lys 157 and Lys 158) and one alanine (Ala 91). As expected from the hydrophobic nature of α-bisabolol, most of the interactions between this ligand and Bid are predominantly Van der Waals interactions. The two unique polar residues of this site, Lys 158 and Lys 157, do not interact with the ligand through polar interaction, but the methyl groups of their side chain appear to have strong hydrophobic contacts with the ligand. Finally, only one polar contact between the ligand and the protein can be observed in the best orientation provided by our docking experiment and corresponds to a hydrogen bond between the hydroxyl group of the bisabolol and the backbone carbonyl group of Ala 91.

Based on the information provided by docking simulations on the putative docking site of α-bisabolol on Bid protein we produced two mutated Bid proteins in order to further examine their capacity to interact with α-bisabolol either by SPR analysis or by fluorescence assay. In the M194Q (Fig. 8A) mutant in which the mutation is located in the putative binding site, methionine 194 is part of a hydrophobic groove present on the surface of Bid that has been shown to be important for the Bid function [26]. The second mutant, with a double mutation G94E/D95E located in the region of the BH3 helix, was selected to study the role played by the functional BH3 domain in the interaction with α-bisabolol. Based on the computer modeling, this region seems not to be involved directly in the binding of this compound. SPR analysis indicated that this double mutant maintains its ability to directly interact with α-bisabolol as predicted, even if with less affinity, whereas the other Bid mutant M194Q fails in it (Fig. 7).

To evaluate further ability of Bid to bind α-bisabolol we have utilized another direct approach based on the measurement of changes in the intrinsic fluorescence of Bid. Mammalian Bid contains a single tryptophan residue, the fluorescence of which dominates the intrinsic fluorescence spectrum of the protein. As in the case of lysolipids, the binding to selected molecules changed the whole spectrum of fluorescence emission of Bid (with excitation at 270 nm) indicative of altered solvent exposure of aromatic residues in the protein. Interestingly, the incubation of Bid protein with α-bisabolol induced a decrease in tryptophan fluorescence emission, indicating a change in the environment of the aromatic residues, while non-ionic detergents like Tween or octylglucoside (not shown) had negligible effects (Fig. 9). When α-bisabolol was added to the M194Q Bid

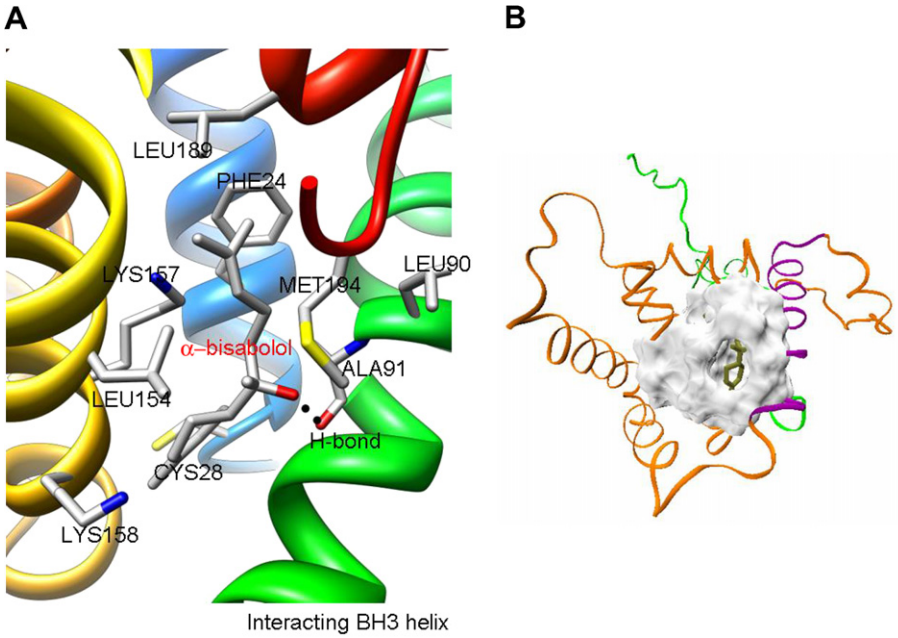

Fig. 8. Computer modeling analysis of interaction between α-bisabolol and Bid protein. (A) Interactions of α-bisabolol within site 1 of Bid; (B) α-bisabolol within site 1 forming a deep cavity.

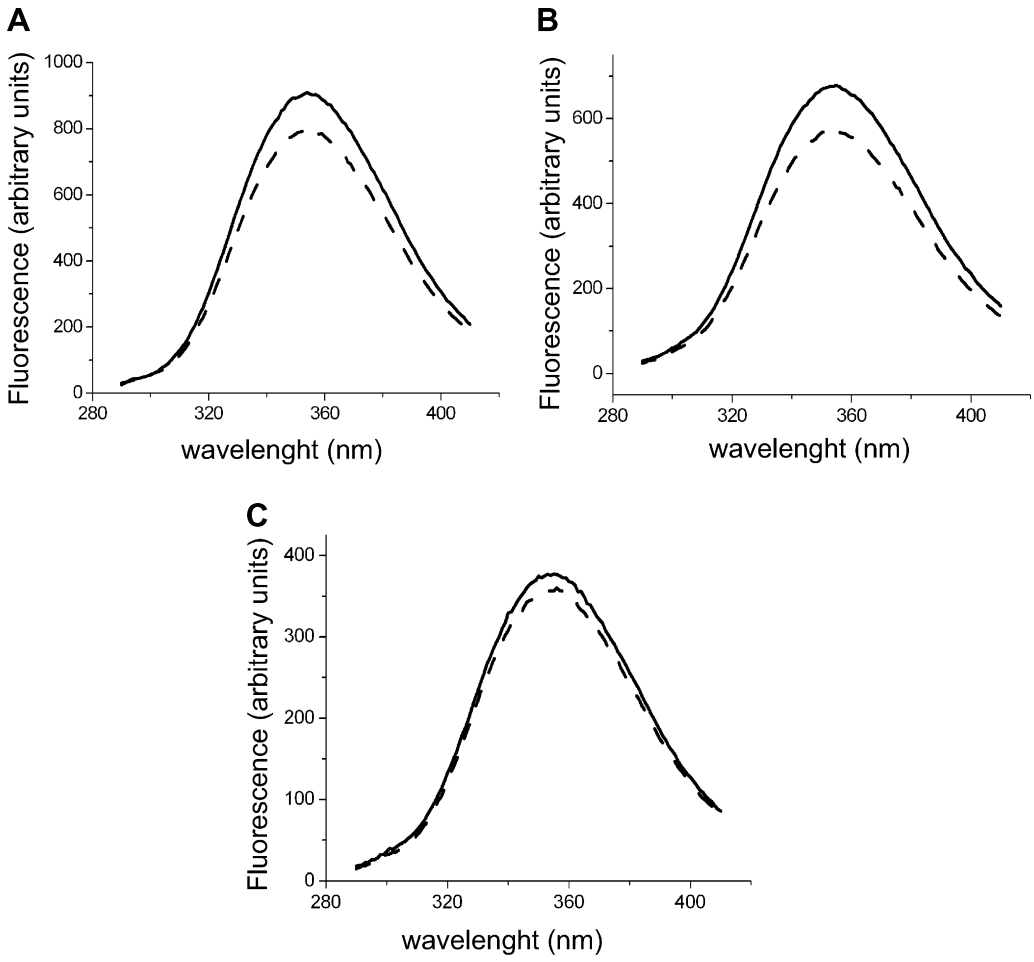

Fig. 9. Changes of intrinsic fluorescence emission of recombinant Bid proteins induced by α-bisabolol. Intrinsic fluorescence of 1 μM recombinant Bid wild type (A), mutant G94E-D95E (B) and mutant M194Q (C) in the absence (solid spectrum) and in the presence (dotted spectrum) of 4 μM α-bisabolol. Measurements were carried out as described under "Materials and methods".

mutant it did not induce changes in the fluorescence emission spectra indicating that this mutant was unable to interact with α-bisabolol at the concentrations utilized in the experiment (Fig. 9), whereas, α-bisabolol induced changes in the fluorescence emission spectra of the double mutant. Therefore, this result, in line with results obtained by SPR analysis, indirectly confirmed that changes observed in the wild type and in the double mutant proteins are specifically induced by the interaction with α-bisabolol at a binding site probably located near to the mutated methionine residue.

Discussion

The present study extends the previous notion on the selective apoptotic action of α-bisabolol towards malignant human and murine glioblastoma cells [5]. Indeed, α-bisabolol rapidly kills, via a mitochondrial pathway, human transformed cells derived from pancreatic carcinoma considered to be one of the most malignant tumors (Figs. 2–4). Furthermore, the ability of α-bisabolol to annihilate glioblastoma cells [5] or pancreatic carcinoma cells (data not shown) represents a very important aspect of the α-bisabolol action considering that few surviving tumor cells after chemotherapy often acquire a more virulent character creating severe problems of chemio-resistance. These data, together with other data showing its action towards many other human malignant carcinoma-derived cell lines [27], suggest that cytotoxic action of α-bisabolol may not be restricted to a narrow spectrum of tumor cell species. Less cytotoxicity of α-bisabolol towards human fibroblasts also confirms our previous report on human and murine astrocytes [5]. These features may be relevant in the future development of α-bisabolol-related compounds to be used in chemotherapy.

The present study further shows that α-bisabolol is preferentially absorbed into malignant cell lines (Fig. 5), indicating that the structural feature of plasma membranes in normal cells is different from that of tumor cells. As shown in Fig. 6, upon contact with tumor cells α-bisabolol was quickly absorbed in a highly specialized region of membranes, i.e. lipid rafts that could represent the docking region of α-bisabolol on plasma membranes of tumor cells. Lipid rafts play an important role in intracellular protein transport and membrane fusion [7]. Many structural and

functional aspects of lipids rafts represent one of the remarkable features of the tumor cells development. On the other hand, lipid rafts may be involved also in tumor cell apoptosis. Indeed, a recent report shows that alkyl-lysophospholipids induced recruitment of Fas/CD95 death receptor and procaspase-8 into lipid rafts, leading to apoptosis. TNF-related apoptosis-inducing ligand receptor-1/death receptor 4 (TRAIL-R1/DR4) and TRAIL-R2/DR5, as well as Bid, are also recruited into lipid rafts, playing a critical role in the formation of the death-inducing signaling complex [26]. A recent report further describes the involvement of Bid in lipid and vesicle trafficking from plasma membranes to mitochondria in the initial phase of cell death [14–17]. The present study demonstrating that α-bisabolol induces a movement of Bid protein to lipid raft regions (supplementary figure) indicates the possible functional link between α-bisabolol and lipid rafts in the rapid apoptotic action of α-bisabolol.

Furthermore, at the structural level, our computer modeling studies highlight the most probable site of interaction of Bid with α-bisabolol. This site, laying deep in the core of the protein, displays strong hydrophobic complementarities between the ligand and Bid. This result is quite interesting because both the hydrophobic nature of the ligand but also its apparent strong solubility in the lipidic layer is a key requirement for the molecular mechanism of α-bisabolol.

Moreover, based on the characterization of this site, the directed mutagenesis and SPR experiments indicate that wild type Bid directly interacts with α-bisabolol and the mutant M194Q, designed to modify the structure of a putative binding site, looses its ability to interact with α-bisabolol. This mutant also displays a reduced binding of lysophosphatidylcholine as indicated by intrinsic fluorescent measurement (data not shown) in line with a partial overlapping of the binding sites for these molecules. Furthermore, the double mutant G94E-D95E, designed to modify the functional BH3 region far from the putative binding site, maintains its ability to interact even if with less affinity (Fig. 6). These two mutated residues that are near binding site 1 are outside the binding cavity, so they do not importantly interfere with the α-bisabolol binding. It is possible to speculate that α-bisabolol, once incorporated into lipid rafts, could directly interact with Bid protein at the site predicted by the computer modeling analysis (Fig. 7) and successively be transported to mitochondrial membranes. The role played by the interaction between α-bisabolol and Bid in inducing apoptosis through the mitochondrial pathway remains to be elucidated. In this context we are going to clarify if α-bisabolol is directly targeted to mitochondria or if Bid activation is induced by α-bisabolol binding.

In conclusion, α-bisabolol, a well-known but poorly studied sesquiterpene alcohol, is able to rapidly, efficiently and selectively induce apoptosis in malignant tumor cells by targeting lipid rafts on cell membranes. Thereafter, α-bisabolol could interact with Bid protein recruited in lipid rafts region after α-bisabolol treatment, which may be involved in the transduction pathway from plasma membranes to intracellular compartments including mitochondria. Absence of toxicity towards normal cells or in animals (data not shown) may represent a powerful feature useful in the development of the clinical trial. Furthermore, structure/function correlation studies, which are currently under way in our labs, will be of fundamental importance for the development of safe and efficient anti-tumor drugs aimed not only to be used in the prevention of tumor growth but also to treat patients bearing tumors either alone or in combination with other anti-tumor drugs. Who knows if the sweet dream brought on by chamomille flowers for hundred/thousands of years is bringing nice news to those millions of suffering people with tumors?

Acknowledgments

Authors thank technical assistance of Francesco Pasini for GC–MS analysis. SPR was partly performed at Tokyo University of Science (Chiba, Japan) with a kind help of Prof. Tanuma. The manuscript was carefully proofed by Michelle Strauss. The present work was financially supported by Cariverona Foundation (Cariverona project 2003 to H.S.) and 60% (to H.S. and M.C.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2008.02.004.

References

[1] Y.D. Burke, Lipids 32 (1997) 151–156.
[2] J.H. Joo, G. Liao, J.B. Collins, S.F. Grissom, A.M. Jetten, Cancer Res. 67 (2007) 7929–7936.
[3] I. Adany, Cancer Lett. 79 (1994) 175–179.
[4] A. Rioja, FEBS Lett. 467 (2000) 291–295.
[5] E. Cavalieri, S. Mariotto, C. Fabrizi, A. Carcereri de Prati, R. Gottardo, S. Leone, L.V. Berra, G.M. Lauro, A.R. Ciampa, H. Suzuki, Biochem. Biophys. Res. Commun. 315 (2004) 589–594.
[6] S. Budavari (Ed.), The Merk INDEX, twelfth ed., 1996, p. 208.
[7] S.K. Patra, S. Bettuzzi, Oncol. Rep. 17 (2007) 1279–1290.
[8] Y.T. Hsu, R.J. Youle, J. Biol. Chem. 272 (1997) 13829–13834.
[9] Y.T. Hsu, R.J. Youle, J. Biol. Chem. 273 (1998) 10777–10783.
[10] B. Antonsson, S. Montessuit, S. Lauper, R. Eskes, J.C. Martinou, Biochem. J. 345 (2000) 271–278.
[11] B. Antonsson, S. Montessuit, B. Sanchez, J.C. Martinou, Biol. Chem. 276 (2001) 11615–11623.
[12] M. Suzuki, R.J. Youle, N. Tjandra, Cell 103 (2000) 645–654.
[13] F. Sandra, M. Degli Esposti, M. Magnus, D. Knight, K. Ndebele, R. Khosravi-Far, Cancer Res. 65 (2005) 1–12.
[14] M. Degli Esposti, J.T. Erler, J.A. Hickman, C. Dive, Mol. Cell. Biol. 21 (2001) 7268–7276.
[15] M.R. Rippo, F. Malisan, L. Ravagnan, B. Tomassini, I. Condo, P. Costantini, S.A. Susin, A. Rufini, M. Todaro, G. Kroemer, R. Testi, FASEB J. 14 (2000) 2047–2054.
[16] W.J. van Blitterswijk, A.H. van der Luit, R.J. Veldman, M. Verheij, J. Borst, Biochem. J. 369 (2003) 199–211.

[17] S. Ouasti, P. Matarrese, R. Paddon, R. Khosravi-Far, M. Sorice, A. Tinari, W. Malorni, M. Degli Esposti, Cell Death Differ. 14 (2007) 453–461.
[18] L. Perbellini, R. Gottardo, A. Caprini, F. Bortolotti, S. Mariotto, F. Tagliaro, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 812 (2004) 373–377.
[19] D.W. Marquardt, J. Soc. Indust. Appl. Math. 11 (1963) 431.
[20] G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, J. Comput. Chem. 19 (1998) 1639–1662.
[21] J.J. Chou, H. Li, G.S. Salvesen, J. Yuan, G. Wagner, Cell 96 (1999) 615–624.
[22] R. Dennington II, T. Keith, J. Millam, K. Eppinnett, W.L. Hovell, R. Gilliland, GaussView, Version 3.09, Semichem, Inc., Shawnee Mission, KS, 2003.
[23] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, J.A. Montgomery Jr., T. Vreven, K.N. Kudin, J.C. Burant, J.M. Millam, S.S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G.A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.E. Knox, H.P. Hratchian, J.B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R.E. Stratmann, O. Yazyev, A.J. Austin, R. Cammi, C. Pomelli, J.W. Ochterski, P.Y. Ayala, K. Morokuma, G.A. Voth, P. Salvador, J.J. Dannenberg, V.G. Zakrzewski, S. Dapprich, A.D. Daniels, M.C. Strain, O. Farkas, D.K. Malick, A.D. Rabuck, K. Raghavachari, J.B. Foresman, J.V. Ortiz, Q. Cui, A.G. Baboul, S. Clifford, J. Cioslowski, B.B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R.L. Martin, D.J. Fox, T. Keith, M.A. Al-Laham, C.Y. Peng, A. Nanayakkara, M. Challacombe, P.M.W. Gill, B. Johnson, W. Chen, M.W. Wong, C. Gonzalez, A. Pople, Gaussian 03, Revision C.02, Gaussian, Inc., Wallingford, CT, 2004.
[24] S.J. Korsmeyer, M.C. Wei, M. Saito, S. Weiler, K.J. Oh, P.H. Schlesinger, Cell Death Differ. 7 (2000) 1166–1173.
[25] C. Gajate, F. Mollinedo, Blood 109 (2007) 711–719.
[26] B. Becattini, S. Sareth, D. Zhai, K.J. Crowell, M. Leone, J.C. Reed, M. Pellecchia, Chem. Biol. 11 (2004) 1107–1117.
[27] E. Darra, G. Lenaz, E. Cavalieri, R. Fato, S. Mariotto, C. Bergamini, A. Carcereri de Prati, L. Perbellini, S. Leoni and H. Suzuki, Ital. J. Biochem., in press.