Etoposide- and BMY-40481-Induced Sensory Neuropathy in Mice*

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ABSTRACT

The effects of high toxic doses of the anticancer drugs, etoposide and its phosphate derivative, BMY-40481, on the nervous system of female CD-1 mice were examined by light microscopy (LM) and transmission electron microscopy. Mice were euthanatized 4 wk following a single iv injection of either 0, 50, 100, or 150 mg/kg of BMY-40481 or 44 or 88 mg/kg of etoposide. Mice treated with 100 or 150 mg/kg of BMY-40481 or 88 mg/kg of etoposide had clinical symptomology of progressive ataxia, impaired righting reflex, and splaying and paresis of fore- and hindlimbs at day 8. Similar, dose-related LM changes were observed with both drugs at all doses and consisted of degeneration of dorsal root ganglion cells and axonal degeneration of their distal and proximal processes in peripheral nerves, dorsal spinal roots, and dorsal funiculi of spinal cord. Axonal degeneration was characterized by LM as shrinkage, swelling, and fragmentation of axon cylinders accompanied by secondary demyelination. Degenerative changes in ganglion cell bodies included eccentric nuclei, cytoplasmic vacuolation, central chromatolysis, and peripheral clumping of Nissl's bodies. Ultrastructurally, ganglion cell bodies had focally extensive dilation of the rough endoplasmic reticulum, mitochondrial swelling, increased numbers of phagolysosomes and prominent aggregations of microfilaments (globular filamentous bodies). Ultrastructural axonal changes occurred primarily in large, myelinated fibers and consisted of axonal swelling or loss, thinning of myelin sheaths, and a decrease in the number of organelles. This is the first report of etoposide-related sensory neuropathy in laboratory animals, a model that may be useful for the study of etoposide-related peripheral neuropathy in humans.

Keywords. VP-16; VePesid; etoposide analog; neurotoxicity; light microscopy; electron microscopy; intravenous; single dose

INTRODUCTION

Etoposide (VP-16, VePesid®, Bristol-Myers Squibb) is a semisynthetic derivative of podophyllotoxin and is designated chemically as 4'-demethylepipodophyllotoxin-g-(4,6-O-ethylidene-~-3-D-glucopyranoside) (1). Etoposide has been used successfully alone and in combination with other antineoplastic drugs to treat a variety of small and non-small-cell lung neoplasms and testicular neoplasms. The clinical use, chemistry, pharmacology, pharmacokinetics, and adverse effects have been extensively reviewed (9, 11, 15).

BMY-40481 is a water-soluble phosphate derivative and prodrug of etoposide. BMY-40481 had antitumor activities comparable to etoposide in several preclinical tumor models (19). Because of its water solubility, BMY-40481 has potential clinical advantages as a chemotherapeutic agent over etoposide, which has very poor water solubility.

The dose-limiting toxicity of etoposide in humans is myelosuppression, especially leukopenia and, less commonly, thrombocytopenia. Other adverse effects associated with etoposide therapy include alopecia, nausea, vomiting, diarrhea, mucositis, and rarely bronchospasm and hypotension (9, 10). A mild peripheral neuropathy was reported in 1–20% of patients receiving etoposide alone (1, 4, 8, 18), although this finding has been challenged by others (14). The possible enhancement of vincristine-induced peripheral neuropathy by etoposide has also been questioned (12, 25). A single case of etoposide-induced central nervous system toxicity, character-
ized by acute dystonia, an extrapyramidal side effect, has also been reported (2).

Sensory neuropathy is not a recognized feature of etoposide-induced toxicity in experimental animals (1, 21–25), although ataxia has been reported in rats (23, 24). Clinical neuropathy in mice was induced by both test articles in recent studies that compared the effects of BMY-40481 and etoposide. The objective of the present study was to evaluate etoposide- and BMY-40481–induced neuropathy in mice by light microscopy (LM) and electron microscopy (EM).

**MATERIALS AND METHODS**

*Experimental Animals*

Sixty female CD-1 (Crl: CD-1 [ICR] BR) VAF mice, obtained from Charles River Laboratories (Kingston, NY), were used. The mice were approximately 6 wk old and weighed between 19.2 and 25 g at the start of the study. They were housed 5 mice per cage and were offered pelleted chow (Rodent Laboratory Chow #5002; Purina Mills Company, St. Louis, MO) and fresh drinking water ad libitum. All animal care and use were carried out in accordance with the Bristol-Myers Squibb and National Institutes of Health guidelines for the care and use of laboratory animals.

*Experimental Design*

The test mice were ranked by weight and then randomly divided into 6 groups of 10 mice each, with groups having approximately equal average body weights. Each group received a single iv injection of either saline, BMY-40481 at a dose of 50, 100, or 150 mg/kg, or etoposide at 44 or 88 mg/kg. Doses were compared on a mol/mol basis with a BMY-40481, molecular weight of 668.5, and etoposide, 588.5. The iv injections were administered via a tail vein at a rate of approximately 0.05 ml/sec, using a 27-gauge needle and syringe. Mice were observed daily for viability and changes in general health and behavior. Body weights were determined once a week.

*Test Articles*

Etoposide and BMY-40481 (etoposide phosphate) were prepared for dosing by dissolution in physiologic saline (0.9% sodium chloride injection; USP, Baxter Healthcare Corp., Deerfield, IL). Both drugs were assayed, and stability of dosing solutions was established at the start of the study. Drug concentration of BMY-40481 solutions was assayed on day 1. Etoposide (VePesid®) was a commercial preparation, and dosing solutions were not assayed for drug concentration. The concentration of dosing solutions and dose volumes for each treatment group are presented in Table I.

**Necropsy**

Twenty-eight or 29 days after the single dose, all surviving mice were euthanatized with a lethal ip injection of sodium pentobarbital. After reaching a deep surgical plane of anesthesia, mice were perfused via the left ventricle with a heparinized saline solution. Tissues of 3 control mice (Group I), 1 mouse treated with 100 mg/kg of BMY-40481, 2 mice treated with 150 mg/kg of BMY-40481, and 3 mice treated with 88 mg/kg of etoposide were fixed in situ by vascular perfusion with modified Karnovsky’s fixative (10). Tissues of the remaining mice were fixed in situ by vascular perfusion with formalin–alcohol–acetic acid (FAA) solution. Mice that were sacrificed in moribund condition prior to scheduled necropsies were also perfused with heparinized saline followed by FAA. The brain, the entire spinal cord (within the spinal column), sciatic nerves, and plantar nerves were collected.

*Morphology*

**LM.** Following demineralization, representative sections of cervical, thoracic, and lumbar spinal column were prepared so that the dorsal root ganglion, spinal cord, and spinal nerve roots could be examined in transverse sections of the column. Representative sections of brain, sciatic nerve (ischiatic nerve), plantar nerve, and demineralized sections of spinal column with cord were processed by routine methods, embedded in paraffin, and stained with H&E. Selected tissues were also stained with Bodian’s stain.

**EM.** After whole-body perfusion with Karnovsky’s fixative, the sciatic nerve, lumbar dorsal root ganglia, and dorsal spinal nerve roots were transferred to phosphate buffer, postfixed in osmium tetroxide, dehydrated in a series of graded methanol baths, and embedded in epoxy resin. One-μm-thick sections were cut, stained with Toluidine blue, and examined by LM to delineate areas to be examined by EM. Ultrathin sections, stained with uranyl ac-
RESULTS

Clinical Observations

Eight of 10 mice administered 150 mg/kg of BMY-40481 died on study days 3–9. The only clinical sign observed in mice given 50 mg/kg of BMY-40481 was slight, generalized alopecia between days 7 and 22 and rarely thereafter. Mice administered 100 mg/kg of BMY-40481 exhibited a hunched body, rough haircoat, generalized alopecia, and ptosis as early as day 4. Three of these mice had splayed rear legs beginning at day 8. Clinical signs observed in mice given 150 mg/kg BMY-40481 included hunched body, rough haircoat, decreased activity, ptosis, and generalized alopecia. Ataxia, weakened and/or splayed rear legs (Fig. 1), weakened front legs, paresis, and impaired righting reflex were observed in 6 of 10 mice. Dehydration, tremors, hypothermia, soiling, and moribundity occurred prior to death.

Seven of 10 mice treated with 88 mg/kg of etoposide died or were euthanatized on study days 6 and 8. Mice administered 44 mg/kg of etoposide had generalized alopecia between days 6 and 20 and rarely during the remainder of the study. Mice receiving 88 mg/kg of etoposide exhibited decreased activity, increased respiration, ataxia, prostration, hunched body, piloerection, twitching, and flushing immediately after dose administration. All mice were clinically normal on day 2. The majority of the mice had a hunched body, rough haircoat, decreased activity, ptosis, and generalized alopecia commencing on days 3 and 4. Weakened and/or splayed rear legs, weakened front legs, paresis, and impaired righting reflex were observed in 5 of 10 mice beginning on day 8. Dehydration, tremors, hypothermia, soiling, and moribundity occurred prior to death.

The average weight gain of each group was as follows: Group I (controls), 22.8%; Group II (50 mg/kg of BMY-40481), 25.2%; Group III (100 mg/kg of BMY-40481), 11.5%; Group IV (150 mg/kg BMY-40481), −21.0%; Group V (44 mg/kg of etoposide), 18.7%; and Group VI (88 mg/kg of etoposide), 4.5%.

Necropsy Findings

No drug-related macroscopic alterations were observed in the nervous system of etoposide- or BMY-40481–treated mice. BMY-40481–related changes in other organs were limited to the high-dose group and included adrenal enlargement, red discoloration of the small intestine, dark intestinal contents (presumably bile), splenic atrophy, red fluid in the thoracic cavity, thymic atrophy, generalized congestion, and dehydration. These changes were observed only in mice that died prior to day 29.

Etoposide-related gross changes in organ systems other than the nervous system were observed in high- and low-dose mice. Changes in high-dose etoposide-treated mice were red discoloration of duodenum and jejunum, splenic atrophy, necrosis of the tip of the tail, thymic atrophy, and generalized congestion. Partial, focal alopecia was observed in low-dose etoposide-treated mice. The drug-related changes observed in the high-dose group were observed only in mice that died or were sacrificed in moribund condition prior to day 29.

Morphology

Similar, drug-related changes of axonal degeneration in the sciatic nerve, dorsal spinal nerve roots, spinal cord, and plantar nerves, accompanied by degeneration and necrosis of dorsal root ganglia, were observed in etoposide– and BMY-40481–treated mice. These changes occurred at all doses with dose-dependent severity and were generally more severe in etoposide– than in BMY-40481–treated mice at comparable molar doses (100 and 50 mg/kg BMY-40481 vs 88 and 44 mg/kg of etoposide, respectively). Drug-related changes were not observed in the brain or ventral spinal nerve roots with either drug.

Drug-related axonal degeneration in the sciatic nerve, by LM, was “Wallerian-like” and characterized by axonal shrinkage, swelling, and fragmentation; digestion chambers, consisting of secondary myelin degeneration (myelin ellipsoids); and small numbers of vacuolated macrophages (Fig. 2a). Drug-related axonal degeneration in the plantar nerve of mice treated with either drug was similar to, but less severe than, that observed in the sciatic nerve. Predominant ultrastructural changes in the sciatic nerve
included a variable decrease in the size and number of large, myelinated fibers, axonal swelling accompanied by a thinning of the myelin sheath, and disruption and disintegration of axonal components accompanied by an infiltrate of a small number of macrophages (Fig. 2b). Additional ultrastructural axonal changes, occasionally observed, included high volumetric swelling of the inner mitochondrial compartment and a variable decrease in the number of organelles, predominantly microfilaments, microtubules, and cytoplasmic vesicles.

Drug-related axonal degeneration in the cervical, thoracic, and lumbar regions of the spinal cord was limited to the dorsal funiculi (fasciculus gracilis and fasciculus cuneatus). Axonal degeneration in the spinal cord was characterized by axonal swelling, fragmentation, and loss and by mild distention of myelin sheaths. Axonal degeneration occurred with greater incidence and severity in the cervical and thoracic spinal cord as compared to the lumbar region. Axons in the spinal cord were not as severely affected as their counterparts in the sciatic nerve.

Etoposide- and BMY-40481-related axonal degeneration in spinal nerve roots was limited exclusively to the dorsal roots of the cervical, thoracic, and lumbar regions and resembled axonal degeneration observed in the sciatic nerve (Fig. 3). Axonal degeneration induced by either drug was most common and severe in mice that survived until day 29.

Drug-related degeneration of dorsal root ganglia and necrosis of ganglion cell bodies was observed in the cervical, thoracic, and lumbar regions of the spinal cord and were generally more severe in mice that died or were sacrificed prior to day 29. Degeneration of ganglion cell bodies, observed at all dose levels, was characterized by eccentric nuclei, cytoplasmic vacuolation (large and medium-sized clear vacuoles), central chromatolysis, peripheral clumping of Nissl's bodies, and an increase in Schwann cells (Fig. 4a). Ultrastructurally, numerous free ribosomes were dispersed among large stacks of granular or rough endoplasmic reticulum near the cell periphery, which corresponded to the light microscopic changes in Nissl's bodies. The ultrastructural correlate to the prominent cytoplasmic vacuoles observed by LM were large, single membrane-bound vacuoles that contained fine granular to fibrillar material. These were interpreted to be focally extensive dilations of the endoplasmic reticulum (Fig. 4b).

The ultrastructural correlate to the dense cytoplasmic bodies observed in semithin sections by LM were globular filamentous bodies, dense ovoid clusters composed predominantly of microfilaments and (to lesser extent) of microtubules (Fig. 5). High volumetric swelling of the inner mitochondrial compartment and increased numbers of phagolysosomes were also observed in ganglion cell bodies. Ultrastructural changes previously described in the sciatic nerve were also observed in the nerve fibers of the dorsal root. Ganglion cell necrosis, observed at the intermediate and high dose, was characterized...
FIG. 3.—Light photomicrograph of a nonremarkable ventral spinal nerve root and a severely affected dorsal spinal nerve root from a 150-mg/kg BMY-40481-treated mouse. Changes in the dorsal spinal nerve root include marked axonal swelling and disintegration, demyelination, and a mononuclear cell infiltrate H&E. × 233.

by pyknosis with karyolysis or karyorrhexis and liquefactive necrosis.

DISCUSSION

In the present study, mice given a single iv high dose of either etoposide or BMY-40481 developed clinical signs and light and electron microscopic lesions, consistent with a diagnosis of a primary sensory neuropathy. This is the first report of etoposide-induced neuropathy in laboratory animals. Neuropathy was not observed in several species (rat, dog, rhesus monkey) in previous etoposide toxicity studies, which varied by the route and the length of exposure (Table II). Any of several factors may have been responsible for the development of neuropathy in this mouse model, including species susceptibility, single high dose, route of administration, and an extended observation period prior to tissue collection. Intravenous administration presumably produces greater tissue bioavailability, as compared to oral administration. Because both drugs are poorly absorbed from the gastrointestinal tract in mice (unpublished data). Poor gastrointestinal absorption may also explain the lack of neurotoxicity in mice and rats following a single oral dose of 1,000 mg/kg etoposide (unpublished data). In another mouse study (1), neurotoxicity was not observed following an acute iv LD_{50} dose of 0.2% etoposide solution (118 mg/kg). The lack of a sufficiently long obser-
Fig. 5.—Transmission electron micrograph of dorsal root ganglion from a mouse treated with 88 mg/kg etoposide in which the ganglion cell body contains a large, discrete aggregate (arrowheads) of microfilaments and microtubules known as a globular filamentous body, adjacent to the nucleus (N). Several lipid droplets are present in the ganglion cell cytoplasm. Lead citrate and uranyl acetate. ×3,150.

The anatomic distribution of the pathologic changes in this study strongly suggests that dorsal root ganglion cells, including their distal and proximal processes, are the primary targets of etoposide- and BMY-40481-induced neuropathy. Other compounds that induce similar selective damage to the dorsal root ganglion cells in laboratory animals include doxorubicin (adriamycin) (7), methylmercury (5), and vitamin B₆ (pyridoxine) at megadoses (13). The biochemical mechanism of etoposide- and BMY-40481–induced neuronal damage most likely resides in their ability to induce DNA strand breaks in cells by inhibiting DNA topoisomerase II, a strand-rejoining enzyme (6). Factors responsible for the selective damage to dorsal root ganglia in this study are speculative and may involve the blood–brain barrier, which is normally absent around these ganglia (16). Previous studies have documented that the penetration of the blood–brain barrier by etoposide is minimal (11). The observation that large myelinated axons were much more severely affected by both drugs as compared to small fibers suggests that proprioception may be a more sensitive clinical indicator of toxicity as compared to loss of deep pain sensation.

The morphologic changes in the dorsal root ganglion cells, consisting of eccentric nuclear location, loss of perinuclear rough endoplasmic reticulum (central chromatolysis), and clumping of peripheral rough endoplasmic reticulum, are common responses of the neuronal cell body to injury. Severely dilated rough endoplasmic reticulum with ribosomal detachment was the ultrastructural correlate to the cytoplasmic vacuoles observed by LM. The origin of the fibrillar material within the dilated cisternae of the rough endoplasmic reticulum was not determined. The marked increase in the number of lysosomes and phagolysosomes most likely represented an increased turnover of cellular

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**Table II.**—Preclinical studies of etoposide in animals reported in the literature.

<table>
<thead>
<tr>
<th>Species</th>
<th>Duration</th>
<th>Route</th>
<th>Doses (mg/kg/day)</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Rat</td>
<td>1 month</td>
<td>ip</td>
<td>0.6, 1.8, 6.0</td>
<td>1</td>
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<tr>
<td></td>
<td>6 months</td>
<td>po</td>
<td>3, 10, 30, 5 days/wk</td>
<td>1</td>
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<tr>
<td></td>
<td>1 month</td>
<td>iv</td>
<td>0.15, 0.5, 1.5, 4.5</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>1 month</td>
<td>po</td>
<td>3, 10, 30, 100</td>
<td>14</td>
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<tr>
<td></td>
<td>3 months</td>
<td>iv</td>
<td>0.05, 0.13, 0.5, 1.5</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>6 months</td>
<td>po</td>
<td>1, 3, 10, 30</td>
<td>15</td>
</tr>
<tr>
<td>Dog</td>
<td>6 months</td>
<td>po</td>
<td>0.5, 1.5, 5–6</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>5 days/wk</td>
<td></td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>1 month</td>
<td>iv</td>
<td>0.4, 1.2, 3.6</td>
<td>1</td>
</tr>
<tr>
<td>Mouse</td>
<td>1 day (1-mo observation)</td>
<td>iv</td>
<td>44.88</td>
<td>this study</td>
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</table>
creased turnover of organelles resulted primarily determined from the present study whether the lysosomal number or composition. It cannot be determined if accelerated organelle senescence. Globular filamentous bodies, observed in dorsal root ganglion cell bodies, were composed of spherical aggregates of microfilaments and a smaller microtubular component. These bodies were frequently juxtanuclear in location and were either randomly scattered or arranged in an orderly fashion, forming concentric whorls. Although globular filamentous bodies have been described in toxicologic conditions of the nervous system, e.g., experimental aluminum encephalopathy (20, 26), they are most commonly observed in neoplastic conditions, including tumors of neuronal cell origin (3, 17, 28). The precise significance of this change remains to be elucidated but may represent alterations in intracytoplasmic transport.

References