Internalization of the anti-carcinogenic IBB1, a major Bowman-Birk isoinhibitor from soybean (G*lycine max*), in HT29 colon cancer cells

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HIGHLIGHTS

- IBB1, a major serine protease isoinhibitor of the Bowman-Birk family from soybean, exerts an anti-proliferative effect on HT29 colon cancer cells in a dose-dependent manner.
- IBB1 crosses the membrane of HT29 colon cancer cells over a time-course of 1 h, being localized in cytoplasm.

SUMMARY

Protease inhibitors of the Bowman-Birk type, a major protease inhibitor family in legume seeds, which inhibit potently trypsin- and chymotrypsin-like proteases, are currently being investigated as colorectal chemopreventive agents. Although the therapeutic target/s and the action mechanism/s of Bowman-Birk inhibitors (BBI) have not yet been elucidated, the emerging evidence suggests that BBI exert their chemopreventive properties *via* protease inhibition; in this sense, serine proteases should be considered as primary targets in early stages of carcinogenesis. In this work, we have demonstrated that IBB1, a major protease inhibitor of the Bowman-Birk family in soybean (*Glycine max*), exerts anti-proliferative effect in human colorectal HT29 cancer cells at concentrations higher than 15 µM, in a dose dependent manner. By using confocal microscopy, we have demonstrated that IBB1 is taken up by HT29 colon cancer cells in a time-dependent manner, being the bulk of the internalized protease inhibitor localized in the cytoplasm where might interact with their potential therapeutic target/s.

INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of cancer-related mortality worldwide, with over 1.2 million new cases diagnosed globally per year [1]. In recent years, substantial evidence has pointed to the link between dietary patterns and lifestyle in primary prevention and control of CRC. Within this framework, there is a growing interest in naturally-occurring serine proteases of the Bowman-Birk family due to their potential chemopreventive and/or therapeutic properties which can impact positively in pathological disorders, including cancer, muscle atrophy and neurodegenerative diseases [2]. In particular, several studies suggest that

dietary BBI from different legume sources are effective at preventing or suppressing radiation- and chemical carcinogen-induced transformation *in vitro*, as well as carcinogenic and associated inflammatory disorders within the mammalian gastrointestinal tract [3, 4]. Soybean BBI exerted a protective role in dimethylhydrazine (DMH)-treated rodents when ingested at low concentrations (10 mg/100g diet), decreasing the frequency and incidence rates of colorectal tumours [5]. In patients with active ulcerative colitis, intake of BBIC -a protein extract of soybean enriched in BBI- was associated with a clinical response and induction of disease remission, as assessed by the Sutherland Disease Activity Index [6]. By using colon cancer cells as models, we are currently investigating the action mechanism/s by which BBI might exert a chemoprotective effect in early stages of colorectal carcinogenesis. A significant concentration- and time-dependent decrease in the proliferation of colon cancer cells, following treatment with BBI from several legume sources, including pea (*Pisum sativum*) [7], lentil (*Lens culinaris*) [8] and soybean [9] has been reported; neither BBI affected the growth of non-malignant colonic fibroblastic CCD18-Co cells. We demonstrated that such suppressive effect on growth of colon cancer cells was related to their intrinsic ability to inhibit serine proteases [10]. In order to gain insight about the action mechanism of BBI as colorectal chemopreventive agents, the aim of this study was to determine if IBB1, a major protease isoinhibitor from soybean, is internalized by HT29 colorectal adenocarcinoma cells facilitating the inhibition of intracellular target proteases.

MATERIALS AND METHODS

Materials

BBI from soybean, trypsin (type III) from bovine pancreas, N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), N-benzoyl-L-tyrosine ethyl ester (BTEE), high-glucose Dulbecco's modified Eagle's medium (DMEM), neutral red (NR) and additional high-grade chemicals for cell culture were obtained from Sigma. The Cy5 fluorochrome was purchased from GE Healthcare. The human colorectal adenocarcinoma HT29 cell line was supplied by the Cell Bank of the Scientific Instrumentation Centre at the University of Granada (CIC-UGR). Culture flaks and flat bottom ninety-six-well microtitre plates were purchased from Corning Costar and Nunc, respectively. All other chemicals were of analytical grade.

Isolation of soybean BBI isoinhibitors

IBB1, a major BBI isoinhibitor from soybean, was purified from commercial soybean BBI consisting in a mixture of IBB1 and IBBD2 [9]. The mixture was fractionated on a MonoS 5/50 GL cation exchange column (GE Healthcare), connected to an AKTA FPLC system (GE Healthcare), using a linear gradient of 0-0.22 M NaCl in 25 mM sodium acetate buffer, pH 4.4, at a flow rate of 1 ml/min. The elution was monitored at 280 nm and 0.5 ml fractions were collected. Measurements of trypsin inhibitory activity (TIA) of eluted samples were carried out in flat-bottom microtitre plates by using BAPNA as specific substrate, and assay products measured at OD_{405nm} as previously described [11]. The unbound sample, containing both trypsin and chymotrypsin inhibitory activity –measured by using BTEE as specific substrate [12]-, was dialysed extensively against distilled water and freeze-dried until use.

Identification of IBB1 by peptide mass fingerprinting

The unbound freeze-dried sample (10 µg) was dissolved in NuPAGE® lithium dodecyl sulphate sample buffer (Invitrogen) and separated by electrophoresis on Novex 12% Bis-Tris pre-cast gels using NuPAGE® 2-N-morpholine-ethane sulphonic acid (NuPAGE, MES, Invitrogen) as running buffer. Immediately before use, the sample was reduced with dithiothreitol (DTT) and NuPAGE antioxidant added to the upper buffer chamber to prevent reduced proteins from re-oxidation during electrophoresis. The electrophoretic band was excised from Colloidal Blue

(Invitrogen)-stained gels and subjected to in-gel trypsin digestion. Peptide fragments from digested proteins were desalted and concentrated using C-18 ZipTip columns (Millipore) and then directly loaded onto the matrix-assisted laser desorption/ionization (MALDI) plate, using α-cyanohydroxycinnamic acid as the matrix for MALDI-MS analysis. MS spectra were obtained automatically in a 4700 Proteomics Analyzer (Applied Biosystems) operating in reflecton mode with delayed extraction. Peptide mass data were used for protein identification against the MS protein sequence database (www.matrixscience.com).

Fluorescent covalent labeling of IBB1 protein

IBB1 was covalently labeled with the Cy5 fluorochrome using the minimal labeling protocol according to manufacturer´s instructions. This method ensures that only a single lysine residue per protein molecule is labeled, avoiding a major effect on its protease inhibitory activity. Briefly, 200 µg of freeze-dried IBB1 was dissolved in 200 µl of lysis buffer (30 mM Tris-HCl, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5), and mixed with 1,600 pmol of dye and leave on ice for 30 min in the dark. The reaction was stopped by adding 4 µl of 10 mM lysine followed by incubation for 10 min at 4 °C on the dark. Samples were then centrifuged through an Amicon® Ultra-0.5 3K device (Millipore) at 14,000*g* for 30 min. The concentrated Cy5-labeled IBB1 protein (~50 µl) was diluted in ultrapure H_2O to a final volume of 500 µl and then centrifuged again as above. This step was repeated three times and the final retentate was transferred to a microcentrifuge tube and stored at −80 °C in the dark until use. To check the quality of labeling, 1 µg of Cy5-labeled IBB1 protein was electrophoresed by SDS-PAGE according to standard procedures and visualized in a Pharos Molecular Imager (Bio-Rad) by exciting at 635 nm. The specificity of the fluorescence signal was confirmed by scanning the same gel at 532 nm.

Western blotting

A polyclonal Ab was generated by immunizing a healthy rabbit using purified IBB1 as antigen. The immunization and antiserum collection was carried out by Biomedal SL. The polyclonal anti-IBB1 Ab was affinity-purified by using a protein-A column (BioRad) following standard protocols. In order to evaluate its specificity, equals amounts of IBB1, IBBD2 and Kunitz inhibitor from soybean were separated by SDS-PAGE and electrotransferred to a PVDF membrane using a Trans-Blot® Turbo™ Transfer System (BioRad). When the transfer was completed, the nonspecific binding sites were blocked by immersing the membrane in 1% (w/v) BSA in Tris-buffered saline (TBS), 0.3% (v/v) Tween 20 (TBST) at 4 °C for 2 h. The membrane was washed with TBST and then incubated using the rabbit polyclonal anti-IBB1 Ab (diluted 1:1000) at 4 °C overnight. After three washes with TBST, the membrane was incubated with a secondary goat anti-rabbit IgG Alexa Fluor 488-conjugated Ab (diluted 1:2500) for 2 h at 4 °C in the dark. The image was visualized in a Pharos Molecular Imager (Bio-Rad).

Cell viability assays

Human colorectal adenocarcinoma HT29 cells were maintained by serial passage in 75 cm^2 plastic culture flasks. HT29 cells were cultured in DMEM, supplemented with 5% (v/v) fetal bovine serum, 2 mM glutamine and 1% (v/v) antibiotic-antimicotic solution (Sigma), all at final concentration. Optimal assay conditions for colonic cells were reported previously [7]. Briefly, ninety-six-well microtitre plates were inoculated at a density of 2,000 HT29 cells *per* well in 200 µl of growth media. Plates were incubated under 5% CO_2 in humidified air for 24 h to allow the cells to adhere to the wells. Purified IBB1 was dissolved in growth media at a range of concentrations (15-93 µM) and added to the cells under sterile conditions. Control cells received no IBB1. At the end of the growth period (96 h), the viability of HT29 cells was assessed by the NR (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) cytotoxicity assay, based on the ability of viable uninjured cells to incorporated and actively bind NR, a supravital dye, into lysosomes. Cells were stained with NR solution (2 h at 37 °C), followed by cell fixation [0.5% (v/v) formaldehyde, 0.1% (w/v) $CaCl_2$ for 30 s] at room temperature. Plates

were washed by two brief immersions in PBS (0.01 M-sodium phosphate buffer, 0.15 M NaCl) and the dye extracted from the viable cells using an acidified ethanol solution [50% (v/v) ethanol, 1% (v/v) acetic acid] at 4 °C overnight. The absorbance of the solubilized dye was quantified at 550 nm using a BioRad Model 550 microplate reader (BioRad). Cell viability data, expressed as a percentage of the values determined for control cells grown in the absence of IBB1, were obtained from at least three independent experiments, having each at least three technical replicates. The concentration of IBB1 that reduced cell viability by 50% (IC_{50}), as compared with untreated controls, was calculated by non-linear regression fit using the GraFit software (Erithacus Software Ltd.). The data were analysed statistically by the Bonferroni's test to compare means and statistical significance was set at $P<0.05$.

Time-lapse confocal laser scanning microscopy

To study the internalization of IBB1 into human colorectal adenocarcinoma HT29 cells, we carried out time-lapse confocal laser scanning microscopy (CLSM) experiments in triplicate. For this purpose, HT29 cells were grown *in vitro* as described above. A 1µ-slide microscopy chamber (Ibidi) was inoculated at a density of 25,000 HT29 cells per well in 700 µl of growth media. Microscopy chambers were incubated overnight under 5% CO_2 in humidified air to allow the cells to adhere to the surface of the chamber. Fifty µg of Cy5-labeled IBB1 was added to the microscopy chamber and immediately placed on a C1 confocal laser microscope (Nikon). Z-series images of HT29 cells were recorded at different time (min) intervals after the onset of the experiment and processed with the software EZ-C1 Gold v2.10 build 240 (Nikon). Fluorescence images were obtained by exciting the sample with a red diode (633 nm). The signal corresponding to the Cy5-labeled IBB1 protein was visualized as discrete red fluorescent spots.

RESULTS AND DISCUSSION

Purification and molecular characterization of a major soybean BBI isoinhibitor, IBB1

Commercial soybean BBI, consisting in a mixture of IBB1 and IBBD2, was fractionated by MonoS cation exchange chromatography. The elution pattern of IBB1 and IBBD2, monitored by TIA measurements, are shown in **Fig 1**. At pH 4.4, IBB1 was not retained by the MonoS column, whereas IBBD2 was bound and eluted as a single broad chromatography peak in the range 0.04-0.13 M NaCl. The chromatographic fractions containing IBB1 were pooled and analysed by SDS-PAGE, showing a single band of appropriate molecular mass (12 kDa) (**Fig 2**, lane 1). The polyclonal Ab recognized the electrophoretic band corresponding to IBB1 (**Fig 2**, lane 2). Further studies by peptide mass fingerprinting were carried out in order to confirm the identity of IBB1. The purified protein, corresponding to the unbound chromatographic peak, was identified as Bowman-Birk proteinase inhibitor (Swiss-Prot entry: IBB1_SOYBN). The amino acid sequence of IBB1 is shown in **Fig. 3**, where the peptide sequences that contributed to protein identification by MS are indicated. Like others BBI proteins, IBB1 contain 14 Cys residues in conserved positions [3], with Lys and Leu in position P_1 in the N- and C-terminal inhibitory domains, respectively. In agreement with the identity of the P_1 residues, IBB1 inhibited both trypsin and chymotrypsin (data not shown). IBB1 was labelled with Cy5 fluorochrome in order to determine if the protease inhibitor is internalised by HT29 colorectal adenocarcinoma cells in a time-dependent manner. Cy5-labelled IBB1 was electrophoresed by SDS-PAGE and visualized in a Pharos Molecular Imager by exciting at 635 nm (**Fig 2**, lane 3); only an electrophoretic band was visualized, revealing the effectiveness of the minimal labelling protocol. To confirm the specificity of the signal, the same gel was also scanning at 532 nm (**Fig 3**, lane 4) in which the signal was completely abolished.

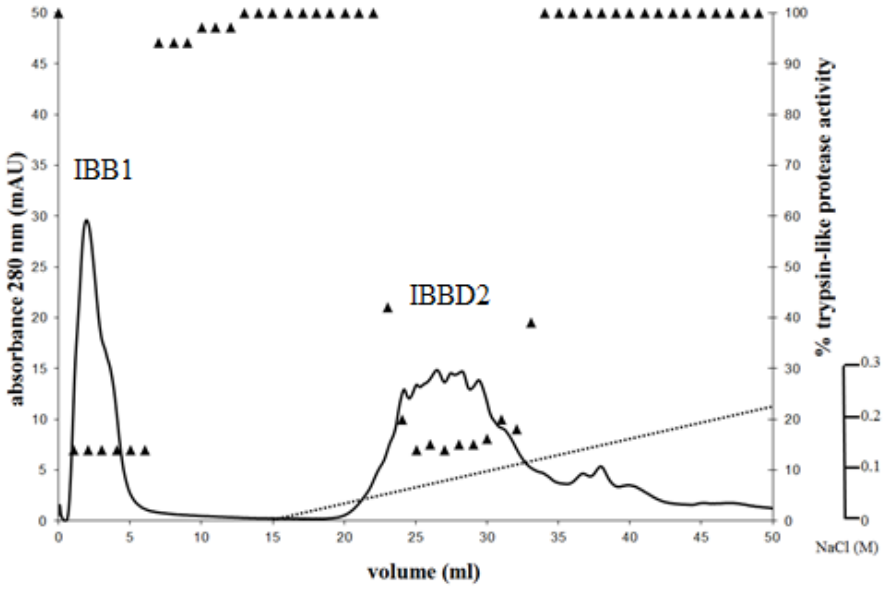

Figure. 1. Elution profile of a mixture of Bowman-Birk iso-inhibitors, IBB1 and IBBD2, from soybean on a MonoS 5/50 GL cation exchange column. Absorbance (mAU) at 280 nm of the chromatographic elution and the linear gradient of NaCl (0-0.22 M) are shown (solid and dotted lines, respectively). Using BAPNA as specific substrate, the trypsin inhibitory activity, measured on every fraction, is shown.

Effects of IBB1 on the proliferation of human colorectal adenocarcinoma cells HT29

The effect of IBB1 on the growth of human colon adenocarcinoma HT29 cells was determinated by comparing the cell viability of cells cultured in the absence or presence of IBB1 (15-93 µM), monitored by the cytotoxic NR cell assay. IBB1 exerted a significant effect on growth of HT29 cells at concentrations as low as 15 µM. A stadistically significant ($p<0.05$) and dose-dependent decrease of the growth of HT29 colon cells was observed (**Fig 4**); the IC_{50} value for IBB1 was 47 ± 5 µM, in agreement with those obtained for BBI from others plant sources, including lentil [8] and pea [10].

Internalization of IBB1 into human colorectal adenocarcinoma cells HT29

HT29 human colorectal adenocarcinoma cells were incubated with about 50 µg of Cy5-labelled IBB1 for 50 minutes (**Fig 5**). Fluorescent images were obtained by exciting the sample with red diode (633 nm), being the signal corresponding to IBB1 visualized as red fluorescent spots. Although additional biochemical assays are necessary, these results clearly demonstrated that IBB1 is internalized by HT29 cells. To further analyse the IBB1 internalization dynamics, images were taken at different time intervals (19, 32, 43 and 54 minutes). Labelled IBB1 crossed the cellular membrane of HT29 cells very rapidly and was gradually accummulated, forming fluorescent patches randomly distributed across the cytoplasm (**Fig 6**).

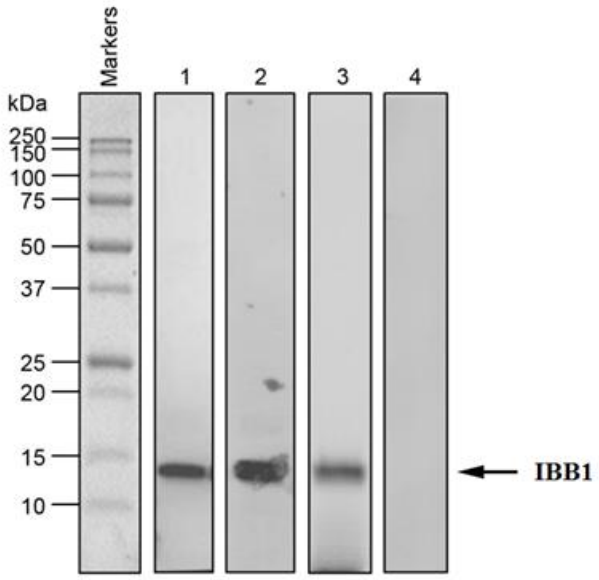

Figure 2. Electrophoretic characterization and fluorescent covalent labelling of IBB1. ***Lane 1***, analysis of the electrophoretic mobility of IBB1 by SDS-PAGE. One microgram of purified protein was loaded on a 4-20% gradient gel. A single band of about 12 kDa was visible on the gel after Coomassie staining. Molecular weight markers are shown on the left. ***Lane 2***, IBB1 protein was blotted to a PVDF membrane and probed with a rabbit polyclonal anti-IBB1 Ab followed by a secondary goat anti-rabbit IgG Alexa Fluor 488-conjugated Ab. The primary Ab was able to bind to the IBB1 protein. Negative control with preimmune serum did not show any signal (data not shown). ***Lanes 3-4***, fifty micrograms of IBB1 were covalently labelled with the Cy5 fluorochrome using the minimal labelling protocol. One microgram of Cy5-labelled IBB1 was electrophoresed by SDS-PAGE and visualized in a Pharos Molecular Imager by exciting at 635 nm (lane 3). The specificity of the signal was confirmed by scanning the same gel at 532 nm (lane 4).

IBB1 *DDESSKPCCDQCACTKSNPPQCRCSDMRLNSCHSACK*SCICA**LS**YPAQCFCVDITDFCYEPCKPSEDDKEN

Figure 3. Identification of IBB1 protein by peptide mass fingerprinting. The protein band from the Commassie-stained gel (Figure 3, lane 1) was excised and processed for MALDI-TOF/MS analysis. Amino acid sequences of inhibitory domains are underlined. The reactive peptide bond sites are in bold. **K** (Lysine) determines specificity for trypsin whereas **L** (Leucine) determines specificity against chymotrypsin. The peptides that contributed to protein identification are indicated in italics.

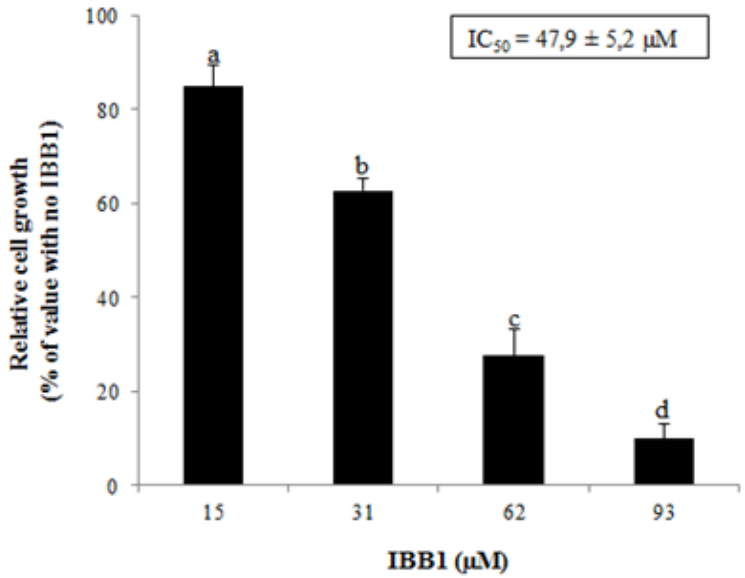

Figure 4. Effect of IBB1 on the *in vitro* growth of HT29 human colorectal adenocarcinoma cells. Growth media were supplemented with concentrations of IBB1 in the range 0-93 µM and cells harvested after a period of 96h. Data are means of three experiments, each having three technical replicates; bars represent SD. Mean values with different letters were significantly different ($p<0.05$; Bonferroni's test).

Soybean BBI has been shown to inhibit, specifically and potently, the chymotrypsin-like proteasomal activity in MCF7 breast cancer cells *in vitro* and *in vivo* [13]. The ability of soybean BBI to inhibit the proteasomal activity in intact MCF7 cells reveals that the cellular membranes are permeable to soybean BBI facilitating the inhibition of intracellular target proteases. Soybean BBI has been demonstrated to be taken up by colonic epithelial cells in a time-dependent manner, being the bulk of the internalised protease inhibitor present in the cytosol in active form [14]. These authors showed that soybean BBI was present in the cells for 12 h following 2h incubation. It has been also reported that soybean BBI is internalised into NIH/3T3 mouse embryo fibroblastic cells and is localized in the nucleus, even after simulated gastrointestinal digestion [15]. More recently, confocal microscopy studies have demonstrated that black-eyed pea (*Vigna unguiculata*) BBI crosses the membrane of breast MCF7 cancer cells, likely *via* endocytosis, and co-localizes with the proteasome in cytoplasm and in nucleus, inhibiting the chymotrypsin-, trypsin- and caspase-like activities of the 20S proteasome [16]. In order to determine the mechanism of internalization of BBI in human hepatoma Hep G2cells, fluorescein isothiocyanate-labelled buckwheat (FITC-BTI) (*Fagopyrum esculentum*) protease inhibitor was used [17]. FITC-BTI colocalised with labelled transferrin implying that BTI enters Hep G2 cells by clathrin-dependent endocytosis. Further studies to determine the internalization mechanism and correct localization of BBI in colon cancer cells will be relevant in order to identify serine proteases as potential therapeutic targets.

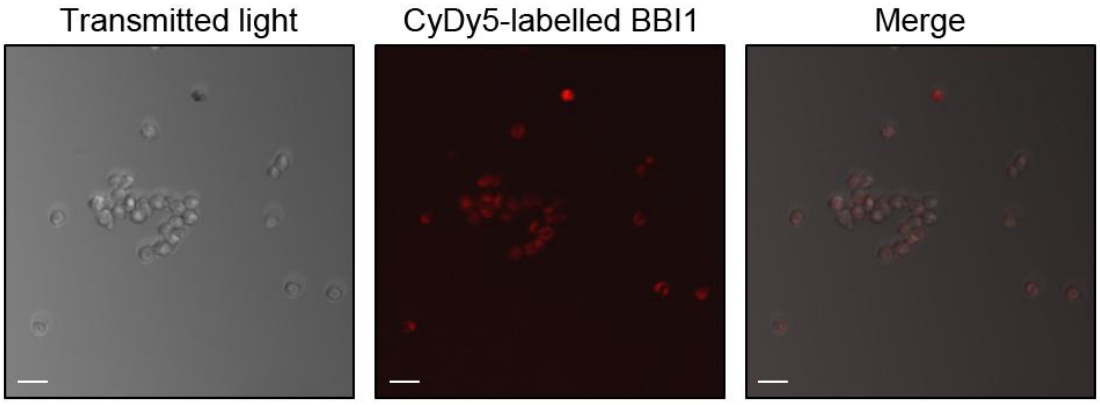

Figure 5. Internalization of IBB1 into human colorectal adenocarcinoma HT29 cells. About fifty micrograms of Cy5-labelled IBB1 were added to the culture medium. Transmitted light (left), fluorescence (middle) and merge (right) images represent a Z-stack projection of 20 optical sections of a group of HT29 cells cultured *in vitro* for 50 min. Images were captured using a confocal laser scanning microscope C1 (Nikon). Fluorescent image was obtained by exciting the sample with a red diode (633 nm). Red fluorescence corresponds to IBB1. Bars= 50 µm.

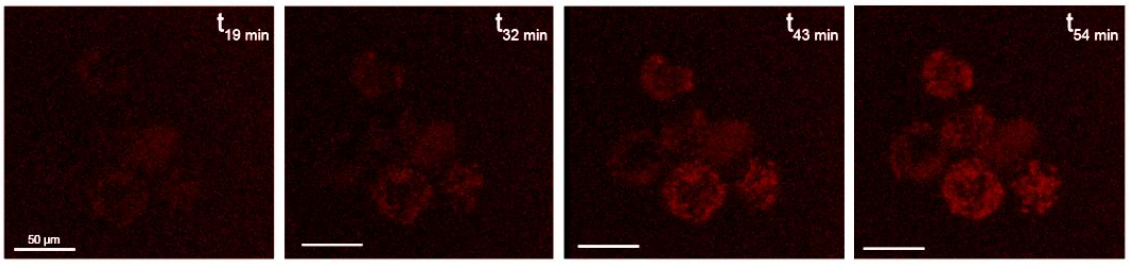

Figure 6. Analysis of IBB1 internalization dynamics in human colorectal adenocarcinoma HT29 cells using time-course CLSM microscopy. About fifty micrograms of Cy5-labelled IBB1 were added to the culture medium. Transmitted light (A) and fluorescence images (B-D) represent Z-stack projections of 20 optical sections of a group of HT29 cells cultured *in vitro*. Images were taken at different time (min) intervals using a C1 confocal laser scanning microscope (Nikon). Fluorescence images were obtained by exciting the sample with a red diode (633 nm). The signal corresponding to IBB1 was visualized as red fluorescent spots. We observed that the labelled protein entered the HT29 cells very rapidly (data not shown) and gradually accumulated, forming fluorescent patches randomly distributed across the cytoplasm. Bars= 50 µm.

CONCLUSIONS

1. **IBB1, a major Bowman-Birk isoinhibitor from soybean, exerts anti-proliferative effect on HT29 human colorectal adenocarcinoma cells.**
2. **IBB1 crossed the membrane of HT29 colon cancer cells over a time-course of 1 h and is localized in cytoplasm, where might interact with their potential therapeutic targets.**

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MY OWN IDEAS

Marta Soria

Hello, my name is Marta and I am studying in the Lux Mundi School, in Granada and I have been involved in the PIIISA project. This project has given me the opportunity to know in depth a little part of the scientific world. I have enjoyed this scientific project so much, being an awesome experience. I have worked in a research group having incredible people and everybody gets along very well with each other. I have made really good friends, and I wouldn't have met them if I had not been part of the project. Being in the laboratory was an awesome experience and I felt super comfortable when I was working there. It was my favorite part of

the project without a doubt! The project gives you the idea of how do people work in the laboratories. When I heard the word 'science' I used to think of a laboratory, but I have learnt that there are so many things besides the fact of being in a laboratory using goggles. I don't know yet what I want to study. However, being part of the PIIISA project has helped me to have it a little bit clearer. Hopefully one day I will be part of this huge scientific world. I'm so thankful I had this opportunity as it has made me grow. It has helped me to put my shyness aside. It has taught me a very important idea, and it is that if you want to fulfill your dreams you must run after them. You have to work really hard. If you really want to achieve your goals the effort will worth it. And the most important thing in life: you should never give up just because you failed the first time.

Patricia Porras

I'm Patricia Porras and I participated in PIIISA Project 2014. Before enrolling, I heard a lot of good things about PIIISA, so I didn't hesitated in becoming part of this. In my opinion, PIIISA is a fantastic opportunity for young people who want to study a scientist career. I learnt a lot here, such as laboratory techniques, working in a team a how a scientist's life is. In addition, PIIISA helped me to practice my English and it taught me how to explain what I did and the results of my work. At last, I want to thank PIIISA for give me this opportunity, the researches for thinking all time about us and my teammates for doing this experiment more joyful.

Francisco J. Moya

Initially I chose this project because it seemed very interesting and is closely related to what I want to study in a few years so since I thought this was going to serve me to know more closely the procedures, tools and discipline of scientific research, although at first I was a little frightened by the thought of having to make a presentation to many people in the same field of study. My first day on the project PIIISA research center Zaidin was fabulous, I felt like a little boy in a sweet shop , as the days passed I was giving me realize that my interest in matters related to my project was greater than I thought. Why not? Undoubtedly imagine me working on this, discovering new drug against deadly diseases and going to conferences around the world. Are already drawing conclusions from our research and about ten Congress expected the PIIISA in which we will reveal these fantastic data obtained after much effort. Now I can say I do not feel those nerves that I felt at the beginning of the project and no doubt I would repeat next year, because it has been a very constructive and didactic experience in which I have met wonderful people and researchers fantastic which have enabled us better understand these materials. I want to thank everyone who has made this project could take over.