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# Lipid lowering effects of *Momordica charantia* (Bitter Melon) in HIV-1-protease inhibitor-treated human hepatoma cells, HepG2

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**1** Hyperlipidemic effects of HIV-1-protease inhibitors (PI) are associated with increased hepatic production of triglyceride (TG)-rich lipoproteins, rather than lipoprotein clearance. PI are known to increase apolipoprotein B (apoB) secretion, apoC-III mRNA expression and decrease apoA-1 secretion. Nutritional therapy remains an important strategy to manage PI-associated hyperlipidemia.

**2** This study investigated the *in vitro* efficacy of Asian vegetable, *Momordica charantia* or bitter melon (BM) to ameliorate PI-associated apoB and lipid abnormalities in HepG2 cells.

**3** Our study demonstrates that bitter melon juice (BMJ) significantly reduced apoB secretion and apoC-III mRNA expression and normalized apoA-I expression in PI-treated HepG2 cells. BMJ also significantly reduced cellular TG and microsomal TG transfer protein, suggesting that lipid bioavailability and lipidation of apoB assembly may play a role in decreased apoB secretion.

**4** Identifying molecular targets of BM may offer alternative dietary strategies to decrease PI-associated hyperlipidemia and improve quality of life among HIV-1-infected patients.
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**Keywords:** Ritonavir; lopinavir; HAART; bitter melon; triglycerides; apoB; apoC-III; apoA-I; apolipoproteins; hyperlipidemia

**Abbreviations:** ATCC, American Type Culture Collection; ATP, adenine nucleotide triphosphate; BM, bitter melon; BMJ, bitter melon juice; CE, cholesterol esters; HAART, highly active antiretroviral therapy; HDL, high-density lipoproteins; HFD, high-fat diet; LPV/r, lopinavir/ritonavir; MTP, microsomal triglyceride transfer protein; NRTI, nucleoside analog reverse transcriptase inhibitors(s); PI, protease inhibitor(s); RTV, ritonavir; sLDL, small low-density lipoproteins; TG, triglycerides; VLDL, very low-density lipoproteins

## Introduction

Treatment of HIV-1 infection with highly active antiretroviral therapy (HAART) has resulted in major improvements in survival, immune function and decrease in the incidence of opportunistic infections (Palella *et al*., 1998). Unfortunately, HAART is complicated with metabolic complications including hypertriglyceridemia, hypercholesterolemia and lipodystrophy (Leow *et al*., 2003; Sekhar *et al*., 2005). HARRT generally consists of a combination of nucleoside analog reverse transcriptase inhibitors (NRTI) plus a protease inhibitor (PI) and non-nucleoside reverse transcriptase inhibitors (NNRTI) (Young, 2005). Although HIV-1 infection *per se* is associated with an atherogenic lipid profile (El-Sadr *et al*., 2005), use of PI-containing regimens, specifically ritonavir (RTV) and lopinavir/ritonavir (LPV/r, Kaletra), further accentuates these lipid abnormalities (Badiou *et al*., 2003a; Carpentier *et al*., 2005; Montes *et al*., 2005). LPV/r is a coformulation of lopinavir with low-dose RTV which elicits a favorable drug–drug interaction between the two resulting in sustained increase in plasma levels of lopinavir (Kaplan & Hicks, 2005). As compared to other proteases, or RTV alone, LPV/r combination has demonstrated significantly greater therapeutic efficacy to treat HIV-1 infection (Kaplan & Hicks, 2005).

Among HIV-1-infected patients on HAART, hypertriglyceridemia is generally associated with perturbations and/or redistribution of apolipoproteins (apo) within plasma cholesterol esters (CE) and triglyceride (TG), resulting in increased apo CIII in TG-rich lipoproteins, decreased high-density lipoproteins (HDL), and increased levels of very low-density lipoproteins (VLDL) and small dense low-density lipoproteins (sdLDL) (Badiou *et al*., 2003b; Carpentier *et al*., 2005; Rimland *et al*., 2005). Clinical studies among HIV-1-infected patients on HAART demonstrate increased levels of apoB and apoC-III and decreased levels of apoE lipoproteins (Lenhard *et al*., 2000; Bonnet *et al*., 2001; Fauvel *et al*., 2001; Mooser & Carr, 2001). Such an atherogenic profile is likely to increase the risk of cardiovascular complications including myocardial infarction and premature atherosclerosis (Carr, 2003; Mehta & Reilly, 2005).

Management of HAART-associated hyperlipidemia include switching PI regimens, exercise and/or conventional therapy

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such as statins or fibrates (Dube *et al*., 2000). However, statins may impair T-cell response to HAART (Narayan *et al*., 2003), while other lipid-lowering drugs can cause adverse drug–drug interactions (Fichtenbaum & Gerber, 2002). HAART-associated metabolic disorders have a tremendous negative impact on quality of life among HIV-1-infected patients, leading to decreased HAART compliance and ultimately virological failure (Sax & Gathe, 2005). Hence, there is an urgent need to develop new therapeutic approaches that are equally or more effective, and have minimal side effects. For the past few years nutritional therapy is becoming an integral part of treatment recommendations for HIV-1-infected patients, with and without metabolic complications (Nerad *et al*., 2003). However, efficacies, long-term safety and food–drug interactions need further considerations for their effectiveness and tolerability of HAART regimens.

High consumption of vegetables and fruits is associated with lowering of plasma lipids (Koebnick *et al*., 2005). We recently demonstrated that the Asian vegetable, *Momordica charantia* (commonly known as BM, bitter gourd, balsam pear or karela) reduces cellular TG synthesis and secretion as well as apoB secretion in HepG2 cells (Nerurkar *et al*., 2005). Bitter melon juice (BMJ) reduces adiposity in rats fed a high-fat diet (HFD), as well as lowers serum insulin and leptin levels and normalizes glucose tolerance (Chen *et al*., 2003). BM supplementation significantly decreased total serum cholesterol, hepatic total cholesterol and TG in control and streptozotocin-induced diabetic rats, as well as in rats fed a high-cholesterol diet and increased HDL cholesterol in diabetic rats (Jayasooriya *et al*., 2000; Ahmed *et al*., 2001; Virdi *et al*., 2003). Therefore, the goal of this project was to test the effects of BMJ on cellular lipids and apolipoprotein expression and secretion in HepG2 cells treated with PI. Our results indicate that BMJ significantly decreased cellular TG and cholesterol mass and apoB secretion in HepG2 cells treated with RTV and LPV/r for 24 h. In addition, BMJ significantly decreased microsomal TG transfer protein (MTP) and apoC-III mRNA expression and increased apoA-I expression. Understanding the mechanisms of functional foods (foods that may provide health benefits beyond basic nutrition), such as BM, may help to identify new molecular targets for the treatment of HAART-associated metabolic disorders.

## Methods

### *Cell lines and culture conditions*

The human hepatoblastoma cell line, HepG2, obtained from the American Type Culture Collection (ATCC; Manassas, VA, U.S.A.), was maintained in T75 flasks at 37°C and 5% $CO_2$ in minimum essential medium Eagle's (MEME) supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 50 U $ml^{-1}$ streptomycin and 100 IU penicillin and used up to 10 passages. Typically, cells were seeded in six-well culture plates, grown to 80–90% confluency, and then incubated in serum-free media containing 1% bovine serum albumin (BSA) for 24 h. After 24 h, cells were further incubated with either the control medium (MEME with 1% BSA) or experimental media (MEME with 1% BSA $\pm$ 0.8 mM oleate and PI $\pm$ BMJ) for 24 h. All PI were added at 10 $\mu$M concentrations, corresponding to the steady-state peak plasma levels ($C_{max}$) found among HIV-1-infected individuals on HAART (Roche *et al*., 2002). We also tested the effects of low RTV concentrations (2.2 $\mu$M) equivalent to $C_{max}$ plasma levels among HIV-1-infected patients on LPV/r regimens. At the end of each experiment, media was harvested to measure cellular cytotoxicity, apoB and apoA-1 levels, whereas the cells were used for analyzing cellular lipids and mRNA expression of MTP, apoC-III, apoA-1 and apoE.

### *Preparation of PI solutions and BMJ*

RTV and LPV/r were kindly provided by Dr Jennifer Frank. Both PI were dissolved in sterile DMSO, filtered, aliquoted and stored at –20°C until used. Final concentration of DMSO in all cultures was below 0.01%. Chinese variety of young BM (raw and green), was obtained from local farmer's market, washed and deseeded. BMJ was extracted according to the published protocols (Raza *et al*., 1996; Nerurkar *et al*., 2005). The supernatant BMJ was aliquoted and stored at −80°C until further analysis. BMJ aliquots were thawed once and discarded after each use. Based on our published cytoxicity data and the effects of varying concentrations of BMJ on apoB secretion (Nerurkar *et al*., 2005), 1% BMJ was used in all the subsequent experiments, with and without PI.

### *Cytotoxicity assay*

Cell viability was assayed fluorimetrically by measuring the release of lactate dehydrogenase (LDH) from cells with a damaged membrane (Nerurkar *et al*., 2004). Briefly, the released LDH was measured with a 10-min coupled enzymatic assay that resulted in the conversion of resazurin into resorufin, using the commercial CytoTox-ONE assay kit (Promega, Madison, WI, U.S.A.) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm using the Perkin-Elmer multiplate reader, Wallac Victor2 (model 1420-011, multilabel counter, Perkin-Elmer Life Sciences, Boston, MA, U.S.A.).

### *Analysis of cellular lipids*

To measure cellular total TG and cholesterol levels, HepG2 cells were washed with PBS and lysed with 0.5 N sodium hydroxide (NaOH). TG and cholesterol levels were measured using the Infinity TG Liquid Stable Reagent and Infinity Cholesterol Liquid Stable Reagent commercial kits (Thermo-DMA, St Louisville, CO, U.S.A.), according to the manufacturer's instructions, and absorbance was read at 540 nm using a Perkin-Elmer multiplate reader, Wallac Victor2 (Perkin-Elmer Life Sciences). Total TG and cholesterol levels were normalized to mg of protein as determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

### *Measurement of apoB and human serum albumin secretion*

ApoB levels in the culture media was quantified by using a commercially available apoB Microwell ELISA Assay kit (AlerCHEK Inc., Portland, ME, U.S.A.) according to the manufacturer's instructions (Scharnagl *et al*., 2001). Albumin in the media was measured with commercial Human Serum

**Table 1** Primer sequences and cycling conditions for semiquantitation of target gene expression

| *Primer* | *Sequence* | *Amplicon size (bp)* | *Cycling conditions* |
|---|---|---|---|
| MTP-(F) | 5′ GGA CTT TTT GGA CAA AAG TGA C 3′ | 699 | 94°C 40 s, 53°C 1 min, 68°C 2 min; 29 cycles |
| MTP-(R) | 5′ GGA GAA ACG GTC ATA ATT GTG 3′ | | |
| ApoC-III (F) | 5′ CTC AGC TTC ATG CAG GGC TAC 3′ | 319 | 94°C 40 s, 54°C 1 min, 72°C 1 min; 26 cycles |
| ApoC-III (R) | 5′ GGT AGG AGA GCA CTG AGA ATA CTG TC 3′ | | |
| ApoA-I (F) | 5′ ATG AAA GCT GCG GTG CTG ACC 3′ | 456 | 94°C 40 s, 60°C 1 min, 72°C 1 min; 21 cycles |
| ApoA-I (F) | 5′ CAC CTT CTG GCG GTA GAG CTC 3′ | | |
| ApoE (F) | 5′ ACT GGC ACT GGG TCG CTT T 3′ | 163 | 94°C 40 s, 52°C 1 min, 72°C 1 min; 30 cycles |
| ApoE (R) | 5′ GTT GTT CCT CCA GTT CCG ATT 3′ | | |
| GAPDH (F) | 5′ AGT CAG CCG CAT CTT CTT TTG C 3′ | 298 | Changed according to different target genes |
| GAPDH (R) | 5′ CTC CTG GAA GAT GGT GAT GGG A 3′ | | |

Albumin (HSA) ELISA kit (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.) (Casaschi *et al*., 2004b).

### *Semiquantitation of MTP, apoC-III, apoA-I and apoE mRNA gene expression by RT–PCR*

Total RNA from HepG2 cells was extracted using RNAzol™ B (TEL-TEST Inc., Friendswood, TX, U.S.A.). RNA, 2 $\mu$g was reverse transcribed into complementary DNA (cDNA) and gene expression levels of MTP, apoC-III, apoA-I and apoE were quantified by reverse transcriptase–PCR (RT–PCR) using primers and PCR cycling conditions mentioned in Table 1. Various cycling conditions were initially used to standardize the cellular gene expression in the log phase of amplification. Final cycling conditions for each gene were based on, half of maximum saturation of the amplicons. The PCR amplicon was size-fractionated on a 2% agarose gel and stained with ethidium bromide. The amount of mRNA expressed for each cellular gene in both treated and untreated cells was semiquantitated using the Kodak 1D image analysis software that captures the intensity of the amplicons and calculates the pixel counts by normalizing against the background. The intensity of the amplicon was then expressed as a ratio of the gene of interest against a housekeeping gene, GAPDH.

### *Immunoblot analysis of apoA-I*

Effects of BMJ and PI on secretion of apoA-I were analyzed by Western blotting. HepG2 cells were treated with or without PI $\pm$ BMJ for 24 h and media was collected and stored at $-80$°C until further analysis. Protein concentrations of media and cell extracts were measured using Bradford method according to the manufacturer's instructions (Bio-Rad Laboratories). Cell culture media proteins (10 $\mu$g) were separated on 12% SDS–PAGE and transferred to nitrocellulose membrane, blocked overnight with 1% BSA and incubated in primary antibody (goat anti-human apoA-I polyclonal serum, Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) at room temperature for 2 h. After washing with Tris-buffered saline, blots were probed with donkey anti-goat IgG HRP conjugate secondary antibody (Santa Cruz Biotechnology). Proteins were detected by commercially available electrochemiluminescence (ECL) kit, Pierce SuperSignal™ West Femto Maximum Sensitivity Substrate (Amersham Biosciences, Piscataway, NJ, U.S.A.).

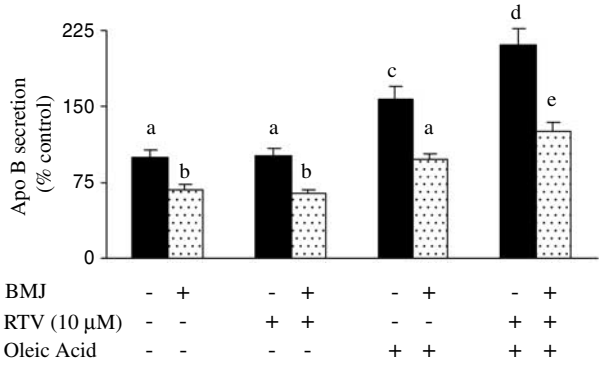


**Figure 1** ApoB secretion in HepG2 cells treated with BMJ with or without PI for 24 h in the presence or absence of oleate. ApoB was measured in the medium by commercial ELISA kit. Data are represented as a percentage of control (untreated cells), set at 100%. Values are mean $\pm$ s.d. from three independent experiments performed in duplicate ($n=6$). Light and dark bars represent cells treated with and without BMJ treatment, respectively. Means with a common alphabet do not differ, $P<0.05$.

### *Statistical methods*

All data are presented as mean $\pm$ s.d. Three sets of each experiment were performed in triplicate and group means were compared using analysis of variance (ANOVA). ApoB modulator, oleate was incorporated into two-way ANOVA model. Normality and homogeneity of variance were determined using multivariate log-normal plots. When appropriate, the data were transformed using a logarithm or square root function. *Post hoc* comparisons were tested *via* the two-stage Ryan-einot-gabriel-welsch multiple range test. *P*-values $\leqslant 0.05$ were considered to be statistically significant.

## Results

### *Effects of BMJ on apoB secretion in RTV-treated HepG2 cells*

As observed in our earlier study (Nerurkar *et al*., 2005), BMJ significantly decreased apoB secretion by 30–50% in HepG2 cells treated for 24 h ($P<0.05$, Figure 1). Under lipid deficient conditions (media without oleic acid) BMJ inhibited apoB secretion by $32\pm5.2$% in control cells and by $39\pm6$% in RTV-treated HepG2 cells. Studies by Liang *et al*. (2001) indicate

that stimulation of neutral-lipid synthesis by oleic acid was associated with increased apoB secretion in HepG2 and rodent McA RH7777 hepatoma cells treated with and without RTV. Consistent with these studies, our data also indicates that treatment of HepG2 cells with oleic acid significantly increased apoB secretion in control cells ($158 \pm 6.5\%$, $P<0.05$) as well as RTV-treated cells ($211 \pm 8.7\%$, $P<0.05$) as compared to untreated control cells without oleic acid (Figure 1). However, treatment of HepG2 cells with BMJ and oleic acid normalized apoB secretion in control cells ($98 \pm 6.9$, $P<0.05$) and RTV-treated cells ($125 \pm 9.6\%$, $P<0.05$) to that of control cells without oleic acid. BMJ did not affect the secretion of the most abundant cellular protein, albumin, as measured by commercially available HSA ELISA Kit', suggesting that the effects of BMJ on apoB secretion are specific (data not shown).

### *BMJ reduces cellular TG and total cholesterol levels in PI-treated HepG2 cells*

As demonstrated in Figure 2a, 10 $\mu$M and 2.2 $\mu$M RTV significantly increased cellular TG by 130 and 25%, respectively, while 10 $\mu$M LPV/r increased TG by 76% as compared to untreated controls ($P<0.05$ as analyzed by one-way ANOVA). Figure 2b demonstrates a 60 and 15% increase in cellular cholesterol levels with 10 and 2.2 $\mu$M RTV, respectively and 75% increase with 10 $\mu$M LPV/r. In contrast, BMJ significantly decreased TG by 30% in untreated control cells ($P<0.05$), and normalized TG levels in RTV and LPV/r-treated groups when compared to the control untreated cells (Figure 2a). Similarly, BMJ significantly decreased cellular cholesterol levels by 65% in untreated control cells and normalized cholesterol levels in PI-treated cells as compared to the untreated control cells ($P<0.05$, Figure 2b).

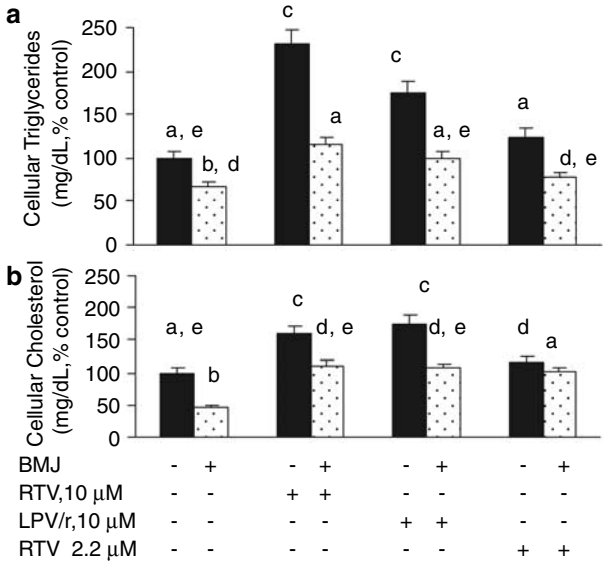


**Figure 2** Effect of BMJ on cellular TG (a) and cholesterol (b) levels in HepG2 cells treated without oleic acid, and with or without PI. Data are represented as a percentage of control (untreated cells), set at 100%. Values are mean $\pm$ s.d. from three independent experiments performed in duplicate ($n=6$). Light and dark bars represent cells treated with and without BMJ treatment, respectively. Means with a common alphabet do not differ, $P<0.05$.

### *BMJ inhibits MTP gene expression in PI-treated HepG2 cells*

Figure 3 depicts representative gels of MTP and GAPDH gene expression (a) in cells treated with and without oleic acid, and the bar graphs (b) represent the intensity of the 699-bp MTP amplicon expressed as the ratio to GAPDH. Overall, PI *per se* had no significant effect on MTP gene expression as compared to their respective controls (Figure 3). Our data indicates that in HepG2 cells treated with 1% BMJ for 24 h without oleic acid, MTP gene expression was significantly decreased by $47.5 \pm 4.3\%$ in control, $59.5 \pm 4.1\%$ in RTV (10 $\mu$M)-treated, $44.5 \pm 4.9\%$ in LPV/r-treated and $52.3 \pm 3\%$ in RTV (2.2 $\mu$M)-treated cells (Figure 3a, $P<0.05$). Similarly, BMJ significantly reduced MTP gene expression in HepG2 cells treated with oleic acid and PI (Figure 3b, $P<0.05$).

### *Effects of BMJ on apoC-III and apoE mRNA expression in PI-treated HepG2 cells*

Figure 4a demonstrates the effects of PI and BMJ on mRNA expression of apoC-III gene. The bar graphs represent intensity of each amplicon expressed as the ratio to GAPDH (Figure 4b). Effects of PI and BMJ, on apoC-III mRNA expression were observed only after 48 h of treatment, rather than 24 h (Figure 4). Among PI, only 10 $\mu$M RTV exhibited a $28 \pm 4.9\%$ increase in apoC-III mRNA expression after 48 h of treatment, while LPV/r and 2.2 $\mu$M RTV had no effect (Figure 4). Overall, 1% BMJ significantly inhibited apoC-III expression by $29.5 \pm 6.2\%$ in control cells and 41–74% in all PI-treated cells without oleic acid (Figure 4, $P<0.05$). In contrast to apoC-III (Figure 4), apoE mRNA expression was unaffected by either PI or BMJ up to 48 h of treatment (data not shown).

### *Effects of BMJ on apoA-I mRNA expression and secretion in PI-treated HepG2 cells*

Our study indicates that after 48 h of treatment, 10 and 2.2 $\mu$M RTV inhibited apoA-I mRNA expression by $23.5 \pm 4.8$ and $12 \pm 3\%$, respectively, whereas LPV/r had no effect (Figure 5a). Although BMJ alone did not increase apoA-I mRNA expression, it normalized the RTV-associated decrease in apoA-I mRNA expression after 48 h of treatment (Figure 5a, $P<0.05$). The bar graphs represent intensity of each amplicon expressed as the ratio to GAPDH (Figure 5b). In contrast, apoA-1 protein secretion was unaffected by either PI or BMJ treatments (Figure 5c).

## Discussion

Prevalence of hyperlipidemia ranges from 28 to 80% among HIV-1-infected patients on PI-containing regimens, which generally includes hypertriglyceridemia in majority of the cases (40–80%), followed by hypercholesterolemia (10–50%) and possibly a decrease in HDL-cholesterol (Carr *et al*., 1999; Mulligan *et al*., 2000; Saves *et al*., 2002). Although the pathophysiological mechanisms involved in PI-associated dyslipidemia are unknown, clinical studies indicate a delay in postprandial clearance of TG-rich lipoproteins among HIV-1-

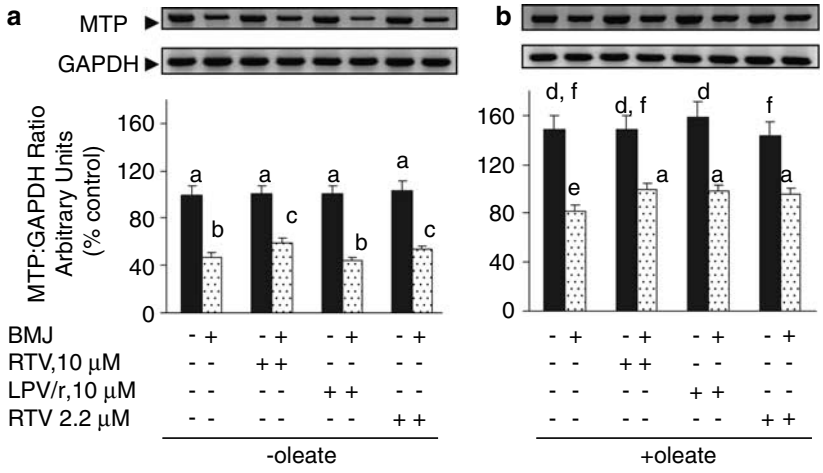


**Figure 3** MTP mRNA expression in HepG2 cells treated with BMJ with or without PI for 24 h in the presence (a) and absence (b) of oleate. Bar graph represents the densitometry scans of 699-bp MTP amplicon and expressed as the ratio to GAPDH. Data are expressed as percentage of control (untreated cells), set at 100%. Values represent the means ± s.d. of three independent experiments performed in duplicate ($n=6$). Light and dark bars represent cells treated with and without BMJ treatment, respectively. Means with a common alphabet do not differ, $P<0.05$.

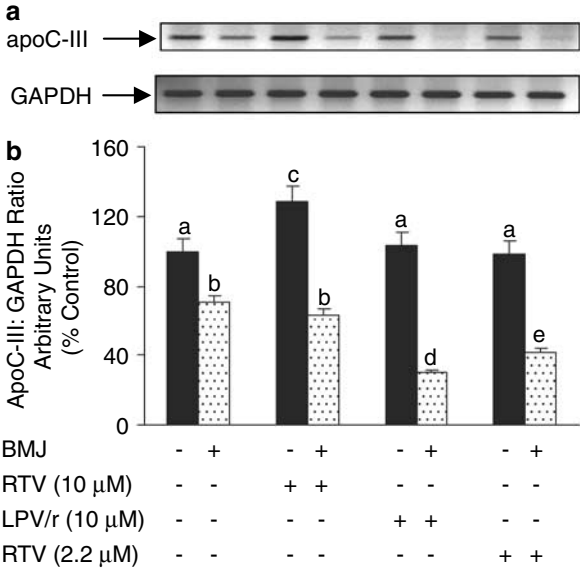


**Figure 4** ApoC-III mRNA expression (a) in HepG2 cells without oleic acid and treated with BMJ ± PI. Bar graph (b) represents the densitometry scans of the 519-bp apoC-III amplicon that is expressed as the ratio to the 298-bp GAPDH amplicon. Data are expressed as percentage of control (untreated cells), set at 100%. Values represent the means ± s.d. of three independent experiments performed in duplicate ($n=6$). Light and dark bars represent cells treated with and without BMJ treatment, respectively. Means with a common alphabet do not differ, $P<0.05$.

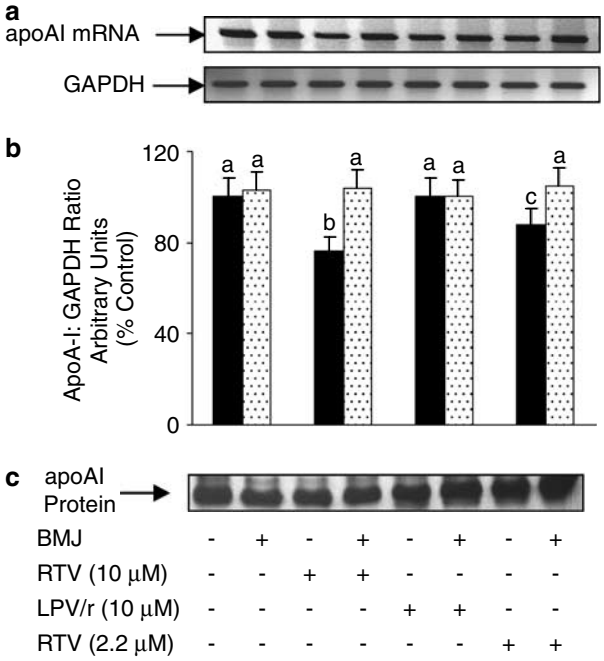


**Figure 5** ApoA-I mRNA expression (a) and protein levels (c) in HepG2 cells treated with BMJ with or without PI for 48 h. Bar graph represents the densitometry scans of 456-bp apoA-I amplicon, and expressed as the ratio to GAPDH (b). Data are expressed as percentage of control (untreated cells), set at 100%. Values represent the means ± s.d. of three independent experiments performed in duplicate ($n=6$). Light and dark bars represent cells treated with and without BMJ treatment, respectively. Means with a common alphabet do not differ, $P<0.05$.

infected patients receiving HAART (Stein *et al*., 2005). In most of these studies, PI-associated dyslipidemia is associated with marked increases in complex lipoprotein particles containing apoB, associated with apoC-III and/or apoE, indicating an atherogenic profile (Bonnet *et al*., 2001). Recent studies indicate that BM lowers plasma and hepatic lipids in animals fed a HFD (Ahmed *et al*., 2001, and Nerurkar *et al*., unpublished observations). Our previous study indicated that BMJ significantly lowered the synthesis and secretion of cellular TG and apoB, *in vitro* (Nerurkar *et al*., 2005). As nutritional management is one of the strategies to address PI-associated metabolic disorders (Hadigan *et al*., 2001; Hadigan, 2003), our study investigated the effects of BM on PI-associated changes in cellular lipids and apolipoprotein expression.

Based on our previous experience (Nerurkar *et al*., 2005) we used 1% BMJ in this study. Lack of clinical data makes it difficult to estimate physiologically effective dose of BM. Clinical studies so far have used about 50–200 ml day$^{-1}$ of the fresh BMJ, 3–15 g of the dried powdered of BM fruit or 300–600 mg of a BM extract (Leatherdale *et al*., 1981; Welihinda *et al*., 1986; Srivastava *et al*., 1993; Ahmed *et al*., 1999). Average single dose of 50–200 ml BMJ day$^{-1}$ (Ahmed *et al*., 1999) administered to a 70-kg person and dissolved in 500 ml of gastrointestinal fluid (Yu *et al*., 2002) would achieve a concentration of 10–40% BMJ in the stomach. The 1% BMJ used in our study is 10- to 50-fold lower than the concentration achieved in the gastrointestinal fluid. However, plasma concentrations of BMJ or its active ingredients, after undergoing absorption and metabolism, are unknown.

Interestingly, in our study, BMJ significantly inhibited not only the PI-associated increases in cellular TG and total cholesterol levels, but also the overall apoB secretion in HepG2 cells treated with and without PI for 24 h ($P<0.05$). Furthermore, BMJ inhibited apoB secretion in both, lipid-rich (containing 0.8 mM oleate) and lipid deficit (serum-free) media. Mechanisms involved in altering TG and apoB metabolism are multifold. We have demonstrated that BMJ significantly inhibits apoB secretion in HepG2 cells, probably due to reduced TG synthesis as measured by the rate of incorporation of [$^3$H]glycerol into cellular TG (Nerurkar *et al*., 2005). BMJ-associated reduction in cellular TG could be affected not only by synthesis but also by $\beta$-oxidation of fatty acids (FA). It was recently demonstrated that BMJ increased mitochondrial transport of FA, and the decreases in hepatic and muscle TG content correlated with the increased activity of key lipid oxidation enzyme, acyl-CoA dehydrogenase (AD) (Chan *et al*., 2005; Chen & Li, 2005). It is therefore possible that BMJ-associated decrease in apoB secretion is influenced by lipid bioavailability as well as oxidation.

We have previously demonstrated that, apoB secretion in HepG2-treated cells with proteasomal inhibitor, *N*-acetyl-leucyl-leucyl-norleucinal (ALLN) was unaffected by BMJ under basal conditions (Nerurkar *et al*., 2005). Interestingly, cotreatment of HepG2 cells with oleate and ALLN significantly increased apoB secretion in BMJ-treated cells as compared to untreated controls, suggesting that BMJ probably reduces apoB secretion by proteasome-dependent pathway with lipid bioavailability (Nerurkar *et al*., 2005). In our study, both RTV and LPV/r caused significant increases in cellular TG and cholesterol mass ($P<0.05$). These increases are similar to those observed by Lenhard *et al*. (2000) in HepG2 cells treated with 10 $\mu$M RTV for 24 h (82% increase in TG and 48% increase in cholesterol, $P<0.05$). Lipid availability is known to increase apoB secretion due to increased translocation and reduced ubiquitination (proteasomal degradation) (Dixon *et al*., 1991). However, Liang *et al*. (2001) demonstrated that RTV causes intracellular accumulation of apoB in HepG2 cells, due to inhibition of proteasome degradation in lipid-deficit media, but increased apoB secretion in the presence of oleic acid. Although we did not measure the intracellular accumulation of apoB, RTV had no effect on apoB secretion up to 48 h in serum-free medium.

Additional factors that regulate the secretion of apoB100-containing lipoproteins by hepatic cells include decrease in MTP enzymatic activity and/or expression, involved in transferring newly synthesized (nascent) apoB proteins to ER lumen for assembly (Wang *et al*., 1999; Borradaile *et al*., 2002; Higashi *et al*., 2002). Liang *et al*. (2001) have demonstrated that PI inhibits MTP enzymatic activity *in vitro*, thereby affecting the transfer of neutral lipids onto nascent apoB particles, while Riddle *et al*. (2002) demonstrate no effect of PI on MTP mRNA expression in mice. As BMJ decreases PI-associated apoB secretion in the presence of lipids, we tested the effects of BMJ on MTP gene expression in HepG2 cells treated with and without PI. Although we did not measure the MTP activity, RTV and LPV/r had no effect on MTP mRNA expression, but BMJ significantly reduced its mRNA expression in the presence and absence of oleic acid ($P<0.05$). Previous studies have demonstrated that BMJ reduces not only plasma lipids in diabetic animals but also lowers VLDL levels (Virdi *et al*., 2003), that probably could result from decreased apoB secretion as suggested by our *in vitro* studies (Nerurkar *et al*., 2005). Reduction of apoB secretion is also affected by a number of lipogenic enzymes, including acyl CoA:cholesterol acytltransferase (ACAT), diacylglycerol acyltransferase (DGAT), hydroxymethylglutaryl coenzyme A (HMG CoA), and MTP (Casaschi *et al*., 2004a, b). While PI have no effect on MTP and/or DGAT activity (Riddle *et al*., 2002), BMJ was demonstrated to reduce MTP (Nerurkar *et al*., 2005). The effects of BMJ on DGAT and ACAT activities have yet to be elucidated. Taken together, our data indicate that although BMJ and PI affect different mechanisms *in vitro*, BMJ may offer novel dietary strategies to overcome PI-associated metabolic abnormalities.

It has been suggested that BMJ parallels metformin action due to its analogous effects on the hepatic activity of enzymes involved in glucose and lipid metabolism, increased expression of GLUT4 in the plasma membrane of skeletal muscle, a tendency to prevent weight gain, lowering serum insulin and favorable effects on serum lipids (Umesh *et al*., 2005). Metformin's beneficial effects on hyperglycemia and lipid metabolism in HepG2 cells have been correlated with its ability to activate AMP-activated protein kinase (AMPK) and to increase insulin receptor phosphorylation and its downstream signaling cascade in HepG2 cells (Zang *et al*., 2004). It is therefore hypothesized that BM may also have the potential to activate AMPK to exert its hypoglycemic and hypolipidemic effects (McCarty, 2004). In the present study, we did not measure either activation of AMPK or insulin signaling cascade in HepG2 cells treated with BMJ and/or HAART. However, unpublished observations form our laboratory indicate that BMJ improves hyperglycemia and lipid metabolism by increasing insulin receptor and insulin receptor substrate-1 (IRS-1) phosphorylation in liver of HFD fed C57BL/6 mice. It is therefore possible that effects of BMJ on HAART-associated lipid abnormalities are regulated through AMPK and/or increased phosphorylation of insulin signaling molecules.

Besides their effect on apoB, PI are also associated with abnormalities of other apo, such as apoC-III, apoE and apo A-I (Malavazi *et al*., 2004). ApoC-III is a major apo regulating the synthesis as well as catabolism of TG-rich VLDL and reduces hepatic uptake of TG-rich lipoprotein by inhibiting the action of lipoprotein lipases (LPL) (Shachter, 2001). RTV and LPV/r therapy in HIV-1-infected patients results in increased TG as well as apoC-III levels (Badiou *et al*., 2003b). Similarly, in our study, RTV increases not only cellular TG mass, but also apoC-III mRNA expression in HepG2 cells *in vitro*.

Inhibition of hepatic apoC-III mRNA as well as protein, is known to correlate with lower plasma TG levels in animal models of dyslipidemia (Haubenwallner *et al*., 1995). Interestingly, BMJ significantly inhibits apoC-III mRNA expression in control as well as PI-treated HepG2 cells ($P<0.05$). ApoC-III also inhibits the interactions between apoE and LDL receptor (LDLR) or with the LDL receptor-related protein (LRP), involved in the hepatic uptake of remnant particles (Hussain *et al*., 1991). Increased expression of apoE promotes the removal of TG-rich lipoprotein by stimulating the uptake of lipoprotein remnants by hepatocytes, thereby decreasing plasma TG (Jong *et al*., 2001). Although PI treatment results in decreased levels of apoE among HIV-1-infected patients on HAART, both, RTV and LPV/r did not affect apoE mRNA expression in HepG2 cells. Furthermore, BMJ also had no effect on apoE mRNA expression *in vitro*.

ApoA-I, the major protein constituent of HDL, is a 28-kDa protein that plays a key role in lipoprotein clearance and cholesterol redistribution. Our study indicates that PI had no effect on apoA-I secretion up to 24 h of treatment. However, 10 $\mu$M RTV significantly decreased apoA-I mRNA expression after 48 h of treatment. Recent studies indicate that changes in apoA-I mRNA expression are not attributable to transcriptional rates or apoA-I promoter activity, but is governed by mRNA stability and half-life (Haas *et al*., 2004). Similarly, apoA-I secretions and protein levels may reflect changes in either translational efficiency of the mRNA or protein-turnover rate (Haas *et al*., 2004). While BM has been demonstrated to increase HDL-cholesterol in diabetic rats, BMJ had no effect on apoA-I secretion in HepG2 cells treated with and without PI for 24 h, but normalized the RTV-associated decrease in apoA-I mRNA levels after 48 h.

PI such as RTV increased TG-rich lipoproteins, but have no effect on the expression of enzymes responsible for TG synthesis and its assembly into lipoproteins, namely DGAT and MTP, respectively (Riddle *et al*., 2002). Furthermore, the increase in hepatic apoB secretion in RTV-treated mice was possibly due to inhibition of proteasome-mediated degradation of apoB in the liver (Liang *et al*., 2001). In contrast, BMJ inhibits apoB secretion in HepG2 cells due to lipid bioavailability as a result of inhibition of TG synthesis, reduction in MTP as well as increased proteasomal degradation under lipid-rich condition (Nerurkar *et al*., 2005). Although BMJ improves PI-associated lipid abnormalities, they both affect lipid metabolism *via* different molecular targets. It is further possible that different molecular targets of lipid metabolism could have been affected if cells were pretreated with BMJ rather than simultaneous treatment with BMJ and PI.

Natural or herbal products are generally used as a single, presumably active, constituent. Animal studies have employed either fresh BMJ or crude organic fractions of BMJ to evaluate its hypoglycemic and hypolipidemic effects (Ahmed *et al*., 1999; Jayasooriya *et al*., 2000; Chao & Huang, 2003; Chen *et al*., 2003). Moreover, clinical studies have used BMJ, BM fruit or BM tea to evaluate its effects in diabetic patients (Leatherdale *et al*., 1981; Welihinda *et al*., 1986; Srivastava *et al*., 1993; Ahmed *et al*., 1999). Compounds isolated from the fruit and seeds of BM plant that are believed to contribute to its hypoglycemic activity include charantin (a steroid glycoside) and polypeptide 'p' or plant insulin (a 166 residue insulin mimetic peptide) (Khanna *et al*., 1981; Marles & Farnsworth, 1995). BM is also known to contain additional glycosides such as mormordin, vitamin C, carotenoids, flavanoids and polyphenols (Anila & Vijayalakshmi, 2000; Raj *et al*., 2005). Purified active fractions of BM are yet to be tested for their hypoglycemic or hypolipidemic effects. However, BMJ preparations from independent laboratories have demonstrated comparable beneficial effects not only on glucose metabolism, but also on plasma and hepatic lipids (Jayasooriya *et al*., 2000; Senanayake *et al*., 2004). Further studies are therefore warranted not only to characterize and identify the active ingredients of BM but also to standardize physiologically relevant dosage in humans.

So far, only a few, nonrandomized clinical studies have investigated the effects of BM in humans (Leatherdale *et al*., 1981; Welihinda *et al*., 1986; Srivastava *et al*., 1993; Basch *et al*., 2003). It is therefore crucial to conduct adequately powered, randomized, placebo-controlled clinical trials before BM can be recommended as an effective alternative and/or complementary therapy for HAART-associated metabolic abnormalities. Additional studies are warranted to test the long-term safety and efficacy of BM. Administration of BM with other hypoglycemic agents must be performed under medical supervision and monitoring, due to its hypoglycemic properties (Welihinda *et al*., 1986; Ahmed *et al*., 1999). Some negative side effects such as diarrhea and hepatotoxicity in humans have been noted (Basch *et al*., 2003), which could be due to excessive consumption. In summary, our study suggests that functional foods such as BM may have multiple molecular targets that may help to lower HAART-associated side effects.

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