Molecular Biology and Clinical Mitigation of Cancer Treatment-Induced Neuropathy

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ABSTRACT: Disruption of microtubule function is the antitumor mechanism of several classes of drugs used to treat cancer today. However, the significant beneficial effect on tumor outcomes is frequently counterbalanced by neurotoxic complications. Despite an abundance of scientific data, our understanding of the biological mechanisms underlying this toxic reaction remains unclear, further hindering attempts to identify and develop effective preventive strategies. The primary goals of this review are to: (1) provide insight regarding the biology of the microtubule, (2) analyze the molecular and biochemical pathways that may be involved in the development of neurotoxicity, and (3) propose a unifying concept linking drug-induced neuropathy, microtubule dysfunction, and vitamin D.

KEYWORDS: centrosome, microtubule inhibitor, neurotoxicity, oxaliplatin, proteasome inhibitor, vitamin D

Introduction

One of the most encouraging trends in oncology over the past 25 years has been the improvement in patient survival rates. While this phenomenon can be attributed to the development and clinical application of a number of novel therapeutic agents, the overall impact of supportive care measures in reducing cancer-related morbidity, and possibly even mortality, should not be underestimated.1 The latter belief is supported, in part, by a better understanding of the complex molecular and biochemical pathways that regulate treatment-associated adverse effects. For example, recognition of the 5-HT3 and NK1 receptor pathways in nausea and vomiting as well as identification of key proteins that regulate granulopoiesis led to the development of agents, which continues to have a substantial impact on clinical oncology.2 However, highlighting some of these notable achievements also exposes one glaring deficiency – the inability to prevent, or even attenuate, cancer treatment-induced neuropathy.

The relevance of this toxicity is further accentuated by the observation that, in some instances, effective therapy must be aborted, not because of drug resistance or disease progression, but rather the negative impact of neurotoxicity on the patient’s quality of life.3 The numerous mechanisms purportedly involved in the genesis of the neuronal toxicity are also important. The list includes, but may not be limited to, the toxic effects on dorsal root ganglia and mitochondria, altered neuronal blood supply, interference of axonal ion channels, and production of reactive oxygen species and inflammatory cytokines.4-8 Notwithstanding reports that neuroprotection could be achieved in the laboratory by targeting or abolishing a number of neurodegenerative events or modulating the balance of critical proteins, clinical trials have not produced conclusive evidence to validate any preventive strategy. Indeed, the ongoing uncertainty regarding the precise pathophysiology of cancer treatment-induced neuropathy has impeded the development of at least one clinically effective protective measure.

Despite the likelihood that different mechanisms exist for different classes of anticancer agents, a unifying concept is proposed in linking treatment-induced neurotoxicity with disruption of microtubule function. As such, this article was sequenced to provide the reader with: (1) a detailed primer of the physiology and dynamics of the microtubule, (2) an abbreviated primer of the beneficial (antitumor) and detrimental (neurotoxic) effects associated with inhibition of this complex polymer, and (3) a scientific rationale for a proof-of-concept clinical study.

As such, numerous publications, many old but of enduring scientific merit, as well as other, more recently, published data were retrieved and extensively evaluated. While portions of the published literature were used to support the accuracy of the textual content, unresolved issues provided the opportunity for reasoned author opinions.
Microtubule-Organizing Center

The centrosome is an intracellular entity, unique in that the organelle is not defined by a distinct membranous boundary. Incorporated within the centrosome are two centrioles, unusual because nine triplet microtubule units form two structurally symmetrical cylinders (Fig. 1). The centrioles, linked by fiber-like appendages and surrounded by pericentriolar material, are comprised most notably of proteinaceous substances. However, the proteins are not of centrosomal origin exclusively but derive also from a labile pool in the cell cytoplasm. Access to this bicompartamentalized source of protein is facilitated by the lack of a clear demarcation between the centrosome and cytoplasm. Of the numerous proteins, the gamma (γ)-tubulin ring complex is among the most well-described components of the pericentriolar material. The importance of this protein is discussed later.

One of the most important activities that occur within the centrosome is nucleation and organization of the microtubules. This centrosomal activity is a manifestation of both the pericentriolar content (which contains the requisite materials) and the centriole (which functions as the assembly platform for microtubule formation). The requisite structural elements of the microtubule are heterodimers of α- and β-tubulin (Fig. 2). Interestingly, while the two protein subunits exhibit less than 50% amino acid homology, three-dimensional diffraction studies describe crystal structures that are strikingly similar with regard to their electron density.9 Formation of the tubulin heterodimers is an equally delicate process that requires additional proteins (ie, chaperones and other cofactors) that assist and provide conformational integrity related to dimer folding and unfolding, dimer interface, and polymerization.10,11 Protofilaments are formed by a unique sequence of events involving heterodimer elongation. Because lengthening occurs with polar opposite α-tubulin linked to β-tubulin, the developing asymmetrical construct engenders the exposed ends that are negative and positive, respectively. In addition, the redundancy of the heterodimeric structure is superficially, though erroneously, simplistic. Not overt is that only guanosine triphosphate bound to β-tubulin is hydrolyzed, despite the presence, and binding, of the purine nucleoside to both tubulin subunits. The

Figure 1. The centrosome. Components include two centrioles of which the more mature is designated mother. Cylindrical in nature, the centriole actually consists of nine triplet microtubular structures surrounded by pericentriolar material. The centrosome is responsible for nucleation of microtubules.
The tenet that inhibition of microtubule function would have important ramifications in oncology is strongly supported by different classes of antimicrotubule agents available for clinical use. Despite chemical and structural differences, the primary mechanism of their antitumor effect is believed to be mediated by either stabilizing or destabilizing microtubule function.

**Microtubule-stabilizing agents.** Laboratory investigation indicates that the taxanes mediate their antitumor activity primarily by blocking microtubule disassembly, thus kinetically stabilizing the polymer. What can be perceived as a rather soft effect is deceiving because crucial cell activities (and cell survival) depend on the unsullied dynamics and function of the microtubule. Seemingly consistent with this mellow disturbance is the finding that the mass spectrum of the microtubule is unaltered. Important also, the stabilizing agents inhibit tumor growth and survival by sequencing two critical events. Initially, drug binding to β-tubulin interrupts metaphase to anaphase transition, thereby interfering with the spindle pole resulting in mitotic arrest. Subsequently, impairment of spindle apparatus activates (by uncertain mechanisms) multiple programmed cell death pathways including the caspases and Bcl-2 family proteins.

The taxanes purportedly bind to a taxoid site, which resides behind the M-loop on the luminal surface of β-tubulin (Fig. 3). Even though this specific locus was believed to be the primary binding site, subsequent studies suggested that taxane binding consisted of a two-site mechanism. Computational molecular modeling with nuclear magnetic resonance showed that binding initially involved an external pore type-1 site followed by sequestration onto the luminal taxoid site. Despite these findings, the dual-site mechanism still remains a hypothesis.

Nascent binding notwithstanding the taxane–microtubule interaction is a very complex phenomenon. Previously, the binding interface of these agents was believed to involve the M-loop and the H1-S2 loop of adjacent β-tubulin monomers, thus increasing the lateral interactions between protofilaments. Other investigators localized taxane binding to a site central to helices H1, H6, H7, and the B7-H9 on the M-loop.

However, in later studies, it was observed that displacement of the M-loop away from H6 in the β-monomer allosterically facilitated drug interaction with the H1-S2 loop. More recently, two studies also showed that the initial M-loop interaction promoted the curved-to-straight conformational change that occurs with tubulin incorporation into microtubules. Nonetheless, while the binding docket for the taxanes resides...
of vinblastine was effectively disrupted by increasing drug concentrations, microtubules depolymerized. At high concentrations, note also, the vinca alkaloids appear to have concentration-dependent effects on polymer mass. At high concentrations, these agents decrease microtubule mass by inhibiting polymerization; at low concentrations, the vinca alkaloids interfere with microtubule dynamics without appreciably affecting the polymer size. Propagation of microtubule chaos results in mitotic arrest between the metaphase and anaphase transition points.

Microtubule-destabilizing agents. Drugs such as vincristine and vinblastine exhibit high-affinity binding to a different, though not necessarily uniquely named, site known as the “vinca domain,” which has been localized to β-tubulin.21 Drug-tubulin or drug-polymer binding disrupts polymerization or induces structural depolymerization, resulting in functional impairment of microtubule dynamics. Notably, the vinca alkaloids can have a negative effect on either process depending on drug concentration. For example, it has been shown that dynamic instability of cells in mediums containing low (ie, nanomolar) concentrations of vinblastine was effectively disrupted by increasing the duration of the resting or pause interval; at 3-log higher drug concentrations, microtubules depolymerized.24 Of note also, the vinca alkaloids appear to have concentration-dependent effects on polymer mass. At high concentrations, these agents decrease microtubule mass by inhibiting polymerization; at low concentrations, the vinca alkaloids interfere with microtubule dynamics without appreciably affecting the polymer size.25 Propagation of microtubule chaos results in mitotic arrest between the metaphase and anaphase transition points.

Neurotoxicity Associated with Conventional Antimicrotubule Agents

Despite what is arguably their target, the microtubule inhibitors are not considered to be true targeting agents as is currently defined. As such, cell damage associated with these drugs is not restricted to the tumor but includes cells of many other (normal) tissues. One major aspect of bystander damage common to all agents that alter microtubule function involves neuronal tissue. While the severity of neuropathy usually varies with duration of therapy, neurotoxicity is the most frequent treatment-limiting adverse effect of these agents. Even though it remains unclear why the peripheral nervous system is at particular risk, a number of alleged biological factors have been proposed. Because of the significant clinical consequences, a critical analysis of existing data was performed to explain the underlying mechanisms of this tactile toxicity.

The neuronal framework is structurally composed of microtubules, neurofilaments, and microfilaments. Each of these separate, yet interactive, polymeric elements have very unique properties. Microtubules have a performance repertoire that includes a functional architectural role as well as serving as a cellular causeway for trafficking proteins and other components. These functions can be further elaborated upon. Although α- and β-tubulin monomers are synthesized in the cell body of the neuron, polymer formation takes place in the distal region of growing axons.26 The restrictive sites of these two processes highlight what appears to be an orderly transport of proteins, including tubulin and other microtubule-associated proteins (MAPs), which are essential for the formation of the platform to support construction of the axonal cytoskeleton.27 In essence, the integrity of the nervous system, which relies on the directional transport of neurotransmitter from perikaryon to synaptic cleft, is largely dependent on a functional microtubule network.

One of the most intriguing (and perplexing) aspects of the axonal cytoskeleton relates to the marked difference in the regenerative ability of axons following injury between neurons of the central and peripheral nervous systems.28 One plausible explanation for the difference was provided by investigators studying traumatically injured axons.29 In their report, Conde et al discovered two distinct phenomena occurring at the damaged site. Subsequent to injury, bulbous swelling followed by internal retraction (hence the descriptive terms retraction bulbs) was observed in the axons of central origin. In essence, the integrity of the nervous system, which relies on the directional transport of neurotransmitter from perikaryon to synaptic cleft, is largely dependent on a functional microtubule network.

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(and indispensable) role of the polymer in reversing growth cone stalling and re-establishing neuronal function.\textsuperscript{31}

These data provide a credible explanation for neurotoxicity associated with microtubule dysfunction. As such, disruption of dynamic instability interferes not only with the cytoskeletal structure and network but also with the axonal transport and directional conduction of electrical impulses away from the cell body. In addition, the morbid (and usually reversible) and mortal (and almost always fatal) consequences associated with peripheral and, sometimes negligent, central administration of vincristine could be explained by the appearance of growth cones and retraction bulbs, respectively.\textsuperscript{32,33}

One other important issue that needs to be addressed relates to the relatively selective injury to the peripheral nervous system. The simplest explanation relates to the likelihood that most of the anticancer agents, including the microtubule inhibitors, do not (or have negligible ability to) cross the blood–brain barrier.\textsuperscript{34} However, a more erudite precis\’ on the topic revolves around the structural integrity of the microtubule after drug-related or trauma-induced injury that determines whether axons survive or die. Based on a several ingenious in vitro and in vivo studies, researchers observed that growth cones from injured peripheral axons exposed to increasing concentrations of a vinca analog underwent transformation to bulb-like appendages, much like retraction bulbs found on the damaged axons of central nervous system origin.\textsuperscript{35} Notably, the transformed growth cones contained conspicuously disorganized (and dysfunctional) microtubules, features identical to the polymeric structures found in the central neuronal retraction bulbs. Likewise, the transformed cones were also devoid of regenerative ability. It is emphasized that the observed transformation was dependent on drug concentration. At high concentrations, growth cones underwent transformation in half of all the neurons compared with the neurons exposed to the control (drug-free) medium. This finding could explain the recovery of peripheral neurotoxic reactions associated with vinca alkaloid therapy.

With a reasonable amount of certainty, the observation that microtubule destabilization generated retraction bulbs would translate to the intriguing possibility that stabilization of the intact polymer should inhibit bulb formation. An ingenious study conducted to test this hypothesis involved conditioning of CNS neurons in taxane-containing or control (saline) mediums.\textsuperscript{35} The laboratory results revealed striking differences. First, even though bulb-like appendices developed on damaged axons bathed in both mediums, the taxane-conditioned cells exhibited significantly fewer bulbous elements. Second, the retraction bulbs of the taxane-treated axons were unusually small in size compared with control. And third, the orientation and arrangement of axonal microtubules in the smaller protuberant bodies were nearly identical to microtubules found in growth cones. These provocative data strongly suggest that microtubule stabilization hinders generation of retraction bulbs and either impedes axonal shortening or promotes axonal regrowth. These findings are even more remarkable when compared with studies using neurons from the peripheral nervous system, which showed no difference in axonal features regardless of the conditioning media. Whereas the collective findings infer that microtubule-stabilizing agents may be less toxic than destabilizing agents, these data also appear to provide insight of the molecular mechanisms underlying the development (and reversal) of drug-induced neurotoxicity. At the same time, it is important to stress the role of other components, including MAPs,\textsuperscript{36} nerve growth factor,\textsuperscript{37} intermediate filaments,\textsuperscript{38} as well as other proteins, such as actin microfilaments and plectin.\textsuperscript{39}

Incidental Microtubule-Damaging Agents

Although frequently associated with drugs that stabilize or destabilize the microtubule, significant neurotoxicity also develops during treatment with bortezomib and oxaliplatin. Not considered traditional antimicrotubule agents, these two drugs were included in this article because their surreptitious effects on the microtubule strengthen the belief that the tubulin complex may be the crux of cancer treatment-associated neurotoxicity.

Proteasome inhibitors. Bortezomib, a proteasome inhibitor, represents an anomaly in oncology.\textsuperscript{40} Proteasomes are cytoplasmic and nuclear constituents of all eukaryotic cells. In conjunction with ubiquitin, the proteasome–ubiquitin pathway is responsible for the constitutive degradation of the majority of cellular proteins. The apparent aberration stems from the finding that targeted inhibition of the 26S proteasome, an essential component of cell metabolism, retards tumor progression by interfering with the orderly degradation of normal, key regulatory molecules. In essence, this pathway plays a pivotal role in regulating the balance between de novo protein synthesis and proteolysis.

Indeed, the link between microtubule and proteasome is strengthened by laboratory studies demonstrating localization of major elements of the proteasome–ubiquitin pathway to the centrosome in cells during interphase.\textsuperscript{41} And perhaps not surprising was the observation that inhibition of the proteasome impinged on spindle dynamics, resulting in the fragmentation of the spindle apparatus.\textsuperscript{42} These intriguing findings led to further studies, one of which is most galvanizing. While it was known that various proteins (of which γ-tubulin is the best characterized) were ensconced within the pericentriolar material surrounding the centrioles,\textsuperscript{43} French investigators discovered that cells treated with proteasome inhibitors accumulated large amounts of centrosomal proteins, including an insoluble form of γ-tubulin with a higher molecular weight.\textsuperscript{44} Because microtubule nucleation is the event that initiates de novo polymerization of α-β tubulin dimers, an important question was to determine whether the accumulated proteins could alter the capacity of the centrosome to nucleate microtubules. The researchers found that not only the proteasome inhibitor, PS-341 (bortezomib), impaired microtubule
nucleation but the drug-treated cells also lacked a discernable microtubule-organizing center. Notably, allowing the cells to recover following drug removal resulted in the normalization of both processes. Still, a couple of uncertainties exist. One relates to the functionality of insoluble γ-tubulin; and two, the precise mechanism underlying the accumulation of the centrosome proteins following proteasomal inhibition. Perhaps the most plausible explanation for the latter is failure of the proteasome to degrade poly-ubiquitylated proteins.

While the importance of proper assembly and the number of centrosomes have been correlated with genetic instability and human cancer, it is conceivable that deregulation of microtubule organization could also be associated with drug-related neurotoxicity.

In addition to the proteasome inhibitors, the platinum compounds have had a major impact on the treatment of various malignancies. Of the latter agents, oxaliplatin has emerged as one of the most important new cytotoxic drugs. Classified as an alkylating agent, this platinum analog contains a sterically bulky ligand (1,2-diaminocyclohexane [DACH]) and a labile oxalate ligand, which distinguish this compound from cisplatin or carboplatin. Formation of site-specific DNA-protein-oxaliplatin as well as inter-DNA-platinum crosslinks is believed to be the basis for inhibiting DNA transcription and replication. Interestingly, although fewer in number (compared with cisplatin), the formed crosslinks are reported to be more potent, a finding possibly related to the DACH ligand.

### Platinum agents

Oxaliplatin, a third-generation platinum agent, is considered one of the two most important new drugs used in the treatment of colorectal cancer. Despite the clinical impact and attendant enthusiasm for this agent, the applicability of oxaliplatin is limited, in large part, by sensory neuropathy. Importantly, neurotoxicity, not tumor progression, is the most frequent reason that forces patients to forego further therapy with this agent. Because the approved use of oxaliplatin has, for the first time in over 50 years, improved overall survival in patients with colorectal cancer, effective prophylaxis against this toxicity should still be considered a priority for innovative prevention research.

The biochemical mechanism of the neurotoxic reaction was initially believed to involve voltage-gated sodium channels through chelation of calcium by a metabolite of oxaliplatin. The apparent, though small, benefit of calcium/magnesium infusions was consistent with the notion that altered calcium homeostasis may have a role in inducing neurotoxicity. However, results of a definitive clinical trial showed that calcium (and magnesium) infusions have no proven benefit against oxaliplatin-induced neurotoxicity.

More recently, studies in animal models strongly suggest the role of transport mechanisms in platinum-induced neurotoxicity. Ciarimboli et al reported that accumulation of drug in, and damage to, neuronal cells was linked to organic cation transporter 2 (OCT2), a protein expressed in the neurons of the dorsal root ganglia. Interestingly, overexpression of OCT2 resulted in a significant (up to 35-fold) increase in neuronal uptake of oxaliplatin, while OCT2 gene knock-out protected against the development of peripheral neurotoxicity. However, a potential obstacle related to targeting OCT2 for preventive therapy is that the protein belongs to a major facilitator superfamily of transporters, which includes OCT1–3 (electrogenic cation transporters), OCTN1–3 (electroneutral cation transporters), and OAT1–5 (organic anion transporters). Indeed, repression of the transporter's direct impact of drug uptake into neuronal cells could, simultaneously, antagonize the antitumor effect since many of these transporters, including OCT2, are expressed in the tissues of the gastrointestinal tract.

### Integrated Hypothesis of Oxaliplatin-Induced Neurotoxicity

Although calcium regulation involves several organs and hormones, vitamin D is known to be an integral component for numerous physiological functions of calcium at tissue and cellular levels. In addition to its well-known effect on bone health, the neuromuscular system is now recognized as another important target for the wide-ranging effects of this hormone. A number of publications have addressed the role of vitamin D in diseases such as multiple sclerosis and Parkinson’s disease. Even more intriguing are laboratory data showing the neuroprotective effects of the hormone in laboratory models of Parkinson’s disease and amyotrophic lateral sclerosis. However, these data are tempered by the understanding that epidemiologic studies suggest correlation rather than causation; while success observed in preclinical models may not fully translate in human trials. Nonetheless, these data do suggest that the neuromuscular system may be another target of the pleiotropic effects of vitamin D. Hence, these data cannot be totally eschewed. What remains as the most relevant issue of this article is to elucidate a link between oxaliplatin and the microtubule and generate a hypothesis to drive a proof-of-concept clinical study.

Intuitively, any neuroprotective strategy aimed at preserving microtubule function would be irrational if both antitumor and neurotoxic effects were invariably linked to microtubule dysfunction. As such, effective preventive therapies for the traditional microtubule inhibitors discussed above would be extremely challenging. However, effective prevention of neurotoxicity may be achievable if disruption of microtubule function was independent of the dominant antitumor mechanism. Such may be the case with the platinum analogs.

Given this principle, and the vitamin’s link to calcium, the supposition that vitamin D may be clinically beneficial appears not only suspicious but also irrational. If so, then what would support the apparent discordance attached to the proposed beneficial effect of vitamin D? The answer may reside in the relationship between vitamin D and the microtubule. As indicated previously, microtubules are relatively simple, yet paradoxically complex, intracellular polymers. Regardless of
manner, deregulation of the microtubule-organizing center or the microtubule itself results in mitotic arrest, the purported mechanism of tumor cell death. However, compelling evidence suggests that alteration of tubulin dynamics also increases the risk of toxicity to peripheral neurons, an adverse effect observed with all microtubule inhibitors. Even though the factors for the increased sensitivity of neuronal tissue to the antimitotic agents remain elusive, a proffered explanation was provided earlier in this article. Briefly, the pathologic process could be described in the following manner. While synthesis of tubulin monomers is restricted to the perikaryon, polymerization occurs distally in regions of axonal growth.26 Limitation of these two processes emphasizes an orderly mechanism for transporting tubulin and other microtubule-essential proteins required for constructing the neuronal cytoskeleton.27 Indeed, the ultimate shape of the cytoskeleton, integrity of the intracellular protein transport system, and function of the nervous system are dependent on the tubulin network.

Having established a biological link between the microtubule and neurotoxicity leaves the large, but not impossible, task of elucidating a connection between oxaliplatin, the microtubule, and vitamin D. Even though not classified as antimitotic agents, platinum compounds have been shown to form platinum-tubulin adducts causing denaturation of tubulin, disruption of polymerization, and inhibition of the microtubule.60 Of particular importance was the finding that disruption of microtubule function has been shown to result in a marked decrease in the formation of the active hormone, 1α,25(OH)2D3, not because of decreased cellular uptake of 25(OH)D3, but rather altered substrate transport. If so, then supraphysiologic concentrations of vitamin D may have the opposite effect of increasing the antitumor effect of oxaliplatin.61

Conclusion
Reasonably compelling evidence suggests an association that exists between cytoskeletal and axonal function, neurotoxicity, oxaliplatin, and vitamin D. And perhaps of equal importance is the possibility of correcting a ubiquitous irony in clinical research. In this context, a vitamin that may modulate numerous protective effects and costs only a few pennies a day can be considered a worthy clinical trial investment. Such a clinical study is currently being conducted to evaluate the validity of this hypothesis.

Author Contributions
Conceived and designed the experiments: GMH, CS. Analyzed the data: GMH, CS. Wrote the first draft of the manuscript: GMH, CS. Contributed to the writing of the manuscript: GMH, CS. Agree with manuscript results and conclusions: GMH, CS. Jointly developed the structure and arguments for the paper: GMH, CS. Made critical revisions and approved final version: GMH, CS. Both authors reviewed and approved of the final manuscript.

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