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SHORT COMMUNICATION

Cytotoxic effect of different statins and thiazolidinediones on malignant glioma cells

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Abstract
Purpose Glioblastoma multiforme is still a tumor with very poor prognosis. Statins are actually used for the treatment of dyslipidemias and thiazolidinediones for improving insulin sensitivity in diabetes. Statins are inhibitors of the cholesterol pathway, while thiazolidinediones are peroxisomal proliferator activator receptor γ (PPAR) agonists. For both, a potent pro-apoptotic activity has been suggested.
Methods We compared the antiglioma effect of simvastatin, atorvastatin, lovastatin, pravastatin, rosuvastatin, rosiglitazone, pioglitazone and their combinations at several concentrations on human glioblastoma cell lines U87, U 138, LN 405 and rat RG II. The cytotoxic effect was assessed using a cell proliferation assay after 48 and 144 h. Caspase 3 activity and the addition of isoprenoids and PPAR-y inhibitor GW9662 were assessed. Experiments were as well conducted under hypoxia for 24 h.
Results We demonstrated a significant cytotoxic effect with a combination of statins plus pioglitazone. The effect was observed after 48 h and dramatically increased after 144 h. The combination of 2 types of statins (synthetic and natural) allowed a fivefold dose reduction. Statin effect was reversed with isoprenoids and partially with PPAR-γ antagonists, while thiazolidinediones effect was slightly affected by PPAR-γ antagonists. A marked increase in caspase 3 activity was achieved by combining atorvastatin with lovastatin. Cytotoxicity of the combination of statins and thiazolidinediones did not decrease under hypoxia.
Conclusion The assessed combination of statins with thiazolidinediones shows a synergistic cytotoxic effect against glioblastoma cells in vitro, which could represent a feasible therapeutic schema.

Keywords Cytotoxicity · Glioblastoma · Statin · Thiazolidinediones

Introduction

Glioblastoma multiforme is the most common malignant brain tumor with a very poor prognosis. Despite the best current therapy, the median overall survival and progression-free intervals are short [23, 31, 32, 37]. New therapeutic approaches are urgently needed.

Statins are structural substrate analogs of β-hydroxy-β-methylglutaryl coenzyme A (HMG CoA) reductase, a rate-limiting enzyme of the cholesterol pathway. Statins reduce serum cholesterol levels as well as the mevalonate synthesis, which is a requisite for the entry of normal and tumor cells into the cell cycle. The production of mevalonate derivates is reduced and accordingly the levels of dolichol, ubiquinone, geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). These metabolites are required for the post-translational isoprenylation of GTP-binding proteins such as Ras and Rho, which regulate cell proliferation [20]. Ras and Rho are thought to play an essential role in the activation of phosphorylation

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cascades such as the MAPKs (mitogen-activated protein kinase) pathway [20]. Statins are currently used for the treatment of dyslipidemias and prevention of cardiovascular diseases, so far as reduction of stroke incidence [2, 12]. In addition to their primary use, the anticancer activity of statins was intensively studied for several kinds of tumors. The overall response rates in these studies, however, were limited [17]. Thiazolidinediones (TDZ) are agonists of peroxisome proliferator–activated receptor γ (PPAR). Besides its primary use in improving insulin sensitivity in type 2 diabetes mellitus, an anticancer activity was found [9, 16, 27, 34].

Recent studies on malignant glioma are restricted to simvastatin and lovastatin, so as to cerivastatin, which is no longer available on the market [4, 6, 7, 13, 15, 20, 21, 24, 33, 36, 38, 41–43]. A comparison of statins has not been reported yet. For thiazolidinediones, some studies have compared the 4 available TDZ with variable results [8, 36]. Agents that may synergize with statins or thiazolidinediones may substantially improve their application in clinical oncology. An increased effect could be demonstrated when combining both medicaments with other anticancer agents [3, 6, 39, 42–44]. Particularly, a combination of both drugs might be reasonable, because both drugs are able to modulate the lipid metabolism. Recently, it has been suggested that an activation of PPARs receptor by statins could be increased by TDZ [48]. The combination of lovastatin and troglitazone has already been proposed for increased antitumor activity [49].

Important differences among pleiotropic effects of different statins have been suggested [29, 40, 50]. For the most effective therapeutic regimen of statins and thiazolidinediones, it would be necessary to establish the most effective drug of each family. Other important considerations should be the clinically achievable concentration in plasma respectively in tumor cells as well as moderate and tolerable side effects. We hypothesized the existence of an important difference in the effects of statins alone when compared with their combinations with thiazolidinediones, which would allow developing an effective chemotherapeutic regimen.

Recently, studies by Akasaki et al. and Cafforio et al. indicate a facilitation of caspase 8 and 9, important activators of caspase 3 in the apoptosis pathway, by TDZ on glioma cells and by statins on myeloma cells [3, 5]. The direct or indirect effect of a combined therapy on caspase 3 activity has not been analyzed as of yet.

Hypoxia, typically found in tumoral environment, has become an important factor in chemo- and radioresistance [19]. An optimal therapy should be minimally affected by hypoxia or even better, it should be enhanced. We also studied the effect of a combination of TDZ and statins under hypoxic conditions for testing the performance in this principally different setting.

Materials and methods

Cell cultures

Three human malignant glioma cell lines (U87, U138, and LN405) and a rat glioma cell line (RG II) were investigated. The cell lines U87, U138, RG II were supplied by American Type Culture Collection (Manassas, VA, USA) and the cell line LN405 from the German Collection of Microorganisms and cell cultures (Braunschweig, Dtd). The cell medium for cell lines U87 and U138 was minimum essential medium Eagle with Earl′s salts and L-Glutamin and for cell lines LN405 and RG II was RPMI 1640 with L-Glutamin. All media were supplemented with 10% fetal calf serum, and the cells were grown at 37°C. The cells were seeded at a density of 2,000 cells per well into 96-well microtiter plates. After a 24 h preincubation in a volume of 100 µl of the appropriate cell culture medium, the cells were treated with study-relevant drugs for 48 or 144 h followed by MTT assay. The experiments were accomplished under normoxia (21% O_2).

Additional experiments were performed in conditions of hypoxia (0.1% O_2) for 24 h. For hypoxic treatment, cells were placed in Modular Incubator Chambers, which were flushed with 94% N_2, 5% CO_2 sealed, and then kept in a regular tissue culture incubator. After incubation, plates were collected and analyzed for surviving cells by MTT assay.

Drugs

Stock solutions of atorvastatin (synthetic statin) and the natural statins lovastatin, pravastatin, simvastatin were prepared from commercially available tablets using standard techniques [50]. The same procedure was applied for pioglitazone and rosiglitazone. Rosuvastatin calcium salt (synthetic statin) was synthesized at AstraZeneca Pharmaceuticals (United Kingdom). Geranygeranyl pyrophosphate, farnesyl pyrophosphate and GW 9662 were obtained from Sigma (St. Louis, MO). Drug stocks were prepared at 1,000-fold greater than experimental concentrations.

Cell viability assay

Cell viability was determined by the colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide assay (Sigma–Aldrich, Germany). After the different times corresponding to the treatment schedule, the cells were incubated for another 2 h at 37°C with the tetrazolium salt to allow formazan formation. Then lysates were prepared by 15-min shaking at room temperature with DMSO, which also serves as a solvent for the formazan dye. The absorbance at 562 nm (reference wavelength 620 nm) was

determined in an ELISA reader (Anthos, Krefeld, Germany). Viable cells were expressed as percentage of absorbance with respect to control/vehicle cells, which was considered as 100%.

Prenoids and PPAR antagonist

In order to assess the possible mechanism by which statins induce cytotoxicity, we applied the prenoids geranygeranyl pyrophosphate and farnesyl pyrophosphate at 10 μM. We hypothesized that statins could also activate PPAR-γ. We tested the effects after application of the PPAR-γ antagonist GW9662 at 20 μM. For that assessment, we repeated the above-mentioned experiments with statin, TDZ and their combinations while adding prenoids and the antagonist GW9662. All substances were applied simultaneously with the tested drug treatment on U87 cells. After 48 h, the cell viability assay was accomplished and compared with controls.

Caspase-3 function assay

Caspase activation of treated U87 cells was determined following the instructions of a caspase-3 detection kit (Bio Vision). Cells were lysed in a lysis buffer, and whole-cell lysates (20 μg) were incubated with 25 μmol/l fluorogenic substrate DEVD-AFC in a reaction buffer (containing 5 mmol/l DTT) at 37°C for 2 h. Proteolytic release of AFC was monitored at $ex = 405$ nm and $em = 500$ nm using a microplate reader (Tecan, Crailsheim). Fold increase in the fluorescence signal was calculated for each treated sample by dividing its normalized signal activity by that of the untreated control.

Statistical analysis

We used SPSS v.14 for Windows and R 2.5.2. All data were expressed as mean ± SD. Cellular viability and caspase activity data were analyzed by one-way ANOVA followed by multiple comparisons using least significant difference (LSD). For drug application under hypoxia, we fitted linear regression models. Statistical significance was $P < 0.05$.

Results

Comparison of cytotoxic effects of statins and TDZ

We tested the different drugs as single treatment on different cell lines. After 48 h, the maximal effect of statins was determined for lovastatin or simvastatin on cell lines U87, RGII, U138 and LN 405 with around 60% surviving cells. The subsequent order of cytotoxicity was identified: lovastatin = simvastatin > atorvastatin > pravastatin > rosuvastatin (Fig. 1). The incubation with statins for 144 h increased the toxicity with minimal surviving cells of 15% (for 5 μM lovastatin on U87 cell line) and of 27% (for 2.5 μM simvastatin on U87 cell line) (Fig. 1).

After 48 h pioglitazone 40 μM was able to reduce the cell survival to 45% (U138), respectively, and to 81% (U87). A mean cell survival of about 80% was observed with 2.5–5 μM pioglitazone (Fig. 1). Rosiglitazone showed lower effect with a mean survival of 85% in all of the assessed cell lines and with concentrations between 0.5 and 25 μM. After 144 h, the effect was significantly increased with a minimal rate of surviving cells of 35% on RG II after 25 μM pioglitazone and 59% on U87 after 25 μM piogliotazone, respectively (Fig. 1).

Combination of statin and TDZ

To improve the cytotoxic effect and to reduce the required dose, we combined one statin and one TDZ. The combinations with pioglitazone were more effective than with rosiglitazone. In comparison with a single drug, a lower dose was required. After 48 h (cell line U87), the most effective combination was lovastatin 5 μM + pioglitazone 40 μM (47% surviving cells), followed by atorvastatin 1.5 μM + pioglitazone 40 μM (52% surviving cells), simvastatin 2.5 μM + pioglitazone 40 μM (52% surviving cells) and pravastatin 4 μM + pioglitazone 40 μM (69% surviving cells).

After 144 h, the lowest surviving cell fraction of cell line U87 was 6% with atorvastatin 1.5 μM + pioglitazone 40 μM. A similar cytotoxic effect occurred with combinations of pioglitazone and lovastatin or simvastatin, but not for TDZ with pravastatin (40%) or rosuvastatin (15%). Clinically, pioglitazone 40 μM carries a high risk for unwanted side effects. Pioglitazone 5 μM after 48 h showed a modest effect. Its combination with atorvastatin 1.5 μM (around 60% cell survival after 48 h) was discreet less effective than with 40 μM (52%) (Fig. 2b).

Potentiating of statin by statin

The statins can induce different pleiotropic effects, which are variable for each substance. Natural and synthetic statins are basically different from each other. With this premise, we investigated combinations of these. The combination of lovastatin 5 μM + atorvastatin 1.5 μM substantially increased the cytotoxicity after 48 h of incubation (43% surviving cells, cell line U87) as well as the combination of lovastatin 5 μM + rosuvastatin 1.5 μM (32% surviving cells). At a lower dose, a toxicity of 50% was noted. The combination of rosuvastatin

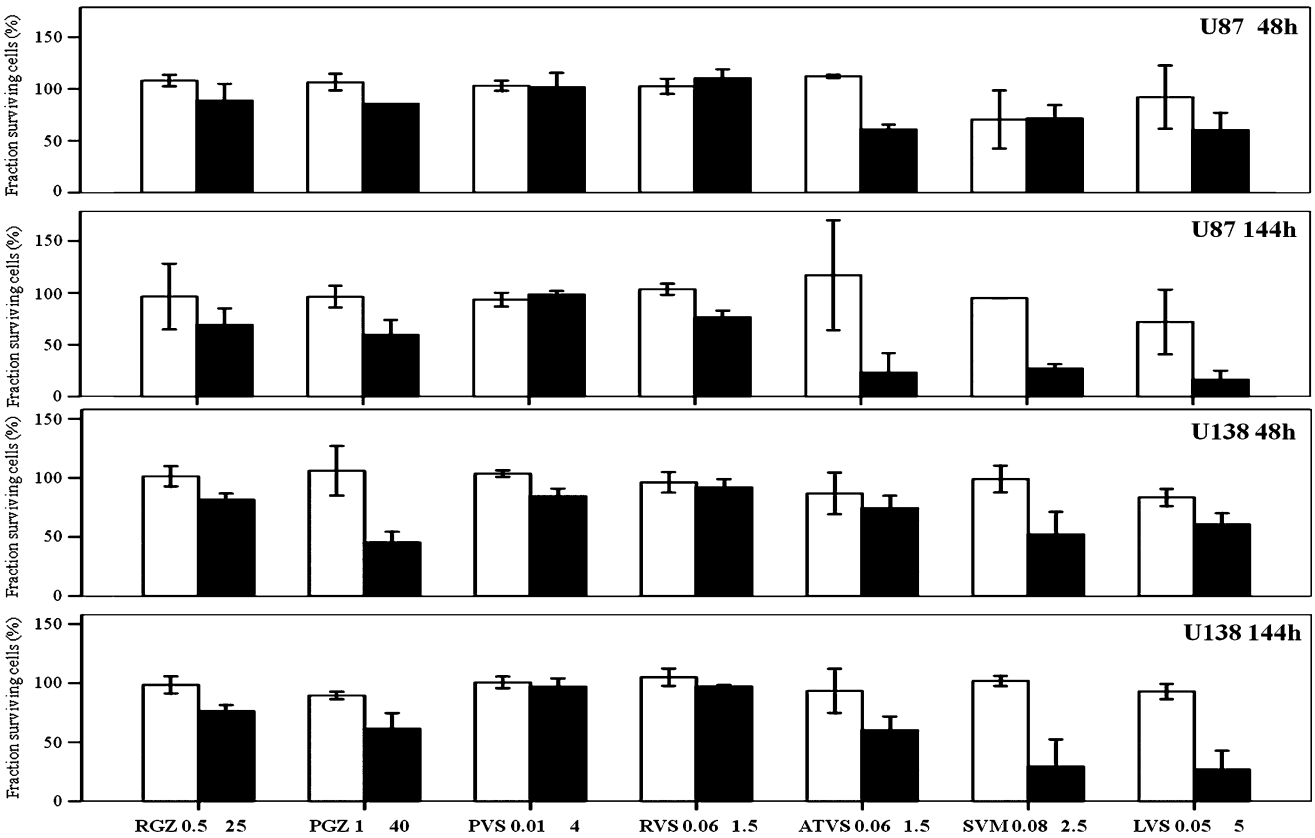

Fig. 1 Effects of TDZ and statins against U87 and U 138 (human) glioblastoma cells in vitro. Cells were seeded in 96-well plates (2,000 cells/well) for 24 h and then treated with the indicated concentrations for 48 and 144 h. Cell numbers are expressed as a relative viability (% of control), Mean ± SD. *ATVS* Atrovastatin, *LVS* Lovastatin, *PGZ* Pioglitazone, *PVS* Pravastatin, *RGZ* Rosiglitazone, *RVS* Rosuvastatin, *SMV* Simvastatin

1.5 μM + atorvastatin 1.5 μM (both synthetic) did not lead to more efficiency. All combinations with simvastatin exhibited a lower toxicity than those with lovastatin.

In pharmacological and animal studies, it is shown that a high concentration of a statin is not easily achievable in tumors [21]. Additionally, the risk of unwanted side effects increases with dose. For that reason, we tested combinations of lovastatin and atorvastatin, a lower dose of one and an increasing of the other one. In this way, similar effects like maximal dose (atorvastatin 1.5 μM + lovastatin 5 μM, 43% cell survival) were achieved with atorvastatin at 1.5 μM + lovastatin 0.5 or 1 μM (50% and 42% surviving cells, respectively).

Combination of three substances

Statins and PGZ showed a synergic effect as well as a combination of synthetic and natural statins. By combining three drugs, it was possible to achieve a slightly better effect with an important dose reduction. The combination of lovastatin 5 μM + atorvastatin 1.5 μM + pioglitazone 40 μM reduced surviving cells to 29.6% after 48 h (cell line U87). Lovastatin 2 μM + atorvastatin 0.48 μM + pioglitazone 10 μM reduced the survival to 50.6% of viable cells, which means, respectively, a 2.5-,three and fourfold lower dose than the maximally tested dose each (Fig. 2b).

Reversion of the statins' effect and TDZ

The co-administration of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) reversed the effect of statins in all experiments (cell survival >90%). Interestingly, the pioglitazone effect was slightly augmented with GGPP 10 μM or FPP 10 μM, which caused 53%, respectively, and 64% surviving cell, after 48 h in cell line U87. In case of combinations of two statins, the GGPP or FPP produced an effect reversion as well as increased growth (>100%), but not in combination of statin with TDZ (difference <2%).

The inhibitor of PPAR-γ, GW9662, was unable to prevent the cytotoxicity promoted by pioglitazone. The effects of statins as single drug or combination of 2 statins were reverted by 20 μM GW9662 with a mean cell survival

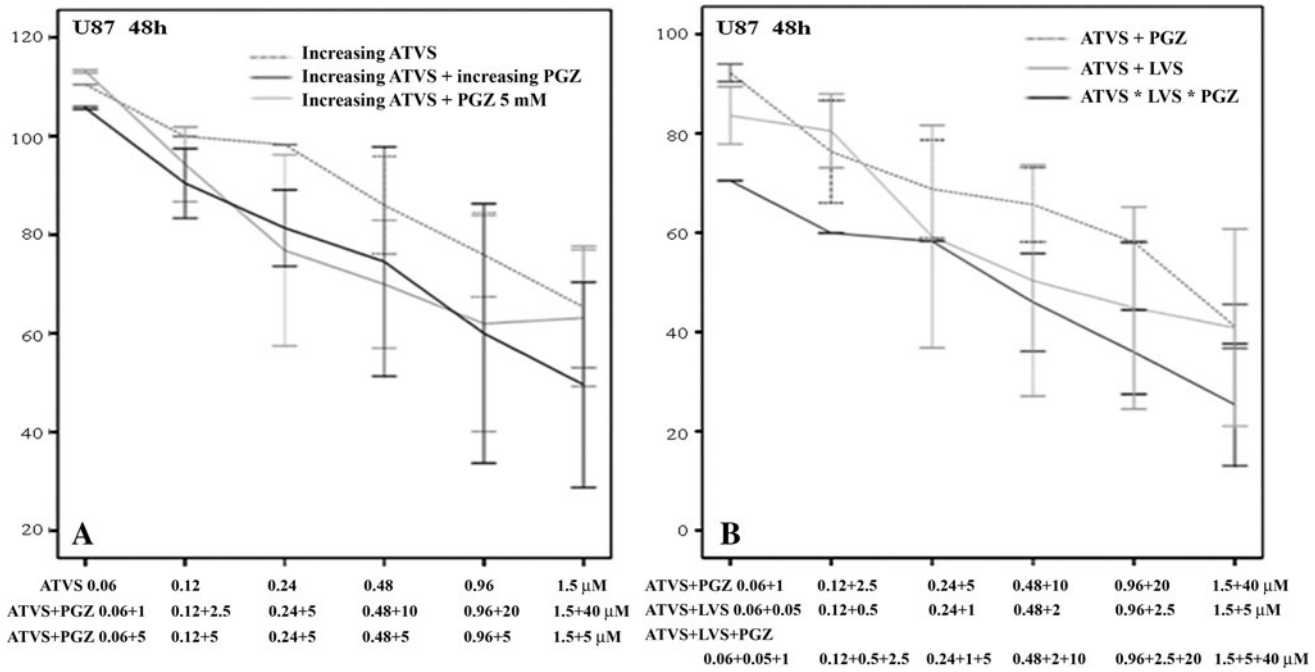

Fig. 2 Synergistic effects on U87 human glioblastoma cells of: **a** Atorvastatin with increasing dose and constant lower dose (5 µM) of pioglitazone and **b** Three substances: atorvastatin + lovastatin + pioglitazone. Cells were treated with the indicated concentrations for 48 h. Cell numbers are expressed as a relative viability (% of control), Mean ± SD. *ATVS* Atorvastatin, *LVS* Lovastatin, *PGZ* Pioglitazone

>90%. Combinations of statin with pioglitazone were not affected by adding GW9662.

Fluorometric caspase 3 activity after 48 h on cell line U87

The maximal increase in caspase 3 activity of 28% was registered for the combination of atorvastatin 1.5 µM with lovastatin 5 µM ($P < 0.001$). Lovastatin at 5 µM alone was unable to induce caspase 3 activity. Pioglitazone 40 µM (13.23%, $P = 0.15$) or atorvastatin 1.5 µM + PGZ 40 µM (12.73%, $P = 0.17$) showed a discrete activation only.

Effects under hypoxia

Some drugs and their combinations were tested under hypoxic conditions of 0.1% O_2 for 24 h. Regarding statins, the effect of lovastatin was not influenced by hypoxia on U87, but reduced on RG II ($P = 0.002$). Hypoxia increased the toxicity of atorvastatin on U87 ($P = 0.01$) and did not influence the results on RG II. For pioglitazone on the cell line U87 and RG II, no different results were obtained under hypoxia. After 24 h, the combination of atorvastatin or lovastatin and pioglitazone as well as lovastatin and atorvastatin did not show differences regarding oxygenation conditions in cell lines U87 and RG II.

Discussion

Despite the best management available, survival of patients with glioblastoma is still very poor until now. This aggressive tumor requires a wide spectrum of therapeutic approaches. Among the molecular mechanisms related to growth of glioblastoma, the Ras-MAPK pathway has been proposed as an important target. Prerequisite for Ras activation are isoprenoids, e.g. geranylgeranyl and farnesyl pyrophosphate [23]. Statins limit their production, and therefore, Ras and similar molecules (Rho, Rac) are regulated [20]. Multiple molecules related to cell cycle regulation are modulated as consequence [7, 13, 33, 41]. Several studies have described the existence of a considerable cytotoxic effect of statins on glioma cells [4, 6, 7, 13, 15, 20, 21, 24, 33, 38, 41]. Apoptosis induction was discussed as main cytotoxic mechanism of lovastatin in glioma cells due to its TRAIL-sensitizing effect [7] and due to the stimulation of the expression of the pro-apoptotic protein Bim [20]. The estimated dose required for achieving this cytotoxic effect is so high that a clinical

application is difficult. It is noteworthy that no activation of caspase-3 with 5 μM lovastatin and only limited activation by combined statins were observed in the present study.

Current studies on different kinds of tumors are controversial, because statins could also enhance the growth of cells [17]. This phenomenon could be related to the specific kind of statin, similar to cardiovascular prevention, immunomodulation and neuroprotection, where intermolecular differences conduce to different pleiotropic effects [29, 40, 50]. With our results, we confirmed this variability of pleiotropic effects of statins as chemotherapeutic agents. We confirmed the efficiency of suggested dose of 5 μM lovastatin previously reported by Yao et al. [49]. It is improbable to achieve this dose in clinical trials without unwanted side effects. Using lovastatin in doses ranging from 10–415 mg/m^2, Holstein et al. could demonstrate a peak plasma bioactivity of 0.06–12.3 μM. Only two patients, however, reached a concentration >5 μM [18]. In the Thibault trial, the mean level was 3.9 μM [47]. In an animal study, Gabryś et al. showed that after administration of lovastatin 50 mg/kg a tumor concentration between 0.023–0.41 μM can be obtained. Nevertheless, with this concentration, a reduction in the tumor volume was reported [13]. This lower dose could be an explanation for the poor results published in the clinical phase I/II trial with anaplastic astrocytoma and glioblastoma multiforme by Larner et al. [22]. In consideration of pharmacological aspects, a similar problem with other statins would be expected. We tested the combination of different statins in order to find a possible synergism related to molecular differences. Rosuvastatin or atorvastatin (synthetic statins) could significantly increase the cytotoxicity, when combined with a natural statin. A dose reduction was possible for the combination with lovastatin (from 5 to 0.5 μM), while the cytotoxic effect remained unchanged. In this way, a combination appears feasible to achieve a clinically tolerable concentration of the statins, for example, lovastatin until maximal serum concentration of 0.5 μM. On primary cultures of cortical astrocytes of rats, we found a dose-dependent light toxicity of the assessed drugs. However, the cytotoxic effect of a high-dose regimen toward malignant glioma cells was much more pronounced (data not shown).

Recently, PPAR-γ was described as a target in the treatment of brain tumors [27]. Some studies have been performed on glioma cells. The results were very promising [3, 8, 16, 28, 30, 35, 36, 38, 39, 42–44, 51]. Our study revealed that the PPAR-γ agonist pioglitazone is more cytotoxic than rosiglitazone. This finding is relevant because pioglitazone is the only TZD that has been shown to significantly pass the blood–brain barrier [28].

Some studies have tried successfully to combine TDZ with other therapies [39, 44]. So far just one study combined lovastatin and troglitazone with a very high synergic effect [49]. A superiority of troglitazone is widely suggested [8, 49]. Its clinical use, however, is quite limited because of its hepatotoxicity, which was the reason for the withdrawal of troglitazone from the market [11]. We tested the combination of different statins with the two currently available TDZ. We found a better potentiating effect with pioglitazone. Using this combination, it was possible to reduce the dose rate, and 5 μM pioglitazone with atorvastatin was nearly as effective as 40 μM pioglitazone plus atorvastatin. After 48 h, a similar effect of one single statin or statin plus TDZ each at maximal dose can be reached by combining two statins and one TDZ, at significantly reduced dose rates (lovastatin around 2.5 μM, atorvastatin 1 μM and pioglitazone 5 μM).

As previously suggested, isoprenoids compensate the statin effects [33]. We could confirm this phenomenon after application of isoprenoids GGPP and FPP. As these molecules are required for the activation of MAPKs-dependent pathways via activation of GTPases like Ras, Rho and similar, we can suggest that this pathway of cell proliferation may be one target of statins in neuro-oncology.

A possible activation of PPAR by statins has been proposed, and studies on monocytes showed an activation of MAPK and PPAR-γ by statins [1, 38]. Accordingly, the PPAR-γ inhibitor GW 9662 slightly affects the statin action in our series. Some studies with TDZ suggested that the antitumor effect is not related to PPAR-γ activation [3, 39]. This suggestion for the administration of PPAR-γ inhibitor was only partially confirmed in this study.

The caspase 3 activity was minimally increased by pioglitazone and unaffected by lovastatin alone. The combination of statin and TDZ produced a slight increase in caspase activity, while it was significantly stimulated by a combination of two statins. TDZ is argued to facilitate caspase 8 and 9 and TRAIL-induced apoptosis [3, 51]; however, this seems not to be the main mechanism. Inactivation of STAT 3 and down-regulation of FLIP and Bcl-2 are additional mechanisms considered for apoptosis induction of TDZ [3, 39]. Up to now, there is scarce evidence of caspase activation by statin on glioma, even if this possibility has been observed with myeloma cells [5]. Experimental evidence showed the proapoptotic properties of statin on several kinds of tumors [4, 45]. The possible mechanism for that is an up-regulation of proapoptotic proteins like Bax and Bim in combination with down-regulation of antiapoptotic proteins (Bcl-2) [10, 20, 46]. In order to fully explain the enhancement of cytotoxicity, especially of the combination of statin with TDZ, other death cell mechanisms need to be assessed because only a weak effect of caspase 3 activation could be detected in this study.

Hypoxia plays a predominant role in tumor development, angiogenesis and growing in many human tumors [19]. The necrotic areas found in glioblastoma look like hypoxic regions, although the contribution of hypoxia to this phenomenon is not clear. Hypoxia, however, can lead to increased tumor invasion, reduced apoptosis, chemoresistance, resistance to antiangiogenic therapy and radioresistance [25]. We therefore assessed the performance of statins and TDZ under hypoxic conditions. Since cell cultures are very critical under hypoxic conditions, these experiments were accomplished for 24 h only. Despite this short incubation time of 24 h with the medication, a modest cytotoxic effect can be observed. The effect of single therapy with statins alone was reduced, while pioglitazone or combinations of statin with TDZ were not influenced. Hence, by an approach combining statins with TDZ, it would be possible to limit the tumor resistance generated by hypoxia.

An additional antitumor effect of statins in vivo may be attributed to immunomodulation. An increased IL-18 production can be stimulated by statins; this cytokine regulates natural killers and other lymphocytic cells [46]. On the other hand, there is evidence for an immunomodulator role of TDZ, in oncology especially important would be IL-2 [26].

In vivo studies showed a weak effect of statin and TDZ as single therapy. In vivo both substances as single therapy can reduce the tumor volume, even if applied orally [13, 16, 44]. It could be possible that our observed effects would not be achieved in vivo, because of the variability in cell lines and the pharmacological properties of the applied drugs. In vivo studies with the combination of statins and TDZ, however, are still missing. This kind of studies and pharmacologic studies in humans are needed to determine the safety of such a combined therapy.

Of course, we cannot fully explain the mechanism of the cytotoxic effect until now; the different pleiotropic effects of statins and TDZ, however, should be considered as relevant for neuro-oncology. Recently, we showed the significant effects of this therapy in meningeoma cells as well [14]. Some antitumor effects of statins alone have been demonstrated in murine models, including nude mice [13] and recently the most appropriate, orthotopic model. The latter exploits syngeneic glioma cells implanted into the brains of normal, immunocompetent mice [52]. In this model, a dual behavior of simvastatin was observed, depending on the dose range, with tumor necrosis and apoptosis being enhanced only for the low-dose regimen [52]. The high-dose regimen increased the vessel diameters, suggesting adverse effects. Our study strongly supports that a combination of namely pioglitazone, lovastatin (natural statin) and atorvastatin (synthetic statin) had to be tested on an in vivo model, preferentially in the orthotopic mouse glioma model, as a feasible approach for glioma therapy.

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