Acetyl-L-Carnitine Prevents and Reverts Experimental Chronic Neurotoxicity Induced by Oxaliplatin, Without Altering its Antitumor Properties

ORLANDO GHIRARDI, PIETRO LO GIUDICE, CLAUDIO PISANO, MARIO VERTECHY, AUGUSTA BELLUCCI, LOREDANA VESCI, SANTE CUNDARI, MARIAROSARIA MILOSO, LAURA MARIA RIGAMONTI, GABRIELLA NICOLINI, CLAUDIO ZANNA and PAOLO CARMINATI

1Research and Development, Sigma-Tau S.p.A. Industrie Farmaceutiche Riunite, Rome; 2Department of Neuroscience and Biomedical Technologies, University of Milan "Bicocca", Monza, Italy

Abstract. Background: Oxaliplatin (OHP) is severely neurotoxic and induces the onset of a disabling sensory peripheral neuropathy. Acetyl-L-carnitine (ALC), a natural compound with neuroprotective action, was tested to determine whether it plays a protective role in OHP-induced neuropathy. Materials and Methods: Peripheral neuropathy was induced in Wistar rats, and the effect of OHP alone or in combination with ALC was assessed, using behavioral and neurophysiological methods. Moreover, ALC interference on OHP antitumor activity was investigated using several in vitro and in vivo models. Results: ALC-co-treatment reduced the neurotoxicity of OHP when it was coadministered. Furthermore, the administration of OHP, once OHP-induced neuropathy was established, significantly mitigated its severity. Finally, experiments in different tumor systems indicated that ALC does not interfere with the antitumor effects of OHP. Conclusion: ALC is effective in the prevention and treatment of chronic OHP-induced peripheral neurotoxicity in an experimental rat model.

One of the most interesting platinum-derived drugs is oxaliplatin [(trans-l)1,2-diaminocyclohexanedioxalatoplatinum(II)] (OHP), an agent with a 1,2-diaminocyclohexane carrier ligand that produces bulkier DNA conjugates than other platinum-derived drugs due to the restricted freedom of motion of the platinum atom (1). OHP is a very promising antineoplastic drug, despite its neurotoxicity.

Acetyl-L-carnitine (ALC) is a member of a family of natural compounds which perform an essential role in intermediary metabolism (10-13). It has been found to be effective as a neuroprotectant in peripheral nervous system disorder models such as diabetic neuropathy (14-16) and motoneuron survival after axotomy (17).

In the present study, the effect of ALC as a putative neuroprotectant capable of preventing the onset of, or enhancing recovery from, OHP-induced peripheral neurotoxicity was studied in rats, using behavioral and neurophysiological methods. Moreover, a lack of interference by ALC with the antitumor activity of OHP in both in vitro and in vivo models was demonstrated.

Materials and Methods

The animals were housed in a limited access animal facility. The animal room temperature and relative humidity were set at 22±2°C and 55±10%, respectively. Artificial lighting provided a 24-hour cycle of 12 hours light/12 hours dark (7 a.m. - 7 p.m.). The care and husbandry of animals were in accordance with European Directives no. 86/609, and with Italian D.L. 116, January 27, 1992. All experiments were approved by the Sigma-Tau veterinarian.
Drugs. ALC was supplied by Sigma-Tau (batch 9747). Oxaliplatin was provided by Debiopharm SA (Lausanne, Switzerland).

Behavioral experiment. Seventy-four 15 to 16-week-old (360-380 g) male Wistar rats (Charles River, Calco, Italy) were used. OHP, dissolved in sterile saline, was administered at a dose of 3 mg/kg i.p. three times weekly for the first week, twice a week for the second week and once weekly for the third week. Control animals were treated with the vehicle alone. ALC was dissolved in sterile saline and administered at a dose of 100 mg/kg/day s.c. The effect of ALC was evaluated both when administered together with OHP and when administered after OHP.

The Randall-Selitto paw-withdrawal test was used to assess mechanical hyperalgesia (18). The nociceptive flexion reflex was quantified with an analgesimeter (Ugo Basile, Varese, Italy), which applies a linearly increasing mechanical force to the dorsum of the rat’s hindpaw. The nociceptive threshold was defined as the force which induced rat paw withdrawal. Rats were habituated to the testing procedure during the week prior to the experiments (19). The test was conducted just before the experiment began (basal) and weekly following OHP administration.

Neurophysiological experiment. Ninety-four 11 to 13-week-old (175-225 g) female Wistar rats (Charles River Laboratories, Iffa-Credo, France) were used for this set of experiments. In all the experimental paradigms, neuropathy was induced by repeated i.v. OHP administration (3 mg/kg twice a week for 4 weeks). Control animals were treated with the vehicle alone.

In the first experiment, ALC was administered i.v. to a group of rats at a dose of 30 mg/kg twice a week for 4 weeks, together with OHP.

In the second experiment, ALC was administered at a dose of 150 mg/kg p.o. in drinking water starting one week before OHP administration.

In the third experiment, ALC was administered to a group of rats at a dose of 100 mg/kg/day s.c., starting on the same day as the first OHP administration. At the end of the treatment, OHP neuropathic rats were divided into two homogeneous groups: one receiving ALC treatment (100 mg/kg/day s.c. for 3 weeks), the other used as a neuropathic control.

Before, during and after the treatment, each animal underwent determination of sensory nerve conduction velocity (SNCV) in the tail (20, 21). Briefly, the antidromic SNCV in the tail nerve was assessed by distally placing recording ring electrodes in the tail, while the stimulating ring electrodes were placed 5 cm and 10 cm proximally, according to the recording point. The latencies of the potentials recorded at the two sites after nerve stimulation were determined (peak-to-peak), and nerve conduction velocity was calculated accordingly. All the neurophysiological determinations were performed under standard conditions. Animal body temperature was maintained constant (37°C) throughout the experiment (BM 70002-type thermoregulator for animals, Biomedica Mangoni, Pisa, Italy).

A summary of the experimental in vivo paradigms is reported in Table I.

Statistical analysis. Statistical comparisons between groups were performed by using repeated measure analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) multiple comparison test. Differences in p-values <0.05 were considered significant.

Table I. Summary of the experimental paradigms in neuroprotection studies.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Single dose</th>
<th>Schedule</th>
<th>Route</th>
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<tbody>
<tr>
<td>OHP</td>
<td>3 mg/kg</td>
<td>three times/week x 1 week</td>
<td>i.p.</td>
</tr>
<tr>
<td>ALC</td>
<td>100 mg/kg</td>
<td>twice/week x 1 week daily</td>
<td>s.c.</td>
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Neurophysiological studies

<table>
<thead>
<tr>
<th>OHP</th>
<th>3 mg/kg in all experiments</th>
<th>twice/week x 4 weeks</th>
<th>i.v.</th>
</tr>
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<tbody>
<tr>
<td>ALC</td>
<td>30 mg/kg</td>
<td>daily (starting on day –7)</td>
<td>o.s.</td>
</tr>
<tr>
<td>ALC</td>
<td>150 mg/kg</td>
<td>daily during treatment</td>
<td>s.c.</td>
</tr>
<tr>
<td>ALC</td>
<td>100 mg/kg</td>
<td>daily during follow-up treatment</td>
<td>s.c.</td>
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Cytotoxicity experiments

In vitro studies. The NCI-H460 non-small cell lung carcinoma (NSCLC), HT-29 colon adenocarcinoma, PC3 prostate carcinoma, A2780 ovarian carcinoma and HeLa cervix uteri carcinoma cells were grown in RPMI 1640 (Sigma Chemicals Aldrich, Milan, Italy). The IGROV-1 ovarian carcinoma cells were grown in DMEM (Life Technologies, Inc.-Invitrogen, Paisley, UK). The LoVo colon adenocarcinoma was grown in Ham’s F12 (Life Technologies). NTERA-2 testicular teratocarcinoma was grown in Vitacell DMEM (American Type Culture Collection, Manassas, VA, USA).

For the cytotoxicity assay, cells were seeded in 96-well tissue culture plates (Corning, New York, NY, USA) and were allowed to attach and recover for at least 24 h. Varying concentrations of OHP alone or combined with ALC (200 µg/mL) corresponding to 1 mM were then added to each well. The plates were incubated for 2 h and then washed before being incubated without drugs for an additional 72 h. The number of surviving cells was then determined by staining with sulforhodamine B (SRB), as described by Skehan et al. (22). IC50±sD values were evaluated using an "ALLFIT" program.

In vivo studies. Swiss nu/nu female mice and BDF1 female mice, 5 to 6 weeks of age (Harlan, Correzzana, Italy), were used for this set of experiments.

L1210 murine leukemia was routinely maintained by i.p. passages (every 10–14 days) of 1x10^5 cells/mouse in BDF1 mice. For anti-tumor activity experiments, 5 mL of Hank's 199 medium were injected i.p. Cells were withdrawn from donor mice, diluted 1:10 (v/v) in the same medium and re-suspended at a density of 1x10^6 cells/mL. Then, 1x10^6 cells/100 µL/mouse were injected i.p. into BDF1 mice. Each experimental group included 8 tumor-bearing mice. Tumors were implanted on day 0. OHP was delivered i.p. in a volume of 10 mL/kg at doses of 12.5 and 6.25 mg/kg according to q4dx3 schedule, and ALC was given at 100 mg/kg p.o. for 14 days; both drugs were administered starting 1 day after tumor injection. Median survival time for each experimental group was analyzed. Increase in Life Span% (ILS%) was evaluated as [(median survival time of treated group/median survival time of control group) x 100] – 100. Cured mice were evaluated 100 days after tumor implantation.
Figure 1. ALC and OHP-induced neuropathy: mechanical nociceptive threshold score (ALC treatment starting from day 1 of OHP administration).

Figure 2. ALC and OHP-induced neuropathy: mechanical nociceptive threshold score (ALC treatment starting from day 24 of OHP administration).
HT-29 human colon adenocarcinoma cells from in vitro cell cultures were injected s.c. using 5x10^6 cells/mouse into the right flank of Swiss nude mice. Each experimental group included 8 tumor-bearing mice. Animals received two i.p. doses (15 and 10 mg/kg) of OHP during the fourth, sixth and tenth day after tumor injection. ALC was administered p.o. for 14 days starting 1 day after tumor implantation. Drug efficacy was assessed as tumor volume inhibition (TVI) in drug-treated versus control mice. TVI percentage was evaluated after final treatments as 100–(mean TVI-control x 100–mean TVI-drug x 100).

The toxic effects of drug treatment were assessed as described below: a) body weight loss (BWL) was calculated as follows: BWL (%) = 100–(mean body weight days x/mean body weight day 1 x 100), where day 1 is the first day of treatment, and day x is any day thereafter. The highest (maximum) BWL is reported in the Tables. Mice were weighed twice a week throughout the experimental period; thereafter. The highest (maximum) BWL is reported in the Tables.

The paw-withdrawal threshold values obtained in the OHP+ALC group were not significantly different from those observed in the vehicle-treated animals at the end of the observation period, while the values obtained in the OHP group were significantly lower.

Statistical analysis. The in vivo experimental results were compared using the Mann-Whitney test. Differences were considered significant at p<0.05.

Results

No side-effects were associated with the administration of ALC i.v., s.c. or p.o.

Behavioral experiment: subcutaneous ALC prevents OHP-induced hyperalgesia and accelerates recovery after onset. The administration of OHP alone induced a significant, early and long-lasting lowering of the mechanical nociceptive threshold compared to the control group (Figures 1 and 2).

As depicted in Figure 1, it can be seen that ALC prevented a lowering of the mechanical nociceptive threshold induced by OHP (vehicle vs. OHP, p<0.001) when the 2 drugs were co-administered (OHP+ALC vs. OHP, p<0.001. The effect of repeated ALC treatments, when ALC administration was initiated 24 days from the first OHP administration, is illustrated in Figure 2.

ALC treatment was able to restore the mechanical nociceptive threshold within a few days (p<0.02 vs. OHP group from days 23 to 51 from the first OHP administration). The paw-withdrawal threshold values obtained in the OHP+ALC group were not significantly different from those observed in the vehicle-treated animals at the end of the observation period, while the values obtained in the OHP group were significantly lower.

Neurophysiological experiments: intravenous, subcutaneous and oral ALC administration prevents OHP-induced SNCV impairment. In the 3 experiments, OHP induced a significant reduction in SNCV with respect to vehicle-treated animals. In the first experiment, SNCV reduction was counteracted by contemporaneous administration of ALC i.v. (Table II) at the determination performed after treatment.

Similarly, OHP-induced SNCV reduction was counteracted by the contemporary administration of ALC, which was started 1 week before the first OHP administration (Table III).

In the third experiment, ALC was administered to a group of rats at a dose of 100 mg/kg/day s.c., starting on the same day as the first OHP administration. In this study as well, SNCV reduction was counteracted by contemporary treatment with ALC (Table IV).

Neurophysiological experiments: subcutaneous ALC enhances the recovery of OHP-induced SNCV impairment. In the third experiment, after the treatment period, neuropathic rats were divided into two homogeneous groups: one receiving ALC...
Table IV. Subcutaneous ALC and OHP-induced neuropathy. Sensory nerve conduction velocity (m/s) in treatment period.

<table>
<thead>
<tr>
<th>Experiment 3.</th>
<th>Treatment group</th>
<th>Baseline</th>
<th>After treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (6)</td>
<td>30.98±0.58</td>
<td>35.80±0.68</td>
<td></td>
</tr>
<tr>
<td>OHP (26)</td>
<td>31.31±0.34</td>
<td>30.69±0.22*</td>
<td></td>
</tr>
<tr>
<td>OHP-ALC (9)</td>
<td>31.44±0.50</td>
<td>33.63±0.85b,c</td>
<td></td>
</tr>
</tbody>
</table>

Values represent mean±SEM
Repeated measures ANOVA:
OHP vs. Vehicle: F(1,30)=27.76; p<0.0001
OHP vs. OHP-ALC: F(1,33)= 12.67; p<0.005
OHP-ALC vs. Vehicle: F(1,13)= 1.54; n.s.
One-way ANOVA followed by SNK test:
*p<0.001, *p<0.05 vs. Vehicle
*p<0.001 vs. OHP

(100 mg/kg/day s.c. for 3 weeks), and the other used as a neuropathic control.

ALC administered at the end of OHP treatment was able to enhance the recovery of SNCV impairment induced by OHP: in fact, during the first 2 weeks of follow-up, SNCV values were significantly higher in the ALC+OHP-treated rats compared to the group treated with OHP alone (Table V).

Cytotoxicity experiments

In vitro studies. The antiproliferative activity of OHP alone and in combination with a high concentration of ALC was also evaluated in a panel of human tumor cell lines (Table VI). In all the cell systems tested, OHP showed marked cytotoxic efficacy. The most sensitive cell lines were the human cell lines IGROV-1, A2780 ovarian, NTERA-2 testicular carcinomas, NCI-H460 non-small cell lung carcinoma, HT-29 and LoVo colon carcinoma, with IC50s in a range between 2.8 and 9.3 μg/mL, followed by HeLa cervix uteri and PC3 prostate carcinoma (IC50s of 16.8 and 58.7 μg/mL, respectively).

However, ALC alone was not cytotoxic for the cells (IC50>200 μg/mL). Moreover, the same concentration of ALC added to cells together with OHP did not influence its growth-inhibitory effect on any human tumor cell lines tested (Table VI).

In vivo studies. In L1210 leukemia-bearing mice, OHP at the approximate maximum tolerated dose of 12.5 mg/kg i.p., given according to the q4d3 schedule, showed significant antitumor activity with treatment starting 1 day after tumor injection, as it prolonged the lifespan by 354% (p<0.05) with 75% LTS (long-term survivors). At 6.25 mg/kg, i.p. a potent effect was obtained again with an ILS of 204% (p<0.001) and 50% LTS (Table VII).

ALC (100 mg/kg p.o.) administered alone daily for 14 days had no antitumor activity, and a combination of ALC with OHP did not influence the antitumor effects of OHP.

The antitumor activity of OHP was also studied in a human tumor xenograft, a colon adenocarcinoma (HT-29). Three i.p. injections of OHP at 15 mg/kg on the fourth, sixth and tenth day after tumor implantation induced a significant reduction in tumor weight (TVI=48%, p<0.01), but were associated with 8 deaths due to toxicity after the last treatment. At a lower dose of 10 mg/kg, OHP showed a significant antitumor activity (TVI=38%, p<0.01) without any notable toxicity. ALC (100
mg/kg p.o.) given daily for 14 days starting 1 day after tumor implantation was inactive against this tumor histotype. Again, a combination of ALC with OHP did not show any difference in tumor growth compared with OHP alone (Table VIII).

Discussion

The prevention of chemotherapy-induced neuropathy associated with the clinical use of platinum-derived drugs such as OHP is a major goal in clinical oncology. Pre-clinical in vitro studies can offer important clues for identifying effective neuroprotective strategies since target side-effects can be tested with different modalities. The peripheral neuropathy induced in rats by repeated administration of OHP has been studied using neurophysiological, pathological, analytical and biochemical methods (9), and it can be safely stated that it is qualitatively similar to that observed in humans, as characterized by the involvement of the sensory nerve fibers secondary to DRG neuronopathy.

In this study behavioral and neurophysiological methods were employed to investigate the possibility that ALC may be able to reduce OHP-induced peripheral neurotoxicity in a chronic i.v. administration rat model. The main action of ALC, as identified to date, is on intermediary metabolism (10-13). Furthermore, different studies have demonstrated a neuroprotective role for this member of the carnitine family in both the central and peripheral nervous systems. Exogenous administration of ALC reduces the severity of experimental diabetic neuropathy (14-16), enhances motoneuron survival after axotomy (17) and modulates control of NGF level in the CNS of adult rats following total fimbria-fornix transection (23) and the rate of transcription of gene coding for the p75NGFR in the basal forebrain and cerebellum of aged rats (24).

Regarding chemotherapy-induced peripheral neurotoxicity, it has already been reported that ALC exerts a protective effect against cisplatin- and paclitaxel-induced neurological impairment in rat models. Moreover, it is noteworthy that the protective effect of ALC was obtained without interfering with the cisplatin or paclitaxel antineoplastic effect in vitro or in vivo, as evaluated on several solid tumor types. In the present study, it was also confirmed that the OHP antitumor effect is not reduced by ALC-co-treatment in a panel of human cell lines and in vivo murine models. The explanation for this different sensitivity to ALC protection in tumor cells and in normal tissues is still only speculative; it is possible, however, that some of the mechanisms and biochemical pathways modulated by this agent may have tissue-specific relevance.

In the present study, ALC was effective in the prevention of OHP neurotoxicity in different experimental paradigms when the neurological impairment was assessed using both behavioral and neurophysiological methods. Interestingly, ALC administration was also able to enhance the reversal from neurological damage in the follow-up period subsequent to OHP withdrawal.

Given the marked similarities between cisplatin and OHP chronic neurotoxicity, our positive results are not surprising, and it is likely that the mechanism of ALC neuroprotection might be the same, although this has not yet been completely clarified.

The pleiotropicity of effects associated with ALC cannot be explained solely on the basis of the above-mentioned effects on intermediary metabolism. Over the last few years, the possibility that ALC may also be involved in nuclear events has emerged. Moreover, the molecular properties of ALC have led

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>BWL%&lt;sup&gt;a&lt;/sup&gt; max</th>
<th>MST&lt;sup&gt;b&lt;/sup&gt; (range of days)</th>
<th>ILS&lt;sup&gt;c&lt;/sup&gt;</th>
<th>LTS&lt;sup&gt;d&lt;/sup&gt; day 100</th>
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</thead>
<tbody>
<tr>
<td>Controls</td>
<td>---</td>
<td>2</td>
<td>22</td>
<td>---</td>
<td>0/8</td>
</tr>
<tr>
<td>OHP</td>
<td>100</td>
<td>0</td>
<td>20</td>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>ALC</td>
<td>12.5</td>
<td>11</td>
<td>100</td>
<td>*&lt;354</td>
<td>5/8</td>
</tr>
<tr>
<td>OHP</td>
<td>100</td>
<td>16</td>
<td>100</td>
<td>***&gt;354</td>
<td>5/8</td>
</tr>
<tr>
<td>ALC + OHP</td>
<td>12.5</td>
<td>6</td>
<td>70</td>
<td>***&gt;218</td>
<td>4/8</td>
</tr>
<tr>
<td>ALC</td>
<td>100</td>
<td>4</td>
<td>70</td>
<td>***&gt;218</td>
<td>4/8</td>
</tr>
<tr>
<td>ALC + OHP</td>
<td>12.5</td>
<td>35</td>
<td>100</td>
<td>***&gt;218</td>
<td>4/8</td>
</tr>
</tbody>
</table>

<sup>a</sup>BWL% = maximum body weight loss during experimental period  
<sup>b</sup>MST = median survival time (days)  
<sup>c</sup>Controls, vehicle-treated group  
Number of long-term survivors on day 100 over the number of mice per group.  
**p<0.001 and *p<0.05 vs. vehicle (Mann-Whitney)
to the suggestion that it may play a role in histone acetylation and, consequently, in facilitating gene expression (25-27).

In conclusion, our experimental results demonstrate that ALC is effective in the prevention and treatment of chronic OHP-induced peripheral neurotoxicity, thus giving further support to the possible role of ALC as a neuroprotectant in chemotherapy schedules.

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References


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