Antitumor effects of the combination of cholesterol reducing drugs

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Abstract. There are a number of potential mechanisms linking cholesterol homeostasis to processes that are tightly linked with carcinogenesis. Statins, which are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR), the rate-limiting enzyme in the mevalonic acid synthesis pathway, exert cytostatic and cytotoxic effects towards tumor cells. It seems that the cytostatic and cytotoxic effects of statins result from blocking protein prenylation, leading to inhibition of isoprenoid compound synthesis. Another compound which affects cholesterol metabolism is the plant alkaloid berberine. The aim of this study was to investigate potential antitumor effects of lovastatin combined with berberine. Combined with berberine, lovastatin appeared to exert potentiated cytostatic and/or cytotoxic effects against human MDA-MB231 breast cancer and murine Panc02 pancreatic cancer cells. The obtained results indicated that the effect of berberine is not dependent on blocking protein prenylation in cells, and the toxic effect of lovastatin combined with berberine is reversed by addition of the substrates of this pathway to the level brought out by lovastatin alone. Lovastatin-berberine combination caused cell cycle inhibition in G1 phase after 48 h of incubation with drugs. In a Panc02 pancreatic cancer model in mice, lovastatin-berberine combination slightly, but significantly, slowed down tumor growth. Taking into account the number of patients treated with the investigated drugs one may suppose that the described interactions may be of clinical value.

Introduction

Cholesterol-reducing agents continuously attract enormous interest not only because of their beneficial cardiovascular effects, but also due to their influence on numerous physiological and pathophysiological processes (1-3). For example, statins, which are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase target mevalonate pathway of cholesterol synthesis and impair production of isoprenoid compounds such as dolichol, ubiquinone and mevalonate-derived prenyl groups that are attached post-translationally to approximately 1% of all cellular proteins (4,5). By affecting cellular cholesterol synthesis statins also influence the formation of lipid rafts which are water insoluble plasma membrane microdomains enriched in receptors and signal transduction molecules (6). The interference with so many critical biochemical processes makes their effects extremely pleiotropic. Although statins have so far been approved only for prophylaxis or treatment of cardiovascular diseases they also show some promising therapeutic activity in the management of Alzheimer’s disease, osteoporosis, graft rejection and cancer (7,8).

Despite alarming results of carcinogenicity studies in rodents (9-11) clinical observations did not show an overall increase in cancer incidence or mortality among statin users (12-14). In fact statins were rather associated with reductions in the incidence of colorectal, prostate and lung cancers as well as in melanoma (15-17). Suggestion that statins could increase the risk of breast cancer development was subsequently called into question by other studies (2,12,13,18). Despite promising preclinical activity of statins in numerous in vitro and in vivo rodent models of transplantable tumors none of the clinical studies showed significant antitumor activity of these drugs. It seems therefore that statins will not become effective antitumor agents used in monotherapy. Nonetheless, these drugs are among the most frequently prescribed therapeutics and are taken on a regular basis especially by elderly people. Therefore, any combinations with other drugs that would show potentiated antitumor activity by addition of statins might become of clinical significance.

The aim of this study was to evaluate antitumor effect of the combination of lovastatin and berberine, a naturally occurring isoquinoline alkaloid, produced by a number of medicinal
plants. *Berberis* (*Berberis vulgaris* from Berberidaceae family) is a plant growing in Europe and Asia and has been used for a long time in folk medicine. Its therapeutic value is attributed to the root, bark, leaves and fruits. Research carried out on the composition of *Berberis* extract enabled to isolate such compounds as berberine, berbamine or palmatine (19). Berberine seems to be the most potent of these alkaloids, responsible for the majority of pleiotropic effects. However, the remaining compounds may also play a certain role (20). Previous studies revealed that berberine not only decreases cholesterol concentration but also displays cytostatic and/or cytotoxic effects against a number of cancer cell lines (21-24).

Materials and methods

Reagents. Lovastatin in the inactive lactone form was obtained from Merck, Sharp and Dohme Research Laboratories (Rahway, NJ). It was converted to the active form by dissolving in ethanol, heating at 50°C in 0.1 N NaOH, and neutralizing with HCl. Distilled water was added to a final concentration of 10 mM. This stock solution was stored frozen (-80°C). Berberine hydrochloride, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP) and mevalonic acid (MA) were purchased from Sigma-Aldrich (St. Louis, MO).

Tumors. MDA-MB231 cells were obtained from ATCC (American Type Culture Collection, Rockville, MD, USA) and Panc 02, a murine pancreatic carcinoma cells were kindly provided by Carsten Ziske (Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany). Cells were maintained in DMEM or RPMI-1640 medium (Invitrogen, Co., Paisley, UK), supplemented with 10% heat-inactivated fetal bovine serum (FBS), antibiotics, 2-mercaptoethanol (50 µM) and L-glutamine (2 mM) (all from Invitrogen), hereafter referred to as culture medium. Cells were cultured in 75 cm² tissue flasks (Sarstedt, Nümbrecht, Germany) at 37°C in a fully humidified atmosphere of 5% CO₂, and were passaged every 3-4 days after short trypsinization with trypsin/EDTA (Invitrogen). Tumor cell viability estimated by trypan blue staining ranged between 95 and 97%.

Mice. C57BL/6 mice, 8-12 weeks of age were used in the experiments. Breeding pairs were obtained from the Animal House of the Medical Research Centre, Polish Academy of Sciences. Mice were kept in conventional conditions with full access to food and water during experiments. The animal studies were performed in accordance with the guidelines approved by the Ethics Committee of the Medical University of Warsaw.

Tumor treatment and monitoring. For in vivo experiments exponentially growing Panc 02 cells were harvested from culture flasks and washed twice in cold PBS (Polfa, Poland). Cells were then washed twice and resuspended in PBS at a concentration of 5x10⁶ cells/ml and injected (0.1x10⁶) into the footpads of the right hind limbs of C57BL/6 mice. Lovastatin was administrated intraperitoneally (i.p.) at a dose of 30 mg/kg daily starting from day 7 after inoculation of tumor cells for 14 consecutive days. Control mice received equal volume of 20% ethanol. Berberine was administrated per os at a dose of 100 mg/kg in the same schedule as lovastatin. Local tumor growth was determined as described (25), by the formula: tumor volume (mm³) = (longer diameter) x (shorter diameter)². Relative tumor volume was calculated as: relative tumor volume = [(tumor volume)/(initial volume)] x 100%.

Cytostatic/cytotoxic assay. The cytostatic/cytotoxic effects were measured using crystal violet staining. Briefly, MDA-MB231 or Panc 02, cells were dispensed into a 96-well plate (Sarstedt) at the concentration of 5x10⁴ cells per dish/100 µl, and allowed to attach overnight. The following day all reagents were added. Then, 48 h after incubation with reagents (berberine, lovastatin, mevalonic acid, FPP, GGPP) the cells were rinsed with PBS and stained with 0.5% crystal violet in 30% ethanol for 10 min at room temperature. Plates were washed four times with tap water and the cells were lysed with 1% SDS solution. Absorbance was measured at 595 nm using an enzyme-linked immunosorbent assay reader (SLT Labinstrument GmbH, Salzburg, Austria). Cytotoxicity was expressed as relative viability of tumor cells (% of control cultures incubated with medium only) and was calculated as follows: relative viability = (A_b-A_0) x 100/(A_b-A_c), where A_0 is the background absorbance, A_b is experimental absorbance, and A_c is the absorbance of untreated controls.

Cell cycle analysis. For the cell cycle analysis 1x10⁶ of tumor cells (Panc 02, MDA-MB231) were scraped off the Petri dishes (Sarstedt) in chilled PBS after incubation with lovastatin (0.5 µM for MDA-MB231 and 0.25 µM for Panc 02), berberine (5 and 10 µM) or both for 48 h. Tumor cells were then washed twice and fixed in 70% ethanol in PBS. Analysis of the cell cycle was performed with propidium iodide staining (Roche, Indianapolis, IN, USA) according to the manufacturer's protocols and analyzed using Vantage FACS (Beckton-Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. Data were calculated using Microsoft™ Excel 2007. Differences in *in vitro* cytotoxicity assays and tumor volume were analyzed for significance by Student's t-test. Significance was defined as a two-sided P<0.05.

The nature of the interaction observed between lovastatin and berberine was analyzed using the CalcuSyn software (Biosoft, Cambridge, UK) which uses the combination index (CI) method of Chou and Talalay, based on multiple drug effect equation (26). The constant ratio combination design was applied to assess the effect of both drugs in combination, in which dose-response curves were determined. The advantage of this method is the automatic construction of a fraction affected-CI table, graph and calculation of dose reduction indices by the software. CIs <1 indicate greater than additive effects (synergism) the smaller the value, the greater the degree of synergy), CIs equal to 1 indicate additivity, and CIs >1 indicate antagonism. The dose reduction index defines the extent of drug dose reduction possible in combination for a given degree of effect as compared with the dose of each drug alone.

Results

Berberine and lovastatin exert synergistic cytostatic/cytotoxic effects against tumor cells in vitro. Cytostatic/cytotoxic effects

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exerted by berberine and lovastatin as well as by their combination were investigated in human breast (MDA-MB231) and murine pancreatic (Panc 02) cancer cell lines using crystal violet staining. Both berberine and lovastatin exerted a dose-dependent growth inhibition against tumor cells. The combination of berberine and lovastatin exerted potentiated cytostatic/cytotoxic effects against both investigated tumor cell lines (Fig. 1A and C). The resulting data were elaborated with a dedicated Calcusyn software to verify potential synergistic interactions between the investigated agents using a Chow and Talalay calculations. In this mathematical model, synergism can be defined when the combination index (CI) is below 1.0 (when CI is below 0.5 the synergism is defined as very strong). We have found that the combination of berberine and lovastatin was highly synergistic when both drugs were used at equipotent concentrations against MDA-MB231 cells (Fig. 1B). A synergistic interaction was also noted for Panc 02 cells (Fig. 1D).

The combination of berberine and lovastatin induces cell cycle arrest and apoptosis. We selected such concentrations of berberine and lovastatin that were highly synergistic at Calcusyn calculations to evaluate the influence of the combination on the cell cycle progression and apoptosis induction in MDA-MB231 and Panc 02 cells (Table I). Lovastatin used at 0.5 and 0.25 µM concentration respectively, for 48-h incubation induced G1 arrest in MDA-MB231 cells only. Berberine at the concentration of 5 and 10 µM did not significantly influence cell cycle distribution or apoptosis induction in investigated tumor cells. However, the combination treatment strongly increased the percentage of MDA-MB231 cells in sub-G1 fraction (from maximal 11.42% in single-agent treated cells to 55.29% in the combination group) (Table I). In Panc 02 cells the combination of berberine and lovastatin resulted in almost 2-fold increase in the sub-G1 phase (from 6.99% in lovastatin-treated cells to 12.80% in the combination group) and significantly increased the percentage of cells in G1 phase of the cell cycle (Table I).

The cytostatic/cytotoxic effects of the combination of berberine and lovastatin do not result from potentiated inhibition of isoprenoid compound synthesis. By inhibiting HMG-CoA reductase, statins decrease synthesis of mevalonic acid-derived isoprenoid compounds thereby influencing post-translational modification of numerous proteins, including Ras and Rho family members. Incubation of Panc 02 and MDA-MB231 cells with mevalonic acid, farnesyl- or geranylgeranyl pyrophosphate abrogated cytostatic/cytotoxic effects of lovastatin (Fig. 2). Accordingly to our previous finding that berberine exerts cytostatic/cytotoxic effects independent of mevalonate pathway (27) we observed that mevalonate, FPP or GGPP do not influence the growth inhibitory effects of berberine (Fig. 2). The products of mevalonate pathway diminished cytostatic/cytotoxic effects of the combination treatment but only to the levels of berberine action indicating that berberine potentiates cytostatic/cytotoxic effects of
Lovastatin independent of inhibition of cholesterol synthesis pathway (Fig. 2).

To further verify the role of protein prenylation in the cytostatic/cytotoxic effects of combination with berberine, we combined the latter with an inhibitor of protein farnesylation (FtI) or geranylgeranylation (ggtI). Inhibition of farnesyl-transferase or geranylgeranyltransferase was ineffective in potentiating cytostatic/cytotoxic effects of berberine against MDA-MB231 (Fig. 3A and B) and panc 02 cells cells (Fig. 3c and D).

The combination of berberine and lovastatin exerts potentiated antitumor effects in vivo. Next, antitumor activity of the combination treatment was evaluated in vivo in a murine model of syngeneic pancreatic carcinoma (Panc 02) growing in C57/B16 mice. Treatment with berberine and/or lovastatin was started 7 days following inoculation of tumor cells. Berberine and lovastatin were used at 100 and 30 mg/kg doses, respectively on days 7-21. Although both drugs only slightly inhibited tumor growth when given alone, their concomitant administration resulted in a significant retardation of tumor growth as compared to all other groups (P<0.05 on days 15, 19 and 35) (Fig. 4).

Discussion

Molecular mechanisms underlying antitumor potential of statins seem to depend on inhibition of Ras and Rho protein...
Prenylation which results from blocking mevalonic acid metabolism. Moreover, statins induce cancer cell apoptosis and this effect was observed in many cancer cell lines (28-30). HMG-CoA reductase inhibitors affect cell cycle causing inhibition in G1 phase of cell cycle (9,28,31,32). Some researches show that this effect may depend on inhibition of Ras farnesylation, but also on inhibition of geranylgeranylation of other proteins, including Rho family members (33-35). Incubation of tumor cells with statins leads to increased levels of p21^CIP1/WAF1 and p27^KIP1 proteins, which are inhibitors of cyclin-dependent kinases, CDK (36,37). Statins can also decrease expression and/or activity of cyclins: A, D1, D3 and E, as well as cyclin-dependent kinases: CDK4 and CDK6 (38-40). Many studies revealed that statins potentiate cytostatic and/or cytotoxic effects in vitro or antitumor effect in experimental tumor models of many chemotherapeutics, including anthracyclines (30,41-43), cisplatin (44-46) or 5-fluorouracil (29,47).

Increasing evidence indicates that berberine, an ancient herbal medicine that decreases cholesterol concentration in humans, exerts potential cytostatic/cytotoxic effects, observed in a number of cancer cell lines (48). It was shown that incubation of tumor cells with berberine leads to cell cycle arrest in G1 phase, followed by apoptosis induction (49). Berberine decreases expression of cyclins D and C, as well as CDK2 and 6. Like statins it is also capable of increasing expression of cyclin-dependent kinase inhibitors (p21^CIP1/WAF1 and p27^KIP1). Berberine can also induce apoptosis, which results from a higher Bax/Bcl2 ratio (50). Apart from the influence on Bax expression it was also observed that berberine enhances expression of other pro-apoptotic proteins.
such as ATF3 or NAG-1 (50). Berberine-induced tumor cell apoptosis may also result from oxidative stress induction, which leads to cytochrome c release, caspase activation and cell apoptosis (51). Some studies suggest that this alkaloid can also increase p53 expression in cancer cells (52,53), but the mechanism of this effect remains unclear. Berberine also displays anti-angiogenic potential (54), and reduces cancer cells invasiveness and their metastatic potential due to inhibition of metalloproteinases (MMP-1, -2, -9) secretion from tumor cells (55-57), and induction of the synthesis of TIMP-2 (tissue inhibitor of metalloproteinase-2) (58). In an animal model berberine decreased the number of LLC lung cancer metastases to the local lymph nodes, which correlated with the inhibition of urokinase plasminogen activator (u-PA) (59), whereas by influencing bone marrow stromal cells berberine reduced the production of stromal-cell derived factor-1 (SDF-1), a chemokine ligand for CXCR4 receptor, regulating migration of leukemic cells (60).

The aim of this study was to verify whether lovastatin potentiates cytostatic/cytotoxic effects of berberine against tumor cells. Combined with berberine lovastatin exerted a synergistic cytostatic and/or cytotoxic effects against MDA-MB231 and Panc 02 tumor cell lines (Fig. 1). It was observed that the effect of berberine does not depend on blocking of the mevalonic acid metabolism, and cytotoxic effect of lovastatin combined with berberine is reversed by addition of the substrates of this pathway to the level brought out by lovastatin only (Fig. 2). Although contrary to previous studies that showed G1 cell cycle arrest in cells incubated with berberine alone (49) the combination of lovastatin and berberine produced G1 cell cycle arrest within 48 h followed by the transition of G1-arrested cells into sub-G1 fraction (Table I). Combination of lovastatin and berberine exhibited antitumor effects in vivo, despite negligible effects observed in single agent-treated mice (Fig. 4).

Based on these observations it can be speculated that the synergetic interaction of these drugs results from geranylgeranylation inhibition by statins and certain undefined mechanism brought out by berberine, independent of blocking mevalonic acid metabolism. The use of statins combined with berberine was previously tested in humans. Simvastatin combined with berberine caused increased expression of receptors for LDL and lower LDL concentration in serum of the patients. Such a combination did not exert any side effect in the study group which points to high safety profile of this drug combination (61). In numerous studies carried out hitherto statins showed a marginal or no antitumor effects both in animals and in humans (17,62,63). Therefore, it seems that statins are unlikely to be used purposefully in oncology as anticancer therapeutics in monotherapy. However, statins have a well-established position in therapy of human diseases, are act as therapeutics capable of potentiating antitumor effects of other drugs, but may also selectively neutralize some of their undesirable effects (41). On the other hand statins might occasionally inhibit or even neutralize beneficial antitumor effects of established anticancer therapeutics (64).

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