Chapter 14
Inhibitors of Mitotic Kinesins for Cancer Treatment: Consequences for Neurons

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14.1 Introduction

Cancer is among the greatest and most expensive medical challenges in the world, and potentially escalating in prevalence as more carcinogens are introduced into the environment. Not surprisingly, there are ongoing efforts to develop novel therapies, as effective treatments would positively impact the landscape for society. Cancer involves abnormal cells that rapidly proliferate, move, and invade tissues of the body [1]. Cell proliferation, movement and invasion all rely on the cytoskeletal elements known as microtubules, which have historically been a primary target cancer therapy. Microtubules are dynamic polymers composed of tubulin subunits, each of which is a dimer of alpha and beta tubulin. Quintessential and crucial for most of the work done by microtubules are their dynamic properties, which are governed by a mechanism known as dynamic instability [2]. A portion of the microtubule mass becomes stabilized in living cells, but a portion remains highly dynamic, undergoing rapid bouts of assembly and disassembly. When their dynamics are frozen, microtubules are no longer able to do many of their assigned tasks, which is why drugs that stabilize microtubules inhibit mitosis. Drugs that depolymerize microtubules are also effective at inhibiting mitosis, as they pare away the mitotic apparatus itself. The problem with any of these therapies is that virtually all cells of the body rely on microtubules for a variety of tasks – such as serving as architectural elements and acting as railways for organelle transport. Cells that are most dependent on microtubules are likely to display the most notable ill effects when patients are systemically treated with drugs that either stabilize or depolymerize microtubules. Consistent with this, neuropathy is among the most common problems with microtubule-altering chemotherapy [3, 4].

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Taxol, its derivatives and related drugs are currently the most commonly used microtubule-based drugs for cancer chemotherapy. Taxol binds to beta tubulin in a pocket on the luminal surface of the microtubule and suppresses the disassembly of the polymer, thus promoting its stabilization [5, 6]. Taxol does not cross the blood–brain barrier, but related drugs such as Epothilone D readily cross and actually accumulate in the central nervous system [7, 8]. Painful, debilitating and sometimes permanent peripheral neuropathies are common in patients treated with taxol and there is concern about parallel effects on the brain with drugs that cross the blood–brain barrier.

About 15 years ago, microtubule researchers began to develop a new microtubule-based strategy to target cancer cells with the goal of prohibiting cell division with no negative consequences on non-diving cells, such as neurons. Neurons are terminally post-mitotic cells that no longer divide, but rely on microtubules for their complex polar morphologies and for the efficient and orderly transport of proteins and organelles within their axons and dendrites. Hyper-stabilization of microtubules leads to a variety of changes in the organization, composition and behavior of the microtubule arrays of the neuron [9]. Therefore, the idea was to develop drugs that would specifically inhibit certain members of the kinesin family of molecular motor proteins believed to be expressed only in cells that divide. These so-called mitotic kinesins were shown to be important for organizing and driving the mitotic apparatus, and hence would presumably not be expressed in terminally post-mitotic neurons. This was considered a breakthrough for the cancer research community and the absence of mitotic kinesins from neurons was enthusiastically touted by the pharmaceutical industry.

A decade and a half later, one might have expected innumerable cancer patients to owe their lives to a rich kit of drugs inhibiting mitotic kinesins. One might also have expected taxol to be long since abandoned in favor of such drugs. In fact, thousands of patients continue to rely on taxol to impede the growth of tumors and continue to suffer the effects of peripheral neuropathies. There are various reasons why the anticipated success targeting mitotic kinesins has not materialized, at least not yet. Here, we discuss a potential concern that should be considered far more than it has been to date. Mitotic kinesins, as it turns out, are expressed not only during mitosis in dividing cells, but also in terminally post-mitotic cells [10–16]. Neurons rely on these kinesins for many of their most important developmental challenges. While the levels of these kinesins diminish in adult neurons, even low levels of these motor proteins may have important work to do throughout the life of a neuron (Fig. 14.1). In addition, there is recurring neurogenesis in the adult brain, which recent research suggests is important for memory and cognition [17]. Drugs that inhibit the so-called mitotic kinesins would, without a doubt, affect the differentiation of these newly born neurons if the drugs were to cross the blood–brain barrier.

Given that chemotherapy is given to patients for discrete periods of time, it may well be that the central and/or peripheral nervous systems can tolerate prohibited function of one or more of the mitotic kinesins in a typical therapeutic timeframe. In fact, inhibiting these kinesins in cultured neurons, while dramatically affecting

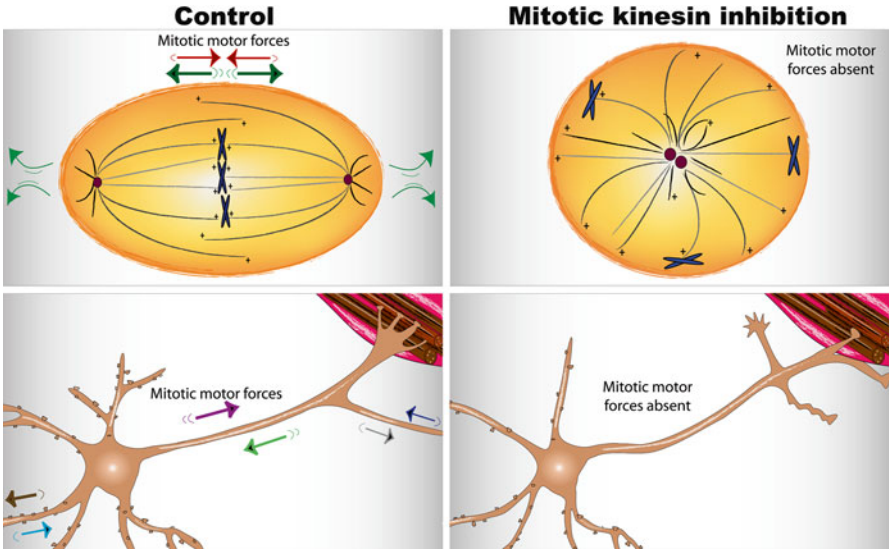

Fig. 14.1 Effects of mitotic kinesin inhibition on mitosis and neurons. In the *left panels*, mitotic kinesins normally generate forces important for the organization of the mitotic spindle in the dividing cell and for the distinct microtubule arrays in axon and dendrites of the neuron. Arrows represent forces generated by different mitotic kinesins In the *right panels*, inhibition of these kinesins yields a failed mitotic spindle and the forces responsible for organizing the microtubule arrays in the neuron are imbalanced, leading to axonal and dendritic architectural and functional abnormalities

neuronal morphology, has no apparent negative consequences on the overall vitality of the neuron [16]. Even so, the concern lies in the mystery of what might potentially happen to patients taking such drugs. Ill effects may be subtle compared to those caused by taxol and may even escape notice in clinical trials, but could nevertheless be insidious. For example, minor changes in dendritic arborization or spines could affect cognition, memory and personality [18–23]. The purpose of this chapter is not to be alarmist about the use of this approach, but to dissuade any temptation for scientists, clinicians and drug developers to be cavalier about ignoring the potential impact of such drugs on the nervous system.

14.2 Neuronal Microtubules

Vertebrate neurons in their post-migratory configuration generally consist of a single axon and multiple dendrites [24, 25]. These are elongated processes (often complex in their branching patterns) that require microtubules as shape-sustaining architectural struts. In addition, the microtubules of axons and dendrites serve as direction-specific railways for organelles and other cargo transport. In a typical vertebrate neuron, nearly all of the microtubules in the axon are oriented with their plus

ends directed away from the cell body [26], whereas the dendrites have a mixed orientation of microtubules [27]. We have recently discussed in detail how these patterns were discovered and the contributions of these microtubule polarity patterns to the distinct morphological and compositional features that define the identity of the axon and the dendrites [28]. Because different types of organelles engage either plus-end-directed or minus-end-directed molecular motor proteins, the polarity pattern of the microtubule array is a major determinant of which organelles/cargoes are transported into each type of process from the cell body, as well as the efficiency and character of anterograde and retrograde organelle movements within the axon and dendrites. This simple scenario can explain, for example, why Golgi outposts appear in dendrites, but not the axon [29]. In addition, the polarity patterns offer an attractive explanation for the differences in morphology and growth properties between axons and dendrites [28].

Contemporary research on neuronal microtubules has yielded a great deal of knowledge on the behaviors of microtubules that underlie the growth and maintenance of the axon, the development and plasticity of dendritic arbors, and the migration of developing neurons to their destinations [24]. Microtubules undergo a variety of behaviors such as dynamic assembly and disassembly, bundling and splaying, and severing [30]. They also integrate at many levels with other cytoskeletal elements, most notably actin filaments [31]. Shorter microtubules undergo rapid transport, and the same forces that transport short microtubules also impinge upon longer stationary microtubules in functionally important ways [32]. These various behaviors are regulated by signaling pathways that impact proteins such as molecular motor proteins, microtubule-severing enzymes, microtubule-depolymerizing enzymes, as well as a variety of other proteins that influence the assembly, stabilization and bundling of microtubules.

While a fraction of the microtubule array of the axon is quite stable, a fraction is not, with dendritic microtubules having an even smaller fraction of stable microtubule polymer than axons [33]. Younger neurons also have more labile microtubules in general than mature neurons, especially in their axonal growth cones, in which highly dynamic microtubules are important for navigation [34, 35]. The stable and labile microtubule fractions in the neuron are not separate microtubules, but rather each microtubule has a stable region toward its minus end with most of these microtubules having a labile region toward its plus end [36, 37]. The stable region has been likened to a microtubule nucleating structure that controls the distribution and polarity orientation of the dynamic microtubule polymer [38]. Without that level of control to restrict where new assembly arises, new microtubules would arise with haphazard organization, thus corrupting the all-important microtubule polarity patterns of axons and dendrites [39].

The structure and function of microtubules explain why microtubule-stabilizing drugs such as taxol have such negative and often permanent effects on neurons. Neurons treated with taxol can display abnormal accumulation and bundling of microtubules, loss of microtubule domain structure, and corruption of normal microtubule polarity patterns (Fig. 14.2). Such effects can cause traffic jams in organelle transport as well as mis-localization of organelles and proteins that should

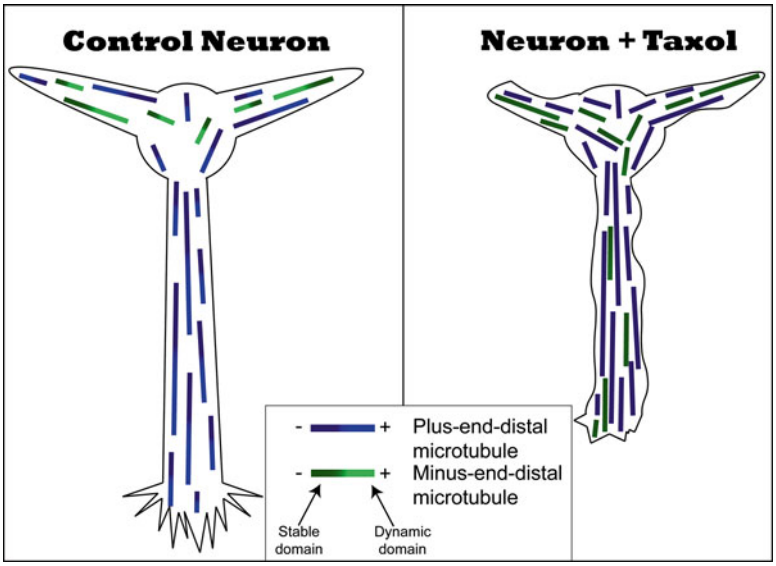

Fig. 14.2 Effects of taxol on microtubule organization in the neuron. In control neurons, microtubules are nearly uniformly plus end distal in the axon. In the dendrite, microtubules have a mixed orientation (*left panel*). Each microtubule consists of a stable domain toward the minus end of the microtubule, with most microtubules also consisting of a dynamic (labile) domain toward the plus end. Dendritic microtubules are less stable than axonal microtubules. In neurons treated with taxol (*right panel*), the density of microtubules increases, the normal domain structure of individual microtubules is lost because the microtubules are stabilized all along their lengths, and flaws arise in the normal polarity patterns of the microtubules. Such abnormalities can lead to degeneration of axons and dendrites

be enriched or exclusive to one type of process or the other. In fact, major abnormalities in axonal microtubule polarity orientation have been documented in cultured neurons treated with taxol and, of particular concern, the abnormalities seem to persist even after removing the drug [40]. Even subtle alterations in the microtubule polarity patterns of axons and dendrites could have profoundly negative consequences over time [41, 42].

Accumulation of certain tubulin post-translational modifications on microtubules is another relevant aspect of microtubule regulation [43–47]. The best studied of these are detyrosination and acetylation, both of which occur on the alpha tubulin component of the tubulin heterodimer and both of which occur only after a tubulin subunit is incorporated into microtubule polymer. Detyrosination is the removal of the C-terminal tubulin residue from alpha tubulin, while acetylation is the addition of an acetyl moiety to lysine 40 of alpha tubulin, which lies at the luminal surface of the microtubule polymer [45, 48]. Some motor proteins, including kinesins, that use microtubules to transport organelles, vesicles or even other shorter microtubules, have been found to have a preference for certain microtubule post translational modifications over others. For example, the depolymerizing kinesins

(kinesin-13 family) interact better with tyrosinated microtubules [49], while conventional kinesin (kinesin-1) interacts better with detyrosinated microtubules [50–52]. Because these microtubule modifications only occur on the polymer (not free subunits of tubulin), and because the modifications accumulate on the polymer over time, the older the microtubule is, the more modified it becomes. The levels of the modified subunits are therefore considered a good indication of the stability of the microtubule, although they do not cause stability, at least not directly. Hyper-stabilization of microtubules with taxol dramatically increases the levels of these modifications on the microtubules, which can be consequential given the role of these modifications in regulating the interaction of the microtubule with such a vast array of proteins.

These various considerations illustrate why drugs that stabilize microtubules have such profound impact on neurons, with any of these effects potentially contributing to the neuropathies experienced by patients using taxol and its derivatives as chemotherapy. As for drugs that inhibit mitotic kinesins, the question becomes whether the potential effects of such drugs on neurons are non-existent (as has been claimed by early advocates for their use), minimal or potentially as bad or worse than the effects of taxol. Due consideration seems warranted, especially in light of the expectation of many drug companies that there will be no effects due to their assumption that mitotic motor proteins are not expressed in neurons.

14.3 Mitotic Kinesins: Function in Neurons

In an ideal world, chemotherapy would be minimally invasive, able to target and eliminate cancer cells, and avoid other cell types. When mitosis was discovered to rely on specialized kinesins, these proteins became an appealing target for drug development. Kinesins were originally discovered as motors that transport membranous organelles, but these specialized mitotic kinesins are different in the sense that they generate forces between microtubules and neighboring microtubules, between microtubules and actin filaments, and can also integrate with other components of the mitotic apparatus in various signaling pathways relevant to cell division [53]. In this way, the so-called mitotic kinesins contribute to the formation of the bipolar spindle, the separation of the half-spindles and cytokinesis. But are these mitotic kinesins really mitosis-specific, as originally believed?

In the early 1990s, a great deal of attention, mainly from our laboratory, focused on identifying the molecular motor proteins that transport and set up microtubule arrays in neuronal axons and dendrites. We figured that at least one molecular motor was needed to transport microtubules with plus ends leading from the cell body into the axon and developing dendrites, and at least one other motor was needed to transport microtubules with minus ends leading specifically into developing dendrites (but not the axon). This was at a time prior to the genome projects and prior to the kinesin tree being established, so we initially thought that entirely new motor proteins were awaiting discovery. It was instructive for us to learn that the mitotic

motors actually have the kinds of properties that we had in mind for such neuronal motors: they do not transport membranous organelles along microtubules, but rather generate forces that move and configure the microtubules themselves [54].

Our first hint that mitotic motors could be repurposed for transporting microtubules into cellular processes came from our collaborative studies with Dr. Ryoko Kuriyama, in which we expressed two different mitotic motors separately into insect ovarian Sf9 cells, which normally do not bear processes. One of these, a motor termed CHO1/MKLP1 (also called Kif23, now known as kinesin-6), had the interesting property of transporting microtubules with minus ends leading toward the plus ends of other microtubules. Notably, expression of a large fragment of this motor in Sf9 cells resulted in the outgrowth of microtubule-rich processes and the concomitant depletion of microtubules from the cell body. CHO1/MKLP1 produced dendrite-like processes (short and tapering) with mixed orientation of microtubules [55–57]. We subsequently showed that a motor protein then called CHO2 (now known as kinesin-14) when expressed as a fragment in Sf9 cells produced axon-like processes with uniformly plus-end-distal microtubules. Kinesin-14 family members are unusual among kinesins in that they move toward minus ends of microtubules, whereas the other kinesins move toward plus ends of microtubules [58]. Thus, like cytoplasmic dynein, kinesin-14 would transport microtubules against a stationary substrate with plus ends leading. These experiments established precedent for the properties of mitotic motor proteins to generate the characteristic microtubule polarity patterns of axons and dendrites by transporting microtubules generated in the cell body into the processes.

We originally considered the work on Sf9 cells as proof-of-principle, not that mitotic motors were actually repurposed in developing neurons. However, when we assayed developing neurons for three different mitotic motors, kinesin-6 (CHO1/MKLP1), kinesin-5 (also called Eg5, KSP, Kif11), and kinesin-12 (also called Kif15), we found each one to be strongly expressed. In mitosis, kinesin-5 generates forces on anti-parallel microtubules during prophase to drive the duplicated centrosomes apart, and then later in mitosis contributes to the separation of the half-spindles [59–61]. Kinesin-12 does not appear to be crucial within itself, but it has overlapping function with kinesin-5 and can compensate for its loss [10, 62–64]. Kinesin-6 generates forces on antiparallel microtubules in the spindle midzone, but also signals through RhoA to the actin cytoskeleton to cause cortical actin filaments to concentrate in the region corresponding to the midzone of the spindle to form the cleavage furrow that ultimately pinches off the two daughter cells [65–67]. In cultured neurons, when we knocked down each of these motors using siRNA, we observed dramatic morphological phenotypes and pronounced effects on microtubule organization and behavior [16].

Studies on these motors in neurons have produced several papers from our laboratory, often as collaborative projects with scientists prominent in the mitosis field. We began our work on kinesin-6, but our work on kinesin-5 has been the most thorough, in part because of availability of drugs that inhibit its function. Such drugs, prototypes for the more sophisticated medications subsequently used for clinical trials on cancer patients, were used to fortify our knockdown approach and enabled

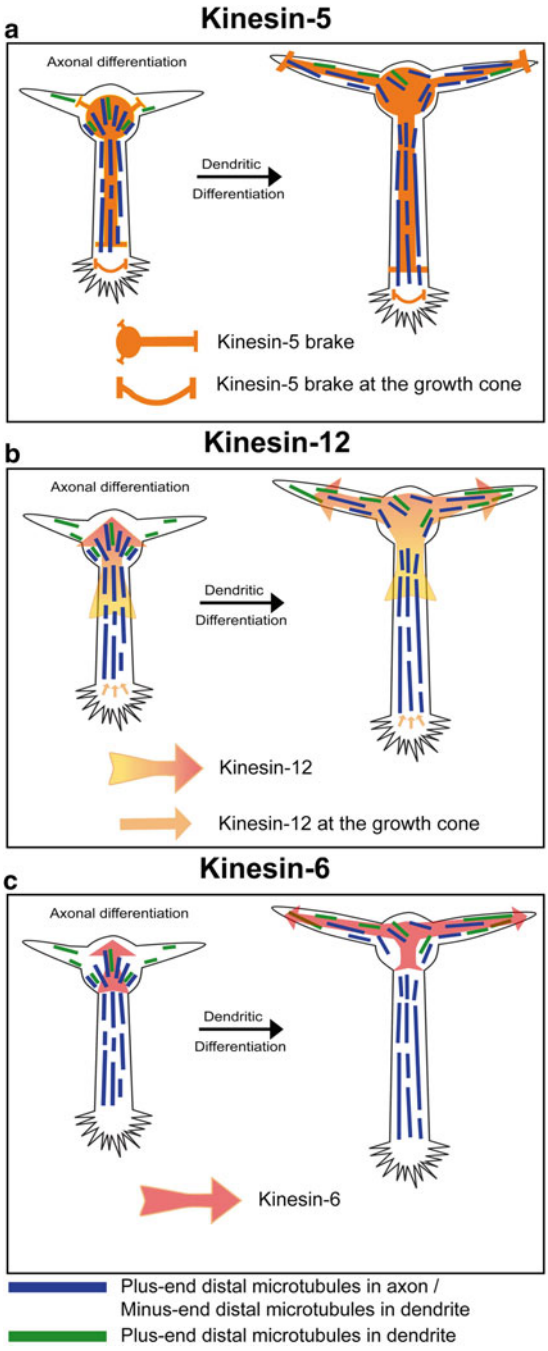

Fig. 14.3 Model for co-regulation of microtubule polarity in axons and dendrites by mitotic kinesins. Figure adapted from previous publication [16]. During axonal differentiation, forces generated by cytoplasmic dynein drive plus-end-distal microtubules into the axon and nascent dendrites (not shown). (**a**) Forces generated by kinesin-5 act as a brake on the transport of microtubules into the axon during axonogenesis. Kinesin-5 also acts as a brake on the transport of plus and minus-end-distal microtubules into dendrites (**b**) Forces generated by kinesin-12 behave

a variety of experiments, such as ones in which we observed the effects on growth cones of a gradient of such a drug [68]. The work with drugs or siRNA produced consistent results, suggesting that such an approach will be useful in exploring the properties of future drugs that become available to inhibit mitotic motors.

Our studies indicate that knocking down any of the three motors causes the axon to grow more rapidly and to increase its content of short mobile microtubules moving in both directions [16]. Knockdown of either kinesin-5 or kinesin-12 causes growth cones to fail to turn in response to environmental cues [13, 68], with no such effect observed with knockdown of kinesin-6 [16]. Knockdown of kinesin-5 results in a more branched axon [12], while knockdown of kinesin-12 results in a less branched axon [14], with this difference presumably owing to the fact that kinesin-12 interacts with actin filaments while kinesin-5 does not [10]. Kinesin-6 knockdown has no effect on axonal branching [16]. Both kinesin-5 and kinesin-6 are present in the axon and the growth cone, but kinesin-6 is undetectable in the axon, suggesting that its effects on axonal growth are probably due to an effect at the level of the cell body that enables more short microtubules to transit into the axon [12, 14, 16]. All three motors are present in the cell body and concentrate in dendrites as they begin to develop, with the knockdown of kinesin-6 or kinesin-12 proving detrimental to the appearance of minus-end-distal microtubules in dendrites [16]. Conversely, knockdown of kinesin-5 increases the proportion of minus-end-distal microtubules in dendrites [69]. Knockdown of any of the three motors causes dendrites to become thinner, with knockdown of kinesin-6 or kinesin-12 causing dendrites to become more axonal in character (Fig. 14.3).

We have also observed notable effects of knocking down these motor proteins on neuronal migration, which is the movement of newly born neurons from their sites of origin near the ventricles through the developing brain, to form its laminar structure. Depletion of either kinesin-5 or kinesin-12 increases the rate of neuronal migration, with either of these motors potentially playing a role in the cessation of migration once the neuron has reached its appropriate location [14, 15]. Knockdown of kinesin-6 prevents newborn neurons from transitioning from multi-polar to bipolar morphology, and prevents the neuron from designating any one process as the leading process [70]. Such neurons are either stationary or are futile in their movements.

Studies on messenger RNA indicate that these three mitotic motors diminish in expression in mature neurons, in some cases to almost undetectable levels [71–73]. At first glance, this seems very good news for the potential use of cancer drugs against mitotic motors, as the developmental events in which the motors are utilized

Fig. 14.3 (continued) similarly to kinesin-6 with regard to introducing minus-end-distal microtubules into the dendrite, but kinesin-12 is also present in the axon and growth cone, pushing plus-end-distal microtubules back toward the cell body. (**c**) Forces generated by kinesin-6 in the cell body oppose the forces generated by cytoplasmic dynein, restricting the transport of plus-end-distal microtubules into the axon. As the neuron matures, kinesin-6 fuels the transport of short microtubules with their minus-end distal into all of the processes except the one designated to remain the axon, thus causing the other processes to differentiate into dendrites. As a result, kinesin-12 behaves like kinesin-6 with regard to dendrites but produces effects more like kinesin-5 with regard to the axon. For more details, see Ref. [16]

will have been long since completed. However, the messenger RNA results do not necessarily mean that protein levels are down by corresponding levels, as our Western blot analyses indicate [14, 74]. Moreover, we have some preliminary indications that expression of certain of these motors may increase when neurons are challenged, for example by injury leading to regeneration (in the case of kinesin-12, according to our unpublished data, Liu, M. and Baas, P.W), or perhaps even in response to normal plastic events such as dendritic remodeling associated with learning. For example, adult neurons may synthesize "gulps" of mitotic motors that could be functionally important, especially during bouts of plasticity. Perhaps more importantly, one of the most provocative areas of neuroscience in recent years has been the discovery that in the adult brain, there is ongoing neurogenesis and the new neurons that are produced are functionally important for cognition and memory [17, 75–77]. When neurogenesis is impeded in the adult brain, rodents have been shown to undergo notable decline in their ability to perform in cognitive and memory tests [78, 79]. Thus, even if adult neurons rely minimally or not at all on mitotic motors, the newly differentiated neurons in the adult brain undoubtedly do, and during the period of chemotherapy, if the drugs cross the blood–brain barrier (or if the drugs are actually used to treat brain cancer), there would presumably be strong effects on these young neurons.

What about other kinesins essential for mitosis? The kinesin-4 proteins (also called Kif4), the so-called chromokinesins, are important in mitosis for condensing and moving chromosomes. Kif4A and kif4B are often redundant in function and sometimes not distinguished in studies, but have essential roles in regulating anaphase spindle dynamics and completion of cytokinesis [80–82]. Depending on the type of cancer, kinesin-4 has been shown to be either overexpressed or downregulated, and has proven to be a valuable prognostic tool in several independent cancer type cases. Loss of kinesin-4 in some studies had been found to induce aneuploidy (abnormal number of chromosomes), which potentially contributes to tumor formation [83], while overexpression of the protein in cervical and lung cancer has potential to become a biomarker and a target for drugs [84, 85]. In neurons, available evidence suggests that these kinesins mediate anterograde translocation and positioning of the cell adhesion molecule L1, ribosomal constituents to axons [86], and are involved in neuronal survival [87]. Kinesin-13 (Kif2A) is a microtubule-depolymerizing motor that is required for bipolar spindle formation [88–91]. Overexpression of this protein has been seen to contribute to metastasis of certain cancers and to play a role in resistance to microtubule-stabilizing drugs [92, 93]. Kif2A has been shown to be present in the neuron, and when inhibited causes impairment of axonal branch formation [94]. Mutations in the Kif2A gene have recently been found to contribute to malformations of cortical development and microcephaly emphasizing the importance of centrosomal and microtubule-related proteins in cortical development [95]. Alterations in levels of these kinesins and other mitotic kinesins are a hallmark of some types of cancers, while certain experimental manipulations of these mitotic motors have been seen to actually

cause cancer [96, 97] or have an effect in resistance to therapy [98]. Potential impact on neurons of inhibiting these kinesins further complicates the landscape.

14.4 Mitotic Kinesins as Targets for Cancer Drugs: Where Do We Stand?

Kinesin-5 was the first mitotic kinesin chosen for cancer therapy, because the knockdown phenotype of a mono-astral spindle was so dramatic and so inconsistent with cell division. Dr. Mitchisons's laboratory developed monastrol as the prototype drug for kinesin-5 inhibition and since then more sophisticated drugs have been developed by companies and put through clinical trials. Some proof-of-principle that anti-kinesin-5 drugs could be affective against cancer comes from gene electrotransfer therapy studies wherein kinesin-5 siRNA drastically reduces outgrowth of subcutaneous melanoma and ovarian cancer lesions in mice [99] and from lipid nanoparticle-driven delivery of kinesin-5 siRNA, which has been shown to cause regression of liver metastases in endometrial cancer in humans [100]. Anti-kinesin-5 drugs such as Ispinesib and ARRY-520 appear to be moderately well tolerated by patients in clinical trials, with common ill effects of mild to moderate neutropenia, fatigue, anemia, nausea, leukopenia, thrombocytopenia, and diarrhea [101–103]. Surprisingly, though, anti-kinesin-5 drugs have elicited mixed results in terms of curtailing various types of cancer, and collectively these results have been rather disappointing (see also, clinicaltrials.gov).

Newer efforts are shifting toward combinatorial therapy with other anti-cancer drugs. One possibility is that in cancer cells, excess expression of kinesin-12 can compensate for inhibition of kinesin-5, so perhaps developing anti-kinesin-12 drugs to be used in combination with anti-kinesin-5 drugs makes sense. Drug companies are also putting attention now on other mitotic kinesins with the hope that the impact on cancer will be more consistent and profound. In terms of nervous system defects, the good news is that we have been unable to find any reports in available literature or clinical trial summaries of the kinds of disorders or defects that concerned us most, given our work on developing neurons. However, it is not clear just how thorough the trials were in terms of seeking potential effects on neurons; and ill effects that might have escaped notice could be exacerbated as the treatment regimes are fortified to have greater effects on cancer.

We will conclude by reiterating a need not for alarm, but for due caution in the use of drugs that inhibit mitotic kinesins. Nature has developed a system of repurposing proteins that while beautiful in its economy presents potential obstacles for cancer therapy that merit consideration. The neuroscience community will hopefully delve deeper into whether mitotic motors serve key roles in adult neurons, and also whether putting adult neurogenesis on hold for periods of chemotherapy is acceptable to the health of the patient.

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