Delta-24 Increases the Expression and Activity of Topoisomerase I and Enhances the Antiglioma Effect of Irinotecan

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Abstract

Purpose: In this study, we sought to determine whether Delta-24 could sensitize glioma cells to the topoisomerase I inhibitor irinotecan (CPT-11) and to identify the mechanisms underlying this enhanced anticancer effect.

Experimental Design: We used human glioblastoma cell lines for the in vitro studies. The expression of topoisomerase I was determined in Western blot analyses, and topoisomerase I activity was determined by measuring the relaxation of a supercoiled DNA. The cell cycle distribution of cells was determined by flow cytometry analysis of the cellular DNA content. Cell viability was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Tissue culture infection dose assays were used to quantitate adenovirus replication. For the in vivo studies, athymic mice received intracranial/intratumoral injections of Delta-24 in combination with CPT-11, after which animal survival was monitored.

Results: Delta-24 infection caused human glioma cells to accumulate in the S phase and induced the expression and activity of topoisomerase I as shown by Western blot and in vitro enzymatic activity assays. Further, we showed that the sequential administration of Delta-24 and CPT-11 to human glioma cell cultures potentiated the CPT-11-mediated anticancer effect in vitro without modifying the replicative phenotype of the oncolytic adenovirus. In vivo experiments showed that the single intratumoral administration of Delta-24 to intracranially implanted human glioma xenografts followed by the systemic administration of CPT-11 resulted in significantly prolonged animal survival.

Conclusions: The combination of Delta-24 treatment with CPT-11 showed an enhanced anticancer effect, which suggests that the interaction between adenoviral and human proteins can be exploited in rational anticancer therapies comprising replication-competent adenoviruses and conventional chemotherapeutic agents.

Tumor-selective replication is one of the most relevant advances in adenovirus-based anticancer therapies. The oncolytic virus is itself capable of lysing the infected tumor cell to eradicate or reduce tumor mass. Replication amplifies the input dose of the oncolytic virus and helps disseminate the agent to adjacent tumor cells. We have described previously such a conditionally replicative adenovirus, called Delta-24, which expresses a mutant E1A protein that is unable to bind to Rb (1). Because of this inability to bind to Rb, Delta-24 behaves like a wild-type adenovirus in cancer cells but does not replicate efficiently in nondividing normal cells. It has been reported that adenoviruses infect primarily quiescent cells and then induce them to enter the S phase of the cell cycle so that viral DNA synthesis can occur (2, 3). This ability to induce quiescent cells to enter the S phase makes these viruses attractive for use with agents, such as topoisomerase I inhibitors, which target cells in the S phase. Of particular interest, Delta-24 induces the accumulation of infected cancer cells in the S phase (1).

Previous studies have shown that the level of topoisomerase I expression correlates with sensitivity to the topoisomerase inhibitor camptothecin in some tumor cells (4). Topoisomerase I inhibitors are a class of agents that interfere with DNA "unwinding" during DNA replication and RNA transcription and stabilize DNA-topoisomerase I complexes through non-covalent interactions to yield enzyme-linked DNA single-strand breaks. The prolonged exposure of replicating cells to these agents produces lethal dsDNA breaks that can trigger programmed cell death (5). Therefore, strategies that up-regulate topoisomerase I protein levels and activity could enhance the effects of topoisomerase I-dependent chemotherapy. It has been reported that adenovirus infection also elevates...
cellular topoisomerase I levels, making adenoviruses even more attractive in combination with S-phase-specific agents (6, 7).

In this study, we sought to determine in vitro and in vivo whether Delta-24 could both sensitize glioma cells to the camptothecin analogue irinotecan (CPT-11) by up-regulating topoisomerase I expression and inducing cancer cells to accumulate in the S phase. Our results showed that the infection of cancer cells with Delta-24 resulted in the marked accumulation of cells in the S phase and in an increase in the levels and activity of topoisomerase I in human glioma cells. Further, we found that the sequential administration of Delta-24 and CPT-11 significantly prolonged the survival of glioma-bearing animals. Our study therefore showed that there is a rational basis for the combination of adenoviral therapy and chemotherapy and that the anticancer effect of the two agents was enhanced when given in combination.

Materials and Methods

Cell lines, adenoviral constructs, and infection conditions. The human glioma cell lines U-87 MG and U-251 MG were purchased from the American Type Culture Collection (Manassas, VA). Cell cultures were maintained in DMEM/F-12 (1:1 v/v; The University of Texas M. D. Anderson Cancer Center Media Core Facility) supplemented with 10% FCS and 1% antibiotic/antimycotic agent (Life Technologies, Grand Island, NY) in a humidified atmosphere containing 5% CO2 at 37°C. The replication-selective adenovirus Delta-24 has been described previously (8). This construct has a 24-bp deletion of the E1a region (nucleotides 92-946, both included), corresponding to amino acids L151-229 (ECHELAFG; a region required for Rb protein binding). As controls, we used the wild-type adenovirus Ad300 (9). Delta-24 virus inactivated by UV light (UVI Delta-24; inactivated by exposure to 200 J UV light), and mock infections with culture medium.

The glioma cells were infected as described previously (1). Briefly, the viral stocks were diluted to the indicated multiplicity of infection (MOI; plaque-forming units per cell), added to cell monolayers (0.5 mL/60-mm dish or 5 mL/100-mm dish), and incubated at 37°C for 30 minutes with brief agitation every 5 minutes. After this, the necessary amount of culture medium was added and the cells were returned to the incubator for the prescribed times.

Drugs. CPT-11 was kindly provided by Pharmacia Corp. (Kalamazoo, MI). Stocks of 20 mg/mL in aqueous solution were kept at 4°C.

Western blot analyses. Glioma cells were infected with 50 MOIs of Delta-24, wild-type Ad300, or UVI Delta-24 or were mock infected. Total cell lysates were prepared 20 hours after infection by incubating the cells in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mmol/L EDTA, and 50 mmol/L Tris (pH 7.4)] for 1 hour at 4°C. Protein (50 μg) from each sample was subjected to SDS-PAGE. The DNA content was measured in samples containing supercoiled plasmid substrate DNA, nuclear extract (5.0 μg/mL protein), and the assay buffer. Positive control samples contained topoisomerase I (5 units). The reaction mixtures were incubated at 37°C for 30 minutes, and the reactions were terminated by adding 5 μL stop buffer gel loading buffer. Proteinase K (Qiagen, Valencia, CA) was added to a concentration of 50 μg/mL, and the mixture was digested for 60 minutes at 37°C. Samples were loaded onto a 1% agarose gel and electrophoresed overnight at room temperature in a running buffer of Tris-acetate EDTA with chloroquine (0.2 μg/mL; Sigma-Aldrich, St. Louis, MO). The gel was stained with 0.5 μg/mL ethidium bromide.

Cell cycle analysis. The DNA content was measured in samples of 10⁶ cells that had been infected with 10 MOIs of Delta-24 or UVI Delta-24 or had been mock infected. Forty-eight hours later, cultures were treated with CPT-11 (4 μmol/L) or vehicle. Cells were trypsinized 3 to 5 days after drug treatment, fixed in 70% ice-cold ethanol, and incubated with propidium iodide (5 μg/mL) and RNase A (1 μg/mL) for 20 minutes at 37°C. All DNA content measurements were done with an EPICS XL-MCL cytometer (Coultier Corp., Hialeah, FL) equipped with an air-cooled argon ion laser emitting 488 nm at 15 mW. A multicycle program (Phoenix Flow System; Phoenix Controls Corp., San Diego, CA) was used for data analysis.

Cell viability assays. The chemosensitivity of the treated glioma cells was assessed by using the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) to measure cell viability. For this assay, 2 × 10⁶ cells per well were seeded in 96-well microtiter plates and infected 24 hours later with Delta-24 (at 1, 2.5, 5, or 10 MOIs) or UVI Delta-24 (10 MOIs) or were mock infected. Forty-eight hours after adenoviral treatment, the cells were treated with various concentrations of CPT-11. Triplicate wells were used for each condition. Sixteen wells seeded with untreated glioma cells were used as a viability control, and 16 wells containing only complete medium were used as a control for nonspecific dye reduction. Medium was removed 72 hours after drug treatment, and 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (1 mg/mL) was added to each well. The plates were then incubated for an additional 4 hours and then read on a Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA) at a test wavelength of 570 nm.

Viral replication assays. U-87 MG and U-251 MG human glioma cells were seeded at a density of 5 × 10⁴ per well in six-well plates and infected 20 hours later with Delta-24 or UVI Delta-24 at a MOI of 1. CPT-11 (5 μmol/L) was added 48 hours later. Three days after drug treatment, we scraped the cells into culture medium and lysed them with three cycles of freezing and thawing. We used the TCIID₂₅₀ method to determine the final viral titration as described previously (8). Briefly, the cell lysates were clarified by centrifugation and the supernatants were serially diluted in medium for infecting 293 cells in 96-well plates. We analyzed the cells for cytopathic effect 10 days after infection. Final titers were determined as plaque-forming units using the validation method developed by Quantum Biotechnology (Carlsbad, CA).

Animal model. To assess the potential therapeutic relevance of the findings from the in vitro studies, we tested the Delta-24/CPT-11 sequential treatment in an in vivo model of a human glioma xenograft implanted intracranially. We selected the U-87 MG cell line for this purpose because it produces gliomas in nude mice with highly predictable growth kinetics and well-characterized pathologic features (12). To perform a reliable multiple-dose experiment, we used an implantable guide-screw system developed in our laboratory that allows for multiple, precise intratumoral administrations of a therapeutic agent (12) and has been validated for testing the antiangiogenic effect of Delta-24 (8). In this study, 5 × 10⁵ cells of the U-87 MG human glioma cell line...
Results

**Delta-24 infection enhanced expression and activity of topoisomerase I.** We investigated whether Delta-24 adenovirus could sensitize glioma cells to the camptothecin analogue CPT-11 by up-regulation of topoisomerase I expression. We first assessed the expression of topoisomerase I in the U-87 MG and U-251 MG human glioma cells after infection with Delta-24. We selected these two cell lines because we had already used them to characterize the antiglioma effect of Delta-24 (1). Western blot analysis showed that endogenous topoisomerase I was expressed at a low level in both glioma cell lines (Fig. 1A). However, treatment with Delta-24 resulted in at least a 4-fold increase in topoisomerase levels in both cell lines compared with mock-infected or UVi Delta-24–infected cells. Cells infected with the wild-type adenovirus exhibited an increase in topoisomerase I expression similar to that seen in the Delta-24–infected cells. We next determined whether Delta-24 infection resulted in increased topoisomerase I activity in glioma cells in culture. A plasmid DNA used as a template for the topoisomerase I reaction incubated with UVi Delta-24–infected nuclear extracts appeared predominantly in the supercoiled form, similar to the finding in the control cells containing the form I DNA plasmid without topoisomerase I. In addition, Delta-24–infected nuclear extracts from both glioma cultures displayed a topoisomerase I activity that caused the plasmidic DNA to relax comparable with the finding in the topoisomerase I–treated positive controls (Fig. 1B). Taken together, these observations indicate that infection with the Delta-24 adenovirus increases topoisomerase I protein levels and activity.

**Cell cycle profile of Delta-24- and CPT-11-treated cells.**

Previous data from our group showed that Delta-24 infections cause cells to accumulate in the S phase of the cell cycle (1). In this study, U-87 MG and U-251 MG human glioma cells were infected with Delta-24 adenovirus and treated 2 days later with CPT-11. Cells were then collected and their DNA content was examined by flow cytometry. As expected, the accumulation of Delta-24-infected cells in the S phase of the cell cycle was striking (>70% of the cells in culture) and statistically significant in comparison with the control cells infected with UVi Delta-24 (P < 0.001; Table 1). We also showed that treatment with CPT-11 resulted in an accumulation of cells in the G2-M phase (>55% of the cells in culture; P < 0.001, compared with vehicle-treated cells; Table 1).

We next investigated the effect of the combination of the Delta-24 adenovirus and CPT-11 on cell cycle progression. Cells infected with Delta-24 and then treated 48 hours later with CPT-11 showed an overrepresentation of cells in the S phase (>65% of the cells in culture) with a dramatic decrease in the G2-M population (<20% of the cells in culture). Thus, cells treated with a combined regimen exhibited a cell cycle profile similar to that of cells treated with Delta-24 alone (Table 1). These data indicate that Delta-24 infection overrides the G2-M arrest induced by CPT-11 and maintains a large population of

<table>
<thead>
<tr>
<th>Cell line/treatment</th>
<th>G0-G1</th>
<th>S</th>
<th>G2-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-87 MG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UVi + vehicle</td>
<td>77 ± 1.7</td>
<td>14 ± 0.86</td>
<td>8.9 ± 1</td>
</tr>
<tr>
<td>UVi + CPT-11</td>
<td>23.7 ± 2.9</td>
<td>20.5 ± 5</td>
<td>55.8 ± 3.9</td>
</tr>
<tr>
<td>Delta-24 + vehicle</td>
<td>21.3 ± 8.4</td>
<td>71.6 ± 9.1</td>
<td>6.8 ± 0.4</td>
</tr>
<tr>
<td>Delta-24 + CPT-11</td>
<td>24.1 ± 6.2</td>
<td>66.5 ± 5.9</td>
<td>9.4 ± 0.28</td>
</tr>
<tr>
<td>U-251 MG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UVi + vehicle</td>
<td>68.1 ± 6.8</td>
<td>21.7 ± 6.6</td>
<td>10.1 ± 3.8</td>
</tr>
<tr>
<td>UVi + CPT-11</td>
<td>13.5 ± 4.1</td>
<td>15.4 ± 8.2</td>
<td>70.9 ± 4.6</td>
</tr>
<tr>
<td>Delta-24 + vehicle</td>
<td>11.3 ± 7.9</td>
<td>85.8 ± 8.5</td>
<td>2.5 ± 1.2</td>
</tr>
<tr>
<td>Delta-24 + CPT-11</td>
<td>9.5 ± 10.4</td>
<td>74.3 ± 8.1</td>
<td>16.1 ± 15.8</td>
</tr>
</tbody>
</table>

*NOTE: Data are median ± SD.*
cells in the S phase, suggesting that Delta-24-infected cells are likely to be particularly susceptible to S-phase-based chemotherapeutic agents.

**Effect of CPT-11 and Delta-24 on proliferation of human glioma cells.** We next ascertained the sensitivity of U-87 MG and U-251 MG glioma cells to CPT-11. In both cell lines, CPT-11 inhibited cell proliferation in a concentration-dependent fashion as assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide bioreduction assay (Fig. 2A). In contrast, no significant inhibition was seen in control cells treated with equivalent concentrations of vehicle and mock infected or infected with a UVi Delta-24 adenovirus. Note the dose-dependent effect of the combined therapy. The shift to the left of the IC50 for CPT-11 is noticeable in control cells treated with equivalent concentrations of vehicle and mock infected or infected with a UVi Delta-24 adenovirus. Note the dose-dependent effect of the combined therapy. The shift to the left of the IC50 for CPT-11 is noticeable in both cell lines treated with input doses of 2 and 5 MOIs. Summary of the IC50s for CPT-11 in single or combined therapy with the oncolytic adenovirus is shown in Table 2. B, effects of a single dose of Delta-24 or CPT-11 and the combination of both on glioma cell growth in culture. Viability is expressed as a relative percentage of cells treated with UVi Delta-24 and vehicle (equal to 100%). Bars, SD.

**Table 2.** IC50s of CPT-11 in human glioma cell lines when given in combination with Delta-24

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mock</th>
<th>UVi Delta-24 (10 MOIs)</th>
<th>1 MOI</th>
<th>2 MOIs</th>
<th>5 MOIs</th>
<th>10 MOIs</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-87 MG (P) *</td>
<td>3.4 ± 0.6</td>
<td>3.4 ± 0.5 (0.7)</td>
<td>3.6 ± 0.1 (0.2)</td>
<td>2.75 ± 1.2 (0.04)</td>
<td>1.8 ± 0.5 (0.001)</td>
<td>1.5 ± 0.5 (0.001)</td>
</tr>
<tr>
<td>U-251 MG (P) *</td>
<td>7.2 ± 1.5</td>
<td>7.8 ± 2.4 (0.3)</td>
<td>4.6 ± 1.6 (0.01)</td>
<td>2.6 ± 1.4 (0.003)</td>
<td>1.1 ± 0.1 (0.001)</td>
<td>1 ± 0.1 (0.001)</td>
</tr>
</tbody>
</table>

*Compared with mock-treated cultures (double-sided t test).
that the sequential administration of Delta-24 and CPT-11 had an anticancer effect in vivo. This constitutes further evidence showing that most tumors, including malignant gliomas, have a better response to combination therapies than to single therapies. Results from this study also indicated that there is a rational basis for the development of more effective cancer therapy consisting of the combined and sequential administration of adenovirus and chemotherapy, specifically the sequential administration of an oncolytic adenovirus and S-phase-specific chemotherapy.

In the clinical setting, the combination of a mutant adenovirus, the dl1520 (also called ONYX-015) oncolytic adenovirus (13), and chemotherapy has already been proven successful. In particular, the combined administration of cisplatin or 5-fluorouracil with dl1520 had a dramatic anticancer effect in patients with head and neck cancer (14). The mechanism by which the adenovirus enhances the effect of cisplatin or 5-fluorouracil has not yet been elucidated, but several factors have been proposed to explain the additive or synergistic effect of these combinations. One factor may be the local production of tumor necrosis factor after the intratumoral injection of the adenovirus (15). Because the dl1520 adenovirus causes cancer cells to enter the S phase, our observations in the present study

Fig. 3. Replication efficiency of Delta-24 alone or in combination with CPT-11 in human glioma cells. Results in U-87 MG and U-251 MG glioma cells infected with Delta-24 (at a dose of 1 MOI) alone or together with CPT-11 (5 μmol/L). Forty-eight hours after adenoviral treatment, CPT-11 was added, and 3 days later, the viral titers [expressed as plaque-forming units (p.f.u.)/mL] were determined by the TCID50 method. Columns, mean of three independent experiments; bars, SD. *, P < 0.1; †, P > 0.1, double-sided t test.

27 days in the control group of animals (treated with vehicle plus UVi Delta-24), and all these animals died by day 32. Treatment with CPT-11 (plus UVi Delta-24) or a single dose of Delta-24 (plus vehicle) extended the survival by an average of 4 and 8 days, respectively (P = 0.001 and P < 0.0001, respectively, compared with vehicle-treated animals). The combination treatment consisting of Delta-24 followed by CPT-11 resulted in the most substantial increase in animal survival (median overall survival of 42 days; Fig. 4A and Table 3). In addition, the overall survival of the animals treated with the combined therapy differed significantly from that in animals treated with either agent alone (P < 0.005, log-rank test), as did differences in the 60-day survival rate (P < 0.012, Fisher’s test). Of further relevance, there were no long-term survivors among the animals receiving a single-treatment regimen; however, 7 of 31 (22.5%) animals treated with the combination of Delta-24 and CPT-11 survived >3 months after tumor implantation without showing any sign of neurologic distress. Examination of the brains of the animals showed that all animals that died had evidence of intracranial tumor. In contrast, microscopic examination of the brains of the long-term surviving animals that received the combination treatment showed complete tumor regression. In these animals, we did, however, identify sequelae of the tumors, including dystrophic calcification and microcytosis, at the tumor implantation site in the right caudate nucleus (Fig. 4B).

Immunohistochemical analyses of the brains of the long-term survivors using both anti-E1A and anti-hexon antibodies failed to detect viral particles (data not shown). Further, we did not observe E1A expression or signs of inflammation in the normal brain tissue.

**Discussion**

Our data revealed, for the first time, that the infection of human glioma cells with a mutant replication-selective adenovirus, Delta-24, up-regulated topoisomerase I and increased chemosensitivity to CPT-11. In addition, we showed
indicate that cell cycle–related chemosensitivity may be another key mechanism. Other anticancer therapies, such as radiotherapy, have also been reported to be potentiated by oncolytic adenovirus, although the underlying mechanisms for this are being investigated as well (16, 17).

In this study, we combined Delta-24 with CPT-11 for several reasons. First, chemotherapy remains part of the treatment for gliomas, with some of the most encouraging results to date seen for chemotherapy-based treatments of malignant gliomas coming from clinical trials of CPT-11 (18). Due to its extremely short half-life, SN-38, the active metabolite of CPT-11, cannot be directly administrated in malignant gliomas coming from clinical trials of CPT-11 (18). Therefore, we opted to perform this study using CPT-11 both in vitro and in vivo. Moreover, it has been already shown that cancer cell lines, including glioma cell lines, express carboxylesterase activity and are able to convert CPT-11 into SN-38 (19, 20). We believe that the use of CPT-11 in both in vitro and in vivo makes this study more internally consistent than using separate compounds in both tested scenarios. Second, the effect of CPT-11 is most marked in cells in the S phase. In particular, flow cytometric analyses of cell death triggered by DNA topoisomerase I inhibitors have shown that, in most cases, S-phase cells were mainly affected by these agents (21). These data corroborated those from previous studies indicating that cells in the S phase are 100 to 1,000 times more sensitive to camptothecin than cells in the G1 or G2 phase (22). Our data are consistent with the data from these studies, further showing that inducing cells to enter the S phase constitutes a clear rationale for the combined use of S-phase-targeted therapies and oncolytic adenoviruses. In addition to possibly enhancing the effect of the drug on cells already in the S phase, the ability of adenoviruses to infect quiescent cancer cells may also render slow-growing tumors more sensitive to S-phase-dependent treatments. On the other hand, replication of the adenovirus in cells treated with topoisomerase I inhibitors is probably possible because the adenovirus encompasses its own topoisomerase I and helicase functions (23, 24) that can lead to efficient replication in a cell-free environment (25). Finally, we chose CPT-11 because, in addition to inducing cells to enter the S phase, adenovirus infection induces increased levels of topoisomerase I (6, 7). Our data confirmed these previous observations and suggest that increased topoisomerase I levels and activity during adenoviral infection could make cancer cells more sensitive to CPT-11. This would be particularly important in those cases in which resistance to the drug is due to low topoisomerase I levels.

Delta-24 is being tested by independent groups in several laboratories (26, 27), and modifications of this construct could be available for clinical use soon. Although, at this stage, the toxicity associated with the combination of oncolytic adenoviruses and topoisomerase I inhibitors is not known, our data encourage further studies of this therapeutic modality. We conclude that there is a clear molecular and cellular rationale for combining topoisomerase I inhibitors and oncolytic adenoviruses for the treatment of gliomas and that oncolytic adenoviruses have much to offer as anticancer agents, especially in combination with the conventional therapies chemotherapy and radiotherapy. As more is learned about the molecular mechanisms of oncolytic adenoviruses, the rational combinations of more potent agents with a greater anticancer action will become clearer.

Acknowledgments
We thank Betty P. Notzon (Department of Scientific Publications, M.D. Anderson Cancer Center) for editorial assistance.

Table 3. Statistical analysis for the in vivo testing of combined Delta-24 and CPT-11 treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>median (days)</th>
<th>95% CI (days)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV + Veh</td>
<td>13</td>
<td>27</td>
<td>27, NA</td>
<td></td>
</tr>
<tr>
<td>UV + CPT-11</td>
<td>23</td>
<td>31</td>
<td>30, 33</td>
<td>0.0013</td>
</tr>
<tr>
<td>Delta-24 + Veh</td>
<td>27</td>
<td>35</td>
<td>35, 38</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Delta-24 + CPT-11</td>
<td>31</td>
<td>42</td>
<td>38, 46</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

NOTE: The P values represent the comparison of every treatment with vehicle treatment.

References
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