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REVIEW

Microtubule destabilising agents: far more than just antimitotic anticancer drugs

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Vinca alkaloids have been approved as anticancer drugs for more than 50 years. They have been classified as cytotoxic chemotherapy drugs that act during cellular mitosis, enabling them to target fast growing cancer cells. With the evolution of cancer drug development there has been a shift towards new "targeted" therapies to avoid the side effects and general toxicities of "cytotoxic chemotherapies" such as the vinca alkaloids. Due to their original classification, many have overlooked the fact that vinca alkaloids, taxanes and related drugs do have a specific molecular target: tubulin. They continue to be some of the most effective anticancer drugs, perhaps because their actions upon the microtubule network extend far beyond the ability to halt cells in mitosis, and include the induction of apoptosis at all phases of the cell cycle. In this review, we highlight the numerous cellular consequences of disrupting microtubule dynamics, expanding the textbook knowledge of microtubule destabilising agents and providing novel opportunities for their use in cancer therapy.

Tables of Links

TARGETS	
Enzymes [2]	**Other protein targets [3]**
ERK	Bcl-2 family
JNK	tubulin
p38	

LIGANDS	
combretastatin A4	colchicine
docetaxel	eribulin
paclitaxel	vinblastine
vincristine	vinorelbine

These Tables lists key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY [1], and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 [2, 3].

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Introduction

Mitosis causes a 20–100-fold increase in microtubule dynamics, providing an increased dependence on proper microtubule function during mitosis. This would suggest that mitosis is the prime time to target microtubules and helps explain why most microtubule targeting agents (MTAs) are frequently called "antimitotic drugs." Defects in spindle assembly or spindle-kinetochore attachment (such as disrupted microtubule function) activate the spindle assembly checkpoint, arresting cells prior to the metaphase–anaphase transition [4]. This effect has been reported for microtubule inhibitors giving rise to the classic G2/M cell cycle arrest seen in cell culture experiments.

It is clear now that there is much more to the mechanism of action of MTAs than simply acting as antimitotic drugs. Some drugs in this class are considered cytotoxics, while others are considered vascular disrupting agents, but, regardless of the terminology, all of these drugs have the ability to kill cancer cells directly and yield clinical outcomes. These microtubule poisons are more successful than any true mitosis-specific inhibitor (e.g. mitosis-specific kinase inhibitors), having the ability to kill mitotic, interphase and quiescent tumour cells. The primary goal of this review is to highlight novel mechanisms of action of microtubule destabilising drugs in which acute activation of signalling pathways can induce apoptosis independent of cell cycle phase.

Microtubules

The cellular cytoskeleton is comprised of three main components: microfilaments (actin proteins); intermediate filaments (various proteins); and microtubules (tubulin proteins). Microtubules are ubiquitous in eukaryotic cells and consist of alpha and beta tubulin dimers. Polymerisation is regulated by GTP hydrolysis within the beta protein of the tubulin dimer and the newly formed microtubule ends often contain stabilising GTP caps [5]. The microtubule-organising centre (MTOC) represents the specific location where microtubules are nucleated, organising the microtubules within the cytoskeleton during interphase. MTOCs are also associated with spindle formation during mitosis forming the centrosomes [6]. It is thought that net growth occurs at the plus end of microtubules with net shortening at the minus end. While the shift between microtubule growth and shrinkage constantly changes, depolymerisation of these structures can occur at rates 100-fold faster from GDP-containing subunits [4].

Microtubules control intracellular trafficking of proteins, organelles and vesicles, and separate chromosomes during mitosis. These structures are also fundamental for cell proliferation, cell shape, migration, secretory processes and vascularisation [7]. They have a very dynamic structure that is perfectly designed for transmitting signals throughout the cell via interaction with numerous signalling proteins and organelles. In addition to microtubule-only functions, actin–tubulin interactions can control cell migration, wound healing, T cell immune response and growth of neuronal cones. Disruption of microtubules can alter the abundance of actin and intermediate filaments impacting cytoskeletal networks. However, there is no backup redundancy for microtubules within the cellular cytoskeleton as they are the only structures that can segregate chromosomes or traffic cellular components [7].

Two classes of motor proteins bind to microtubules and hydrolyse ATP to move steadily along them: dyneins and kinesins. Dyneins move along microtubules (away from the plus end) to traffic vesicles and appropriately localise organelles, while kinesins move in the opposite direction (towards the plus end), playing specific roles in mitotic spindle formation and chromosome separation during mitosis [4, 6, 8]. These two classes of motor proteins work together to provide bidirectional movement of cellular components along the microtubule network.

Microtubule associated proteins (MAPs) impact protein–microtubule interactions, microtubule cross-linking and overall microtubule stability. Microtubule stabilising proteins include cyclin-dependent kinase 1 (CDK1), tau, and MAPs1, 2, and 4, while microtubule destabilising factors include oncoprotein 18, katanin, and the xenopus kinesin-related protein XKCM1 [4]. Microtubules and MAPs associate with protein kinases and phosphatases, mediating rapid signalling networks that can lead to alterations in microtubule stability and function and may even lead to apoptosis. For example, MAPs are phosphorylated to reduce their affinity for microtubules, reducing tubulin polymerisation during mitosis. MLK and KIF3 family kinesin motor proteins interact with c-Jun N-terminal kinase (JNK) and can alter downstream JNK pathways including apoptosis [4]. Microtubules also interact with the voltage dependent anion channel, the main component in the permeability transition core of the mitochondria, yielding another link to the apoptosis machinery [9].

MTAs

Most MTAs bind one of four main sites/domains within microtubules impacting tubulin stability: the laulimalide site (stabilising), taxane/epithilone site (stabilising), colchicine site (destabilising), or the vinca alkaloid site (destabilising; reviewed in [5]). Additionally, binding to a different site on the plus ends of microtubules has been seen with some drugs such as eribulin and maytansine. High concentrations of MTAs alter microtubule mass, while low drug concentrations do not change the overall microtubule mass of a cell but disrupt microtubule dynamics and protein interactions [10]. Vinca alkaloids bind with high affinity to one or a few tubulin molecules at the tip of microtubules but do not copolymerise with them. Low drug concentrations lead to substoichiometric binding at the high affinity binding sites and it is estimated that 1–2% binding could reduce microtubules by 50%. High concentrations can bind stoichiometrically at both high and low affinity sites, enhancing drug impact [11]. High affinity binding of colchicine to tubulin allows it to copolymerise into the structure; this process is slow and quasi-irreversible. The concentrations of microtubule stabilisers and destabilisers that bind tubulin have

consistently correlated with the growth inhibitor concentrations (IC_{50}) in cancer cell line experiments [12].

The bulk of research and development of anticancer therapies targeting the microtubules has focused on agents that bind either the taxane or vinca alkaloid sites, with more recent investigations in the potential of colchicine site binding agents. Here, we focus on the microtubule destabilising drugs because, as will be discussed below, their actions are considerably different from those of taxanes.

Vinca alkaloid site binding drugs

Vinca alkaloids were found by serendipity during diabetes research, in which extracts from the periwinkle plant *Vinca rosea* (now known as *Catharanthus roseus*) were initially studied due to folklore that indicated they had oral hypoglycaemic properties. While no antidiabetic actions were observed, the periwinkle extracts that contained a mixture of natural vinca alkaloids caused a reduction in white blood cell counts and bone marrow destruction in rats. The periwinkle extracts also extended the life of mice transplanted with lymphocytic leukaemia [11]. Initial studies on the biochemical effects of vinca alkaloids revealed disruption of microtubules, elevated oxidised glutathione, alteration in lipid metabolism and membrane lipid content, elevated cAMP, and inhibition of calcium-calmodulin-regulated cAMP phosphodiesterase [11]. The vincas had no impact on cellular respiration, glycolysis, nucleic acid or protein synthesis [13]. Additionally, vinca alkaloids are hydrophobic and can partition into lipid bilayers when uncharged thus altering the structure and function of cellular membranes [11]. Of this list, the most highly documented mechanism of action is the ability to disrupt microtubules via high affinity binding to tubulin.

The vinca alkaloid drug family includes: vinblastine, vincristine, vinorelbine, vindesine and vinflunine, of which the latter three are all semisynthetic derivatives of vinblastine. The first to be approved by the Food and Drug Administration (FDA) was vincristine sulfate (Oncovin) in 1963, followed by vinblastine sulfate (Velban) in 1965 and vinorelbine (Navelbine) in 1994. Vindesine and vinflunine are used in Europe.

The vinca alkaloids are typically administered by intravenous (IV) bolus, but can be given as a longer infusion to maintain high drug levels for a longer period of time. Vinorelbine was the first vinca alkaloid to display oral activity, yet it has similar pharmacokinetics to IV-administered drug. Dose limiting toxicities vary with agent: vincristine is neurotoxic, vinblastine is myelosuppressive, vindesine is both and vinorelbine is myelosuppressive with mild/reversible neurotoxicity [11]. As expected, these side effects are worse with continuous infusions. Vinorelbine is the best tolerated from this family with other common dose-limiting toxicities of granulocytopenia and leucopenia.

Vinblastine was initially administered in 7-day intervals. The response time of solid tumours averaged 10–12 weeks of therapy, yet many early clinical trials only scheduled 2 weeks of therapy and the drug was thought to be ineffective. Even with short trials, early responses were seen in Hodgkin's lymphoma and choriocarcinoma [13]. After further trials, vinblastine was primarily used to treat lymphomas, bladder and breast cancers. Vincristine was primarily used to treat acute lymphoblastic leukaemias and lymphomas because complete remissions of acute leukaemia were achieved in early clinical trials [13]. Vinorelbine has shown an overall response rate of 33% in non–small-cell lung cancer, 52% in untreated advanced breast cancer, 38% in heavily pretreated patients and 90% in untreated Hodgkin's lymphoma [11]. The vinca alkaloid class of drugs is often combined with other chemotherapies with different structures and mechanisms of action to avoid cross resistance [11]. Vincristine dosing is limited by its neurotoxicity, while vinblastine can be administered at higher doses and would be more effective as a single agent. However, vincristine is used more often than vinblastine for drug combinations because many other chemotherapeutics are myelosuppressive like vinblastine.

Dolastatin 10, isolated from the marine mollusc *Dolabella auricularia*, also binds the vinca alkaloid domain but failed to show efficacy in numerous clinical trials and is no longer under development [14–16]. A similar drug that binds the microtubule plus end and competes with vinca alkaloid binding is maytansine from the shrub *Maytenus ovatus* [17]. Many maytansine derivatives are under clinical investigation. Halichondron B binds noncompetitively with the vinca alkaloid domain inhibiting microtubule polymerisation, causing mitotic arrest in murine leukaemia [18]. Low yields and structure complexity of the natural product from the sponge *Halichondria okadai* led to the synthesis of several halichondron B derivatives including eribulin. Unlike its parent compound, eribulin does not directly bind the vinca alkaloid site but instead binds in a noncompetitive manner at a slightly different site which inhibits microtubule growth but does not impact shortening rates as seen with vinca alkaloids [19]. Instead, eribulin forms small unstable globular tubulin aggregates and retains several properties of both vinca alkaloids and taxanes in regards to vascular disruption and antiangiogenesis [20]. Eribulin was FDA approved in 2010 for metastatic breast cancer previously treated with taxane and anthracycline, with phase II studies showing efficacy in numerous malignancies [21].

Colchicine site binding drugs

Colchicine was first extracted from the poisonous meadow saffron *Colchicum autumnale*. Colchicines have a low therapeutic index with several toxicities: neutropenia, gastrointestinal upset, bone marrow damage and anaemia. Thus, colchicine is not the best anticancer candidate; however, it has been FDA approved for the treatment of gout and familial Mediterranean fever [22]. Other agents have been discovered (combretastatins) or designed (BNC105) to target the colchicine domain and are being developed as vascular disrupting agents against cancer.

Combretastain A4 (CA4) isolated from the African bush willow *Combretum caffrum* is the most potent naturally occurring combretastatin in regards to tubulin binding ability and cellular toxicity. Its prodrug CA4 phosphate (CA4P) can inhibit tumour blood flow at concentrations 10-fold lower than the clinical maximal tolerated dose [23]. CA4P can block cell migration and metastasis, with blockage of new vessel formation [24]. It has been suggested that mitotic catastrophe is more likely to be the primary response to CA4P rather than

apoptosis or necrosis, which may occur as a consequence [25]. Sarcomas showed early haemorrhage followed by necrosis in mouse models treated with CA4P. The surviving peripheral tumour cells were able to repopulate within 48–72 h post treatment as perfusion recovered [26]. Another member of the combretastatin family, combretastatin A1 diphosphate (OXi4503), reduced overall tumour burden in a murine systemic leukaemia model and is undergoing clinical trials in acute myeloid leukaemia (AML) [27]. Many derivatives have been made from combretastatins A1 and A4 as they are the most biologically active, but few have reached clinical trials. These drugs tend to have short biological half-lives and poor pharmacokinetic profiles, which may not be a clinical problem as three different dosing schedules indicate that the acute effects of CA4P are more important than chronic exposure/effects [28]. Side effects include neural toxicity, cardiovascular and thromboembolic events. Overall, they have been disappointing as single agents in the clinic.

BNC105, a synthetic colchicine site binding drug, is reported to be 10-fold more potent than CA4 at preventing capillary formation without disrupting preformed capillaries; with low nanomolar concentrations disrupting tubulin. However, BNC105 and CA4 were equipotent in regards to cell blebbing, human umbilical vein endothelial cell permeability and inhibition of tubulin polymerisation. BNC105 treatment led to a decrease in the endothelial cell marker CD31, decreased laminin basement membrane degradation and increased apoptosis. In mice, administration of the prodrug BNC105P induced high levels of tumour necrosis while inhibiting tumour growth in Calu6 xenograft and MDA-MB-231 mammary fat pad murine models [29]. The first-in-human clinical trial of BNC105P was performed in patients with advanced/metastatic solid tumours. Polymerised tubulin was reduced in patient peripheral blood mononuclear cells (PBMCs) 1–7 h postadministration with levels returning to basal by 24 h. The study yielded four patients with stable disease but no objective responses and a short biological half-life as seen with most vascular disrupting agents [30]. Clinical trial results are pending from BNC105P drug combinations in ovarian cancer and renal cell carcinoma.

Nocodazole is a reversible inhibitor of the colchicine binding site that has been used for numerous *in vitro* studies but has not been pursued in the clinical setting [4]. However, several other MTAs that interact with the colchicine binding domain are currently under clinical investigation including plinabulin, ombrabulin and verubulin. Plinabulin was successful in combination with docetaxel in a NSCLC clinical trial [28]. Ombrabulin was unsuccessful in NSCLC or soft-tissue sarcoma trials, while verubulin had limited activity in a glioblastoma trial [31–33].

Consequences of microtubule disruption

It is undeniable that microtubules are essential to mitosis and that MDAs can be toxic to actively dividing cells. However, this is only one of several mechanisms in which MDAs can be utilised to target malignant cells. The fact that neurotoxicity, caused by inhibited trafficking along nerve fibres, is a major side effect of some MDAs highlights the impact of these drugs on nonproliferating cells. High concentrations of MDAs have been shown to halt cells in mitosis and be cytotoxic, yet 10–100 fold lower concentrations of these drugs can interrupt signalling along the microtubule network with various consequences that do not require mitosis [12]. Furthermore, early studies reported cytolytic effects of vinblastine and vincristine in both interphase and mitotic lymphocytes. Cell shape and membrane integrity was altered by vinblastine and colchicine as early as 30 min [34] and these drugs were more toxic to nondividing chronic lymphocytic leukaemia (CLL) cells than normal mononuclear cells [35]. One study in CLL cells demonstrated vinca alkaloid-mediated apoptosis in 48 h [36]. However, we have recently shown that clinical concentrations of vinca alkaloids or BNC105 can induce CLL apoptosis in only 6 h [37–39]. As these cells are almost exclusively in G0 this clearly reflects a nonmitotic activity.

Microtubules require JNK activity throughout the microtubule life cycle and JNK inhibition impacts microtubule growth, shrinkage and rescue [40]. Thus, JNK is naturally linked to the fate of microtubules. The downstream impact of JNK can be complex as many nuclear and non-nuclear targets of JNK have been identified. Among these substrates are members of the Bcl-2 apoptosis family that control intrinsic apoptosis, and 14–3-3, which controls the fate of many apoptosis-regulatory proteins [41]. ERK and p38 kinases also interact with microtubules, giving rise to other potential signalling pathways downstream of microtubule disruption.

Other recent reviews have also suggested that nonmitotic mechanisms are critical in MTA efficacy, including antiangiogenic and vascular disruptive properties [7, 42–44]. However, most of these reviews emphasise taxanes and do not discuss the different mechanisms elicited by microtubule stabilisers *vs*. destabilisers. In this section we will highlight many pathways that are impacted by MDAs with emphasis on the lesser known, yet clinically relevant nonmitotic mechanisms of action. Importantly, many of the mitotic and vascular events may also occur with microtubule stabilising agents. Unfortunately, most studies utilise one family of drugs or the other, not comparing both stabilisers and destabilisers, making it difficult to discern mechanistic differences between them. However, several of the acute, nonmitotic events appear unique to microtubule destabilising agents as highlighted below. These mechanistic pathways are outlined in Figures 1 and 2. The detailed impact of MDAs has been further summarised in Table 1.

Mitotic arrest

A common assumption is that tumours have a greater fraction of actively dividing cells than healthy normal tissues. As microtubules and their dynamic behavior are essential for mitosis, they represent a good target. Thus, apoptosis induced by MDAs is often attributed to dissolution of mitotic spindles, metaphase arrest and eventual apoptosis. However, there have been many reports indicating that MDAs can induce apoptosis at all phases of the cell cycle and even in noncycling cells (i.e., those in the G0 phase of the cell cycle) [35, 45, 46]. The evidence of mitosis as the critical cell cycle phase for the action of MDAs comes from *in vitro* cell culture

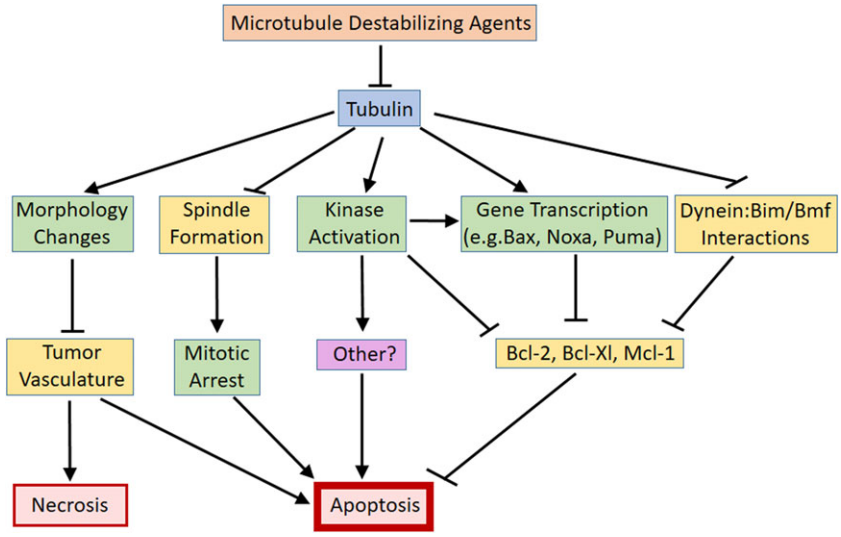

Figure 1

Consequences of microtubule disruption

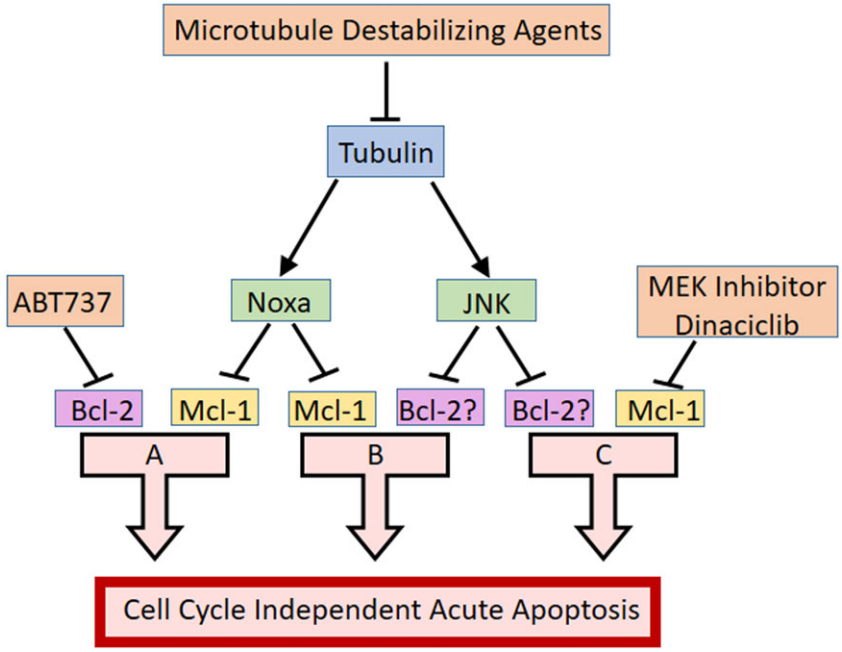

Figure 2

Mechanisms of acute apoptosis. Acute, cell cycle independent apoptosis in response to microtubule destabilising agents (MDAs) may be unique to hematopoietic cells. The target of JNK in this model is currently unknown but it is proposed that Bcl-2 lies downstream of JNK. Bcl-2 phosphorylation has not been seen in this model. (A) MDA + Bcl-2 inhibitor = Noxa-dependent; (B) MDA Alone = Noxa- and JNK-dependent; (C) MDA + Mcl-1 inhibitor = JNK-dependent

and xenograft models. Experimental cell lines have been streamlined for fast doubling times to complete experiments quickly; for example, the median doubling time of the cell lines in the NCI60 tumour panel is 33.1 h. Meanwhile the estimated doubling time for human tumours is 147 days as measured by radiography or serum tumour marker levels. Furthermore, cell cycle analysis shows that mitosis is very short (0.5–2.5 h), suggesting that a low fraction of cells is in mitosis at any given time (reviewed in [6]). Consequently, there are far too few cells in mitosis at any particular time for this mechanism to explain the therapeutic efficacy of these drugs. The lack of mitotic death in tumours could be contributed to other factors such as cell death during other phases of the cell cycle or quiescence, bystander effects, and impact of the tumour microenvironment (e.g. leucocyte activation) [47]. The flaw with estimating tumour doubling time is that is does

Table 1

Impact of microtubule disrupting agents on cellular signaling

Drug	Impact	Target	Reference
MDA impact on cell morphology, mobility and vasculatur			
CA4P	Activation	Rho GTPases	[45]
VB, Col	Alteration	Cell shape, membrane integrity	[11, 34]
CA4P	Alteration	Actin organisation, cell morphology	[45]
CA4, CA4P, BNC	Elevation	Endothelial cell permability	[29, 46]
VB, VC	Elevation	cAMP, oxidised glutathione	[11]
CA4P	Inhibition	Cell migration, endothelial tube formation	[24]
CA4P	Inhibition	Tumour blood flow	[23]
CA4, CA4P, BNC	Inhibition	Capillary formation	[29, 46]
CA4P	Inhibition	VE-cadherin/b-catenin/Akt pathway	[46]
CA4P	Reduction	VCAM1 expression, vessel numbers, metastasis	[45]
BNC	Reduction	Laminin basement membrane degradation	[29]
MDA impact on Bcl-2 family proteins			
VC	Induction	Noxa, Puma	[47]
VB, VC, CA4, BNC	Induction	Noxa	[38, 39, 48]
VB, VC, Col, CA4P	Phosphorylation	Bcl-2, Bcl-Xl	[49–52]
VB, VC, Col, Dol, Noc	Phosphorylation	Bcl-2	[47, 49, 53–59]
UV, JNK activation	Phosphorylation	Bim	[60, 61]
MDA impact on kinases			
CA4	Activation	p38, ERK	[62]
Noc	Activation	Death-associated protein kinase 2	[63]
Noc	Activation	Lyn	[64]
VB	Activation	MEKK1	[65]
VB, VC, Col	Activation	JNK, c-Jun, JunD, Raf, SEK1	[66]
VB, VC, VN, Noc	Activation	JNK	[48, 47, 67–70]
VB, VC, Col	Inhibition	p38, ERK	[71, 66]
VB, VC	Inhibition	Calcium–calmodulin-regulated cAMP phosphodiesterase	[11]

BNC, BNC105; CA4, combretastatin A4; CA4P, combretastatin A4 phosphate; Col, colchicine; Dol, dolastatin; MDA, microtubule destabilising agent; Noc, nocodazole; VB, vinblastine; VC, vincristine; VN, vinorelbine

not take into account that tumours are constantly undergoing cell proliferation, senescence, and cell death. The cell cycle duration of proliferating cells in tumours is more likely to be a few days, even though it takes months for the tumour to double in size. Therefore, mitosis could still be a key target [48]. Furthermore, high drug retention of MDAs would allow cells to be sensitive as they enter mitosis over time.

A high throughput automated time lapse microscopy study using 15 cell lines demonstrated a variety of cell fates during exposure to nocodazole (tubulin destabiliser) or paclitaxel (tubulin stabiliser). The majority of cells arrested in mitosis and then either underwent apoptosis or exited mitosis and continued cycling; thus, apoptosis is not a guaranteed consequence of G2/M arrest. Several cell lines showed more death during interphase than mitosis after drug exposure. This study focused on postdrug, postmitotic interphase death and not *de novo* interphase death. There was no correlation between the duration of mitotic arrest and cell fate, suggesting that the cellular response to microtubule inhibitors may depend upon drug concentration and not duration of arrest [47, 49]. A conflicting report suggests that caspase-dependent apoptosis after mitotic arrest depends on arrest duration and involves DNA damage, degradation and Bcl-2 protein inactivation [50]. Overall, studies suggest that chronic activation of a mitotic checkpoint is not essential for death in response to microtubule inhibitors. Therefore, the mitosis-independent functions of MDAs must contribute to their overall antitumour efficacy.

The antimitotic mechanism of action might explain the MDA side effect of myelosuppression, as bone marrow precursors undergo constant turnover with active mitosis occurring in ~27% of the population at any given time [51]. Neuropathy caused by MDAs, however, is attributed to the disruption

of essential neuron microtubule signalling roles, and not mitosis as these cells rarely undergo mitosis [7, 11, 52]. Morphology changes occur much faster and at lower concentrations than proliferation/death signalling in a cell. This is highlighted by 8–16 fold differences in IC_{50} values for inhibition of cell proliferation *vs*. cell migration and endothelial cell tube formation [24]. The neurotoxicity of MDAs led to the development of alternative mitosis-specific drugs that avoid this toxicity by targeting mitotic kinases CDK1, Plk1 or Aurora A. An alternative approach is to impede mitosis-specific microtubule functions by inhibiting the kinesin KIF [52]. Most of these mitosis kinase-specific inhibitors have been disappointing as anticancer drugs because of low proliferation rates with few mitotic cells found in solid tumours [47, 53]. Furthermore, the mitotic kinase inhibitors suppress their target for short periods of time while MDAs have more durable target inhibition due to high affinity binding even after extracellular drug has been cleared. This highlights another advantage of MDAs over mitosis-specific drugs to target cancer cells.

Involvement of Bcl-2 family proteins

The balance between pro- and antiapoptotic members of the Bcl-2 family dictates mitochondrial outer membrane permeabilisation (MOMP) and release of death-inducing proteins, such as cytochrome c, that activate the apoptosome and caspases. MDAs can upregulate proapoptotic members of this family (e.g. Bad, Puma, Noxa, Bax, Bak), alter the location or activity of proapoptotic members (e.g. Bim, Bmf), or alternatively lead to phosphorylation and inactivation of antiapoptotic members of this family (e.g. Bcl-2, Bcl-Xl, Mcl-1) to tip the balance in favour of apoptosis [9]. Several studies have indicated that overexpression of the antiapoptotic proteins Bcl-2 or Bcl-Xl blocked apoptosis by MDAs without blocking mitotic arrest or microtubule inhibition [4, 54–57]. Post-translational modifications of Bcl-2 family members, particularly phosphorylation, have been reported in response to numerous anticancer drugs. Phosphorylation sites for Bcl-2 include T74, T56, T69, S70 and S87, and the latter three are reported as a consequence of MDAs [58, 59]. Bcl-Xl sites include T47 and S63, and Mcl-1 sites include S159 and S64 [60]. The proapoptotic members of this protein family (e.g. Bim) can also be phosphorylated, modifying their ability to induce apoptosis [61, 62]. Numerous kinases have been reported to phosphorylate Bcl-2 family members including JNK, c-Raf, protein kinase A, p38, protein kinase C alpha, mTOR, CDK1, and GSK3 [60]. The effect of phosphorylation appears to be two-sided, with some sites enhancing and others inhibiting apoptotic function of these mitochondrial proteins. However, the bulk of evidence suggests that Bcl-2/Bcl-Xl/Mcl-1 phosphorylation is inhibitory, thus enhancing apoptosis and tumour sensitivity to many anticancer drugs (for a comprehensive review see [62]). The impact of specific microtubule destabilising agents on Bcl-2 family regulation is summarised below.

Mitotic role of Bcl-2 family. Bcl-2/Bcl-Xl phosphorylation induced by MDAs correlated with a high proportion of cells in G2/M or was directly shown to be only in the G2/M fraction of the cell population in several studies. Vincristine led to phosphorylation of Bcl-2 and Bcl-Xl in leukaemia and lymphoma cell lines within 12 h; this was accompanied by MOMP and caspase activation [63]. CA4P also induced phosphorylation of Bcl-2/Bcl-Xl, inducing apoptosis in leukaemia cells [64]. Similarly, vinblastine, vincristine and colchicine all induced Bcl-2 and Bcl-Xl phosphorylation in cervical carcinoma cells in 16–24 h [55, 65]. Bcl-2/Bcl-Xl phosphorylation was independent of JNK, ERK or CDK1 in response to vinblastine; however, JNK inhibition delayed the onset of apoptosis [65]. Nocodazole treatment led to Bcl-2 phosphorylation in a leukaemia model [56]. Similarly, dolastatin induced phosphorylation of Bcl-2 in numerous cell lines [59]. Bcl-2 was also phosphorylated in response to vinca alkaloids and colchicine between 4–24 h in various cell models, with implications of JNK pathway involvement but no reports on cell cycle phase [55, 57, 58, 66–69]. Phosphorylation of Bcl-2 family members was not seen in leukaemia and lymphoma models that were acutely sensitive to MDAs at all phases of the cell cycle (D. Bates, unpublished data). Therefore, these studies suggest that Bcl-2/Bcl-Xl phosphorylation in response to MDA treatment occurs in mitosis.

Correlations between cell cycle phase, JNK activation and phosphorylation of Bcl-2 family members add a layer of complexity. General cell cycle control studies suggest that Bcl-2 inactivation (by phosphorylation) is necessary for cells to proceed through the cell cycle [70] with Bcl-2 phosphorylation occurring constitutively during G2/M but with conflicting results for JNK involvement [71–73]. Thus, many assume that MDA-mediated Bcl-2 family phosphorylation is simply a consequence of G2/M arrest. While this assumption may be true in some cases, it does not dictate whether Bcl-2 family proteins can be phosphorylated and their apoptotic function altered during other phases of the cell cycle.

Nonmitotic role of Bcl-2 family. Phosphorylation is not the only mechanism by which MDAs impact the Bcl-2 apoptotic protein family. In many cells, such as CLL, Bim is constitutively bound to Bcl-2 but in other cells it may be sequestered by dynein proteins [74]. Bim can be sequestered by dynein light chains 1 and 8 (DLC1/8) that are associated with microtubules while Bmf is sequestered by DLC8, which is associated with actin [4, 75]. Thus, microtubule disruption can cause release of Bim and Bmf from the cytoskeleton network to stimulate the apoptotic cascade. In fact, Bim-deficient lymphocytes were resistant to microtubule targeting drugs suggesting a role for Bim release/activation by microtubule disruption [4]. A similar study reported release of BimL and DLC8 from the dynein motor complex and translocation to Bcl-2 during UV treatment [75], which may also occur during microtubule disruption. Furthermore, MDA-activated JNK can phosphorylate Bim (Ser65) on the microtubules leading to its release and subsequent induction of Bax-dependent apoptosis [76, 77]. MDAs could also act directly at the mitochondria, as nocodazole revealed calcium-dependent MOMP toxicity on isolated mitochondria [9].

Vinca alkaloids can also induce proapoptotic Bcl-2 proteins at the transcriptional level. Increased transcription at the AP-1 transcription site has been reported in response to vinblastine [78, 79]. AP-1 transcription factors include Fos, Jun and ATF proteins; the latter two are activated by JNK

and MDAs [80]. Proapoptotic proteins Noxa and Puma were induced in response to vincristine in a melanoma model [57]. Noxa is also acutely induced by vinblastine, vincristine, CA4 and BNC105 in leukaemia and lymphoma cells, including patient-derived CLL cells. CLL sensitivity to these single agents required both JNK activation and Noxa induction [38, 39, 45]. Furthermore, Noxa induction was required for apoptosis induction by vinblastine plus the Bcl-2/Bcl-Xl inhibitor ABT-737, and, while JNK was activated, it was not required for apoptosis suggesting that JNK activation is redundant when Bcl-2/Bcl-Xl are concurrently inhibited [38]. It is known that JNK can induce Noxa gene transcription, but it is not required in all cell models, suggesting alternative pathways for Noxa upregulation by MDAs. This JNK-Noxa-apoptosis pathway is unique to MDAs as taxanes did not yield any of these acute signals in leukaemia [37, 39].

Kinase activation

JNK-interacting proteins (JIPs) link the JNK pathway kinases and kinesin-1 to vesicles carrying membrane proteins along the microtubule network. JNK activation (via MAP3K and MAP2K) can regulate the linkage between kinesin-1 and cargo via the JIP APLIP1 [8]. Other kinases such as MLK, ASK1 and MEKK1 bind JIP scaffolding proteins, which may explain activation of these proteins in response to MDAs [81]. MEKK1 may also act upstream of JNK in response to MDAs [82]. Blockage of cellular transport is probably a crucial target of microtubule binding drugs, with JNK being a major readout of disrupted microtubule signalling. Additionally, death-associated protein kinase 2 interacts with both tubulin and 14–3-3 and was required for nocodazole-induced apoptosis in HeLa cells [83]. The tyrosine kinase Lyn, part of the B cell receptor signalling pathway, was implicated in nocodazole cytotoxicity that was specific for CLL B cells over normal B or T cells [84].

Several MDAs (vinblastine, vincristine and colchicine) were compared in an epidermal carcinoma cell line and all demonstrated increased JNK, c-Jun, JunD, Raf and SEK1 phosphorylation starting at 2 h with death occurring at 36 h [85]. Vinblastine also activated JNK and induced AP-1 transcriptional activity with corresponding cytochrome c release and caspase-3 activation in cervical epithelial carcinoma cells. In this model kinase activation appeared as cells accumulated in G2/M (8 h), while JNK-dependent apoptosis did not occur until 36 h [78]. Similarly, vinorelbine and nocodazole activated JNK within 3 h, followed by MOMP and apoptosis (at 48 h) in lung adenocarcinoma cells. Apoptosis but not mitotic arrest was blocked by JNK inhibition in this model [86]. JNK is further implicated in vincristine-induced apoptosis in melanoma [57]. These studies all represent models in which early activation of JNK occurs in solid tumours followed by late apoptosis. This MDA-induced apoptosis was JNK-dependent and may require G2/M arrest, although arrest-dependence was not directly studied.

A panel of 15 leukaemia and lymphoma cell lines including patient-derived CLL cells revealed JNK activation in response to vinblastine within 4 h, with the exception of Jurkat cells that have a known defect in their JNK pathway [45]. Several of the mantle cell lymphomas and patient-derived CLL cells were innately sensitive to single agent vinblastine, with JNK activation at 30 min and apoptosis in 4–6 h. Follow up studies demonstrated acute (4–6 h) JNK-dependent apoptosis by vinblastine, CA4 and BNC105 in leukaemia models [37, 39]. Furthermore, JNK was required for apoptosis induced by vinblastine in combination with a CDK 7/9 inhibitor in leukaemia but not normal leucocytes [37]. As CDK 7/9 inhibition causes global inhibition of transcription, this model demonstrates transcriptional independence for acute apoptosis induction. Furthermore, transcriptional inhibition results in rapid loss of Mcl-1. These haematopoietic results are in stark contrast to the solid tumour studies described above in regards to cell cycle. The carcinoma studies may involve and/or require G/M arrest for apoptosis; however, the leukaemic apoptosis is clearly independent of cell cycle phase as it occurs in rapid time frames and in noncycling cells (e.g. CLL). A proposed mechanism for acute apoptosis in haematopoietic cells is outlined in Figure 2.

One study reported that CA4 rapidly induced JNK phosphorylation and that JNK inhibition blocked microtubule destabilisation in breast adenocarcinoma cells [87]. This report of JNK acting upstream of microtubule destabilisation contradicts other findings that microtubule stability is independent of JNK activity [39] and that drug binding to microtubules is required for JNK activation [88].

The ability of vincristine to activate the JNK cascade was tested *in vivo*. CLL patients were treated with a single 2 mg dose by IV injection and blood samples were collected over time to determine drug pharmacokinetic (PK) and pharmacodynamic markers. JNK was activated by vincristine in CLL cells within 5 min; however, other markers of apoptosis were not found [89]. This study suggests that JNK may be a viable biomarker of tubulin disruption but drugs that more potently activate this pathway may be needed for clinical efficacy.

From these highlighted studies, there appears to be a general agreement that MDAs increase JNK activity and subsequently its downstream targets (e.g. c-Jun, ATF2). The impact of MDAs on other kinase pathways is however, controversial. CA4 increased phospho-p38 levels in breast adenocarcinoma cells [87] but vincristine, vinblastine and colchicine all reduced phospho-p38 levels in KB3 cervical carcinoma cells [85]. The same destabilising drugs had no impact on phospho-p38 levels in leukaemia and lymphoma cells lines (D. Bates, unpublished data). Similarly, CA4 increased ERK activity in breast cancer cells [87], while vinblastine, vincristine and colchicine all reduced ERK activity in cervical cells [79, 85]. Furthermore, vincristine, nocodazole and colchicine had no impact on ERK in ovarian and breast cancer cells [88]. None of these studies reported any reliance on p38 or ERK pathways for drug-induced cell death. However, two studies did report enhanced cell death when combining vinblastine with a MEK/ERK inhibitor [45, 79]. This combination was most effective in leukaemia models in which Mcl-1 was suppressed by MEK/ERK inhibition, thereby inducing JNK-dependent apoptosis during all phases of the cell cycle [45]. A separate study demonstrated dependence upon CDK1 and not JNK or p38 for nocodazole-mediated apoptosis in T-cell leukaemia cells [56].

Angiogenesis and metastasis

The growth of new blood vessels towards tumours and tumour cell migration towards metastatic sites are two complex issues

in anticancer therapy. Many angiogenesis inhibitors (e.g. bevacizumab, sorafenib) prevent new blood vessel formation by targeting vascular endothelial growth factor or its downstream targets. This class of drugs is clinically effective but often requires chronic treatment in combination with other anticancer therapies. As microtubule structure and function are essential for cellular shape and motility, altered microtubule dynamics can impact angiogenic and metastatic signalling. MDAs impede angiogenesis by altering cellular contacts, inhibiting cell movement, and sprout formation [90, 91]. Furthermore, metastatic events such as cell migration, attachment and invasion are all reduced by MDAs [91, 92]. A comparative study of drugs that bind the taxane, vinca alkaloid and colchicine microtubule sites showed that the antiangiogenic and antimetastatic effects are not unique to MDAs but can occur with all classes of microtubule targeting drugs [90]. The antiangiogenic and antimetastatic properties of MTAs are reviewed in more detail by Greene *et al*. [93].

Vascular disruption

Many drugs that disrupt microtubule dynamics are being developed as vascular disrupting agents (VDAs) in addition to their activity as cytotoxic drugs. Acute administration of VDAs yields an immediate vascular result in contrast to angiogenesis inhibitors, which require chronic administration. The immediate vascular effects of VDAs include increased vascular permeability, increased interstitial pressure causing plasma leakage, reduction in blood vessel diameter, decreased blood flow and vascular shutdown. Tumour vessels are prime for this approach as they already have increased vascular permeability, abnormal basement membranes, absence of pericytes and irregular vessel diameter [94].

Vascular disruption has been linked to tubulin destabilisation, whereas both microtubule stabilisers and destabilisers may be antiangiogenic as described above. The endothelial cells that make up tumour vasculature undergo morphological changes in response to microtubule-targeting VDAs at low concentrations that do not cause cell death. Microtubule disruption impacts VE-cadherin/β-catenin/Akt signalling that directly impacts adherin junctions that are needed for endothelial cell interactions in the vasculature. Microtubule instability is also associated with Rho GTPases and actin reorganisation, which impact endothelial cell morphology and cell–cell interactions [43]. Low drug concentrations that disrupt tumour vasculature are considered noncytotoxic, while higher concentrations can inhibit endothelial cell proliferation, migration and induce cell death, enhancing the impact of VDAs on tumour vasculature and survival.

A hallmark of VDAs *in vivo* is the rapid collapse of tumour blood flow followed by complete vascular shutdown [43]. Classic assessments for blocked tumour flow include hypoxia and necrosis in the tumour centre. However, the outer rim of tumours appears to be unaffected by VDAs as normal blood vessels may mediate recovery from damage. Reduced blood supply could prevent prolonged drug exposure, which may explain reversible G2/M arrest seen in some *in vitro* models and a lack of reproducibility in animal models. There is some debate as to whether hypoxia is a friend or foe to apoptosis induction in response to VDAs. The rapid vessel destruction can cause hypoxia which could then upregulate genes of resistance and survival, but more studies are needed to address this issue.

Many VDAs have disappointing single agent activity with neurotoxicity, cardiovascular and thromboembolic events as major concerns in the clinic. Even with these concerns, targeting the tumour vasculature with microtubule destabilising agents is attractive as resistance is unlikely and total vascular shutdown will be likely to result in massive downstream tumour death [94]. Furthermore, vascular disruption can prevent angiogenesis by destroying the current vascular platform needed for new vessel growth [93]. Synergy has been reported for drug combinations including VDAs, antiangiogenics and cytotoxics suggesting that there is a lot of potential for VDAs for future anticancer regimens [43]. The biggest issue may be finding an appropriate schedule for antivascular drugs and their combination with cytotoxic drugs to maximise antitumour efficacy.

Other signalling pathways of MDAs

A role for p53 has also been indicated in response to MDAs. Activation of p53 at low concentrations could be attributed to its nuclear accumulation by altered cellular trafficking but high MDA concentrations could block all trafficking having a reduced effect on p53 signalling. The p53 protein is a transcription factor that regulates expression of Bax, Noxa and Puma pro-apoptotic proteins among many other targets; typically, but not exclusively, in response to DNA damage. Furthermore, p53 can also move directly to the mitochondria and activate Bax/Bak [9]. Vinblastine sensitivity increased with p53 inhibition [79], but numerous malignancies are deficient for p53 or express p53 mutants that would be resistant to anti-p53 therapy. Furthermore, Noxa-dependent acute apoptosis in leukaemia cells was p53-independent, negating the need for p53 regulation [45].

The interaction of leukaemic cells with their stromal tumour microenvironment relies on the interaction of integrins with ligands. Very late antigen 4 (VLA4) binds VCAM1 expressing stroma and AML with VLA4 expression have poor prognosis. This presents an issue of cell adhesion-mediated drug resistance, and disruption of the leukaemic cytoskeleton could disrupt this interaction. CA4P reduced VCAM1 expression resulting in reduced attachment of leukemic cell lines to human umbilical vein endothelial cells and AML cell lines underwent apoptosis with IC_{50} values of ~5 nmol l^{-1} CA4P. Additionally, xenograft models showed decreased vessel numbers and largely necrotic tumour in response to CA4P while a systemic leukaemia model showed reduced engraftment into the bone marrow and less infiltration into organs after CA4P treatment [95].

Alternative signalling pathways activated by microtubule stabilising agents

Many of these pathways have also been identified in response to the microtubule stabilising agents paclitaxel and docetaxel, but the two classes (destabilising *vs*. stabilising) are not universal in all model systems. Similar to microtubule destabilisers, there is controversial impact of taxanes on ERK and p38 signalling [85, 87], while JNK activation and

JNK-dependent apoptosis by paclitaxel has been reported by several groups [11, 85, 88, 96, 97]. Phosphorylation of Bcl-2, reduced Bax binding and apoptosis in numerous cell lines has also been shown in response to paclitaxel [66, 69, 73]. In contrast to vinblastine, paclitaxel did not acutely activate JNK or induce Noxa or apoptosis in a panel of haematopoietic tumours, suggesting that haematopoietic tumours may respond very differently to tubulin stabilisers *vs*. destabilisers in the clinic [37].

Mechanism-based drug combinations

Vinca alkaloids are widely used in combination with anthracyclines for both solid and haematopoietic tumours in the clinic but drug scheduling was never optimised. Primary leukaemia cells from 35 patients were treated concurrently with vincristine plus doxorubicin and resulted in 12 cases of antagonism, 19 additive and four synergistic. Doxorubicin antagonised vincristine efficacy *in vitro* by reducing phosphorylation of Bcl-2/Xl, caspase activation and apoptosis [63]. This suggests that more consideration of drug mechanisms is required to design effective clinical drug combinations.

The link between the Bcl-2 family of proteins and the efficacy of microtubule-disrupting drugs is strong. A large small interfering RNA screen identified vinblastine plus the Bcl-2/Bcl-Xl targeting drug ABT-737 as a good drug combination [98]. Studies with primary CLL cells and leukaemia/lymphoma cell lines show enhanced apoptosis when vinblastine was combined with either ABT-737 or drugs that reduce Mcl-1 expression [37, 38, 45, 99]. Thus, clinical combinations of Bcl-2 family targeting drugs with MDAs are of interest. However, currently there are no reported trials of a microtubule destabilising agent such as vinblastine, vincristine, CA4P or BNC105P in combination with a Bcl-2 family-targeting drug. As most of the preclinical evidence points to MDAs being more globally effective in impacting Bcl-2 family homeostasis, these drugs should be considered more seriously for clinical application of drug combinations. Many have steered away from the vinca alkaloids due to their neurotoxic and myelosuppressive effects, but perhaps newer drugs in this class such as BNC105P would have greater efficacy with reduced toxicity.

Drug resistance

Clinical challenges for MDA therapies include low therapeutic treatment windows and innate or required drug resistance. Generally, tubulin extracts from many tissues respond the same to MDAs *in vitro*, so differences *in vivo* are likely to be due to expression of different tubulin isoforms, cellular retention of drug, binding of MAPs, and differences in downstream signalling events. Some tumour cells do not respond to MDAs at all, such as renal cell carcinomas, which are thought to have microtubule-independent trafficking of key oncogenic proteins [7]. These factors should be considered as new generations of MDAs are developed.

The key determinant in pharmacological activity of vinca alkaloids is the cellular retention of the drug. These drugs are subject to cellular efflux by P-glycoprotein (Pgp) and multidrug resistance protein 1–6 efflux pumps. Blocking Pgp enhances vinca alkaloid retention and toxicity but can cause unacceptable tissue toxic effects due to normal Pgp expression in liver, kidney, small intestine and colon (reviewed in [100]). Combretastatins and BNC105 may have more clinical impact as they do not seem to be substrates of Pgp [64, 101].

The expression of different tubulin isotypes and their posttranslational modifications could impact drug binding and efficacy. Overexpression of class III beta-tubulin is an indicator of resistance for taxanes and vinca alkaloids but not colchicine [22]. Most of the sites for tubulin posttranslational modification and sequence divergence between isotypes are located within the C terminus where most MAPs bind [12]. Furthermore, different microtubule interacting proteins and MAPs (e.g. alpha-tubulin, actin, lamin B, HSP90, TCTP, 14–3-3) can impact MDA efficacy by altering drug binding [100]. Overexpression of the antiapoptotic members of the Bcl-2 family or inhibitor of apoptosis protein survivin could also cause MDA drug resistance [56, 57, 102, 103].

The loss of p53 function can impact MDA responses due to increased MAP4 (microtubule stabilising protein) expression [104]. However, p53 can also act upstream of proapoptotic proteins (Noxa, Puma), suggesting that p53 loss could reduce apoptotic potential [105]. Failure to induce Noxa protein led to CLL resistance to BNC105, emphasising the importance of this apoptotic protein in acute MDA signalling [39]. Yet it is important to note that Noxa regulation is not solely dependent on p53, further making the role of p53 in MDA efficacy appear convoluted. More studies are needed to discern the relevance of this tumour suppressor in microtubule targeting therapies.

Clinical future for MDAs

Several techniques have been utilised to improve drug solubility, bioavailability or tumour targeting of MDAs including nanoparticle delivery, covalent linkage to the fatty acid docosahexanoic acid, encapsulation in lipid complexes, conjugation to antibodies and modifications to avoid Pgp efflux. For example, Marqibo, a vincristine sulfate liposome injection, has been developed using sphingomyelin- and cholesterol-based nanoparticle formulation to improve PK and pharmacodynamic properties of vincristine. The goal was to increase circulation time and target tissue delivery by slow release of vincristine from liposomes. The FDA approved Marqibo for adults with advanced, relapsed, refractory Philadelphia-chromosome negative acute lymphocytic leukaemia in 2012 [106]. Dolastatin 10 derivatives (aurostatins) have been conjugated to anti-CD30 antibodies, with Brentuximab vedotin approved in 2015 to treat relapsed/refractory Hodgkin's lymphoma and anaplastic large cell lymphoma. Similarly, a maytansine derivative conjugated to trastuzumab (T-DM1) was approved in 2013 as a second line therapy in breast cancer [107].

While improving drug delivery and overall PK is a good approach for older generation drugs, the future of MDAs probably lies with newer drugs that more potently activate these nonmitotic signalling pathways. The development of potent colchicine site binding drugs, such as BNC105, provides an opportunity for new clinical applications with a higher therapeutic window.

How much of the clinical efficacy of MDAs can be attributed to cytotoxicity *vs*. antivascular properties? Low doses probably impact tumour vasculature and intracellular signalling, which can lead to apoptosis. Meanwhile high doses are required for direct cell growth inhibition and death. Furthermore, is the tumour death *in vivo* a consequence of mitotic arrest, activation of kinases or pro-apoptotic proteins, inactivation of antiapoptotic proteins or other tubulin-initiated signalling events that ultimately cause apoptosis? None of these pathways are mutually exclusive and each tumour type may be more sensitive to one pathway over others in response to microtubule toxins. The key is to take these nonmitotic mechanisms into account and maximise drug efficacy in the clinical setting.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organisation for the submitted work.

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