Microtubules: a Moving Target in Cancer Therapy

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Introduction

Microtubules are a major component of the cytoskeleton. They are important players in many cellular events and play a crucial role in cell division. As such, microtubules are a highly attractive target for anticancer drug design. The tubulin-binding agents (TBAs; also referred to as anti-microtubule or microtubule-targeted agents) are amongst the most widely used chemotherapeutic drugs in cancer treatment. Their efficacy has been demonstrated in the clinic for the treatment of a wide variety of human cancers, including breast, lung, ovarian and prostate, as well as haematological malignancies and childhood cancers. Furthermore, this class of agents, which includes the vinca alkaloids and taxanes, is constantly evolving. Currently, there are numerous novel compounds in the vinca alkaloids and taxanes, is constantly evolving. The epothilones and orally available taxanes, which are showing promise in clinical trials. However, despite their clinical success, the efficacy of TBAs is often limited by resistance. In recent years, there has been a strong focus on identifying and understanding alterations in the cellular target of TBAs, the tubulin/microtubule system, in resistance to improve targeting of these agents.

Tubulin/Microtubule System

The cytoskeleton is made of three distinct components: microtubules (MTs), actin microfilaments and intermediate filaments. MTs are polarised hollow tubes with a 14 nm internal diameter and up to several microns long, which are formed by the polymerisation of α/β-tubulin heterodimers (Fig. 1A). They are highly dynamic structures, with their ends constantly alternating between periods of growth and shortening (Fig. 1C). This dynamic behaviour, defined as dynamic instability, is mediated by GTP/GDP exchange. Indeed, the α-tubulin subunit is always bound to GTP, acquiring a conformation suitable for MT polymerisation, whereas the β-tubulin subunit can be bound either to GTP or GDP, favourable for MT polymerisation or depolymerisation, respectively. Thus, two ends with different dynamic properties can be distinguished within a MT: the (+) end, which is terminated by β-subunits, and the less dynamic (-) end, which is terminated by α-subunits (Fig. 1A).

In eukaryotic cells, the MTs form a highly organised network of intermingled filaments (Fig. 1B). Schematically, the MT (-) ends are anchored at the centrosome, also called the MT organising center (MTOC) (arrow in Fig. 1B), whereas the (+) ends radiate

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**Fig. 1. The tubulin/microtubule network and its dynamic properties.**

A. Scheme representing the microtubule structure. The microtubules are hollow tubes formed by the polymerisation of α/β-tubulin heterodimers. They are highly dynamic structures that are always either in a state of polymerisation or depolymerisation. The (+) end, which is terminated by β-subunits, is more dynamic than the (-) end, which is terminated by α-subunits.

B. Picture of the microtubule network in a human vascular endothelial cell. The (-) ends are generally anchored at the centrosome (arrow) whereas the (+) ends radiate in the whole cytoplasm, towards the cell periphery.

C. Video microscopy images of a living cancer cell expressing GFP-tubulin. The microtubules display dynamic instability, with their ends growing or rapidly shortening. The arrows point at microtubule (+) ends undergoing growing and/or shortening events. These dynamic properties are highly involved in all cellular functions of microtubules and are the main targets of the TBAs.
in the whole cytoplasm, towards the cell periphery. To perform multiple cellular functions, the MT cytoskeleton can undergo profound morphological modifications. During interphase, the MT network has to ensure cell shape maintenance, mediate intracellular trafficking and contribute to cell motility, while during mitosis, it has to completely reorganise to form the mitotic spindle and orchestrate chromosome segregation. The dynamic properties of MTs – treadmilling and dynamic instability – are finely regulated and critical for their different cellular functions (reviewed in (1)). On the one hand, the dynamic instability is characterised by five main parameters: the rates of growth and shortening events, the duration of pause, the frequency of transition from growth or pause to shortening (called catastrophe) and the frequency of transition from shortening to pause or growth (called rescue). On the other hand, the treadmilling is a process by which tubulin heterodimers continuously flux from one end of the MTs to the other.

MT dynamics are exquisitely regulated in cells, both spatially and temporally, and even within different regions of the cytoplasm of a cell. The differential regulation is required for different cellular activities and many endogenous cellular proteins are involved in the regulation of MT dynamics. During mitosis, MT dynamics dramatically increase and the formation and tension of kinetochore MTs is critical for the correct attachment, separation and segregation of chromosomes. In humans, six α- and seven β-tubulin genes have been identified, and their classification is based on their differing C-terminal sequences. Tubulin isotypes are encoded by different genes and display tissue- and cell-specific expression. Tubulin isotypes can undergo a wide variety of post-translational modifications, such as acetylation, detyrosination, polyglutamylation, polyglycylation, phosphorylation and palmitoylation. The tubulin isotypes and post-translational modifications can affect the dynamic properties of MTs both directly and through the modulation of MT-associated protein (MAP) interactions with MTs. Indeed, MAPs and other MT-interacting proteins are the main modulators of MT dynamics (2). The structural MAPs, such as MAP4 and tau, stabilise MTs and favour their polymerisation and rescue, whereas the proteins of the stathmin family destabilise MTs by sequestrating tubulin heterodimers and promoting catastrophe. In addition, several proteins specifically interact with the (+) ends of MTs and can be either stabilising factors, such as the (+) end tracking proteins, or destabilising factors, such as the proteins of the kinesin 13 family (2).

Mechanisms of Anti-Tumour Action of TBAs

The crucial role played by MTs in cell division makes them important cancer drug targets. TBAs are potent mitotic poisons that target cells at the G2/M phase of the cell cycle (Fig. 2). Thus, drugs that interfere with MT functions, such as the TBAs, inhibit the proliferation of cancer cells by preventing the mitotic spindle from forming properly for accurate chromosome segregation and mitotic arrest. TBAs are usually divided into two distinct categories, in reference to their effects on the MT network: MT-stabilising and MT-destabilising agents. TBAs bind to the β-tubulin subunit of α/β-tubulin either on the tubulin heterodimer (MT-stabilising agents) or the MT wall (MT-stabilising agents), causing disruption of MT dynamics, which leads to mitotic arrest and cell death. Clinically useful MT-stabilising agents include the widely used chemotherapeutic drugs taxanes, paclitaxel and docetaxel, as well as newer agents such as the epothilones. MT-destabilising agents include the widely used chemotherapeutic vinca alkaloids and new compounds currently in clinical trials such as 2-methoxyestradiol (2-ME2), dolastatin and the combretastatins. While most of the TBAs can inhibit the proliferation of cancer cells without inducing extensive stabilisation or depolymerisation of the MT network, they can suppress MT dynamic instability in these cells without significantly changing MT polymer mass. Indirect evidence suggests that the common mechanism underlying the anti-cancer activity of TBAs may mainly rely on the inhibition of spindle MT dynamics, which results in the slowing down of the metaphase-anaphase transition, impaired chromosomal segregation, mitotic block and subsequent induction of the mitochondrial apoptotic pathway (reviewed in (1, 3, 4)).

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**Fig. 1. Microtubule structures through the cell cycle.** Metaphase and anaphase microtubules (red). Telophase and interphase microtubules (green). Chromosomal DNA (blue).
Mechanisms of Resistance to TBAs
Associated with the Cytoskeleton

An important mechanism of resistance to TBAs is associated with alterations of the tubulin/MT system (see review in (5, 6)). These alterations include changes in tubulin isotype expression, post-translational modifications of tubulin, acquisition of tubulin mutations and changes in the expression levels of MT-related proteins. Recent studies implicating the actin cytoskeleton in the action of TBAs will also be reviewed.

Alterations of the tubulin/microtubule system

Alterations in tubulin isotypes, and especially alterations in the β-tubulin isotype composition of cells, have been shown to play an important role in cell response to TBAs. In particular, increased expression of the neuronal specific βIII-tubulin has been frequently implicated in drug-resistant cell lines (reviewed in (6)). High levels of expression of this isotype were first reported in paclitaxel resistant ovarian cancers (7) and there is now mounting clinical evidence that βIII-tubulin expression is involved in resistance to taxanes and vinca alkaloids in several cancer types, such as lung, breast and ovarian cancers. For example, high expression of βIII-tubulin is a marker of poor clinical outcome in advanced ovarian cancer patients given platinum/paclitaxel combination treatment (8). We are currently investigating the mechanisms by which β-tubulin isotypes can influence tumour cell resistance to TBAs. Our recent studies demonstrated that βIII-tubulin targeting by siRNA increases the sensitivity of non-small cell lung carcinoma cells towards both MT-stabilising and MT-destabilising agents, supporting a key role of βIII-tubulin in resistance to TBAs (Gar, P.P., Pasquier, E., Kavallaris, M., unpublished data).

In contrast with the βIII-tubulin isotype, to date there is no clear correlation between the expression level of the other tubulin isotypes and tumour resistance to TBA treatment. Increases in the expression level of other β-tubulin isotypes, such as βII, βIva, βIVb, βV and βVI, have been described in certain tumour cell lines resistant to TBAs. However, clinical evidence of the involvement of these tubulin isotypes in drug resistance is limited. Elsewhere, the understanding of the relationship between the expression level of various tubulin isotypes and drug resistance is complicated by the fact that changes in tubulin isotype composition can differentially affect cell sensitivity to TBAs. For instance, two distinct breast cancer cell lines (MDA-MB-231 and MCF-7) that were made resistant to docetaxel in vitro displayed significantly different alterations in the expression levels of β-tubulin isotypes (9). Elsewhere, decreases in the global expression level of both tubulin and total β-tubulin have been also associated with paclitaxel resistance in vitro (10, 11).

Tubulin mutations, affecting either the β-tubulin subunit (which contains the binding sites of almost all the TBAs) or the α-tubulin subunit, have also been reported to be associated with resistance to taxanes, vinca alkaloids, epothilones and 2-ME2 (reviewed in (6)). However, these mutations have only been reported in vitro in tumour cell lines resistant to TBAs and to date, mutations in clinical samples are yet to be reported. Nevertheless, the in vitro studies on tubulin mutations have provided valuable information on the structure/function activity of TBAs. For example, our laboratory developed a series of epothilone-resistant leukaemia cells that acquired mutations in βI-tubulin (12). Using drug binding and molecular modelling tools, we provided the first evidence that the binding of epothilone on β-tubulin differed to that of paclitaxel. Taxanes and epothilones cause MT stabilisation and were predicted to share similar binding sites within the paclitaxel-binding pocket of β-tubulin. Improved knowledge of drug/target interactions has led to enhancements in TBA drug design.

Post-translational modifications of tubulin have been identified in cells resistant to TBAs. Stable MTs often contain post-translationally modified tubulin such as α-tubulin acetylation and tyrosination, although it is unclear whether they are a consequence of or a trigger for increased MT stability (13). Post-translational modifications occur in the carboxy-terminal domain of β-tubulin, and like a number of other isotypes, class I β-tubulin can undergo various modifications, including glutamylation. Class III β-tubulin is the only isotype that can be phosphorylated. Post-translational modifications of the class I β-tubulin were identified in vincristine-resistant leukaemia cells developed in our laboratory (14). These cells had a two-fold increase in MT polymer levels that would be predicted to counteract the effects of a MT-destabilising agent such as vincristine. Whether modifications are the predominant cause of changes in MT polymer levels is not yet known. However, it is known that increased stability of MTs can counteract the effects of a drug such as vincristine (15), which acts by destabilising the MT network.

As mentioned previously, MTs are regulated by a range of proteins that modulate the function and dynamic nature of MTs. Recently, changes in the expression level of MAPs have been reported in drug-resistant cancer cells. For example, we have previously observed an increase in the expression level of the MT-stabilising protein MAP4 in vincristine-resistant leukemia cells (14) and a decrease in the expression level of MAP2c in vincristine-resistant neuroblastoma cells (16). Both these changes would lead to increased stability of MTs and would hence counteract the action of MT-destabilising drugs such as the vinca alkaloids. The expression level of the MT-destabilising protein stathmin has also been associated with drug resistance (16-18). In breast cancer, increased tau expression was associated with decreased response to paclitaxel (19). Whilst tau is a MT-stabilising protein, its expression would be expected to enhance MT polymerisation induced by paclitaxel. However, investigators demonstrated that tau reduced the ability of paclitaxel to reach its drug-binding site on tubulin on the MT wall. Therefore, low tau expression may be used as a marker to select patients for paclitaxel therapy and raises the possibility that inhibition of tau function might be exploited as a therapeutic strategy to increase sensitivity to paclitaxel in breast cancer.
Actin cytoskeleton and resistance

It is well established that the intracellular target of TBAs is the tubulin/microtubule system, although the specific pathways involved in the cytotoxic action of these agents are not well understood. Our high resolution proteomic analysis of leukemia cell line models of drug response and drug resistance revealed that acquired resistance to vinca alkaloids correlated with altered expression of proteins associated with both the tubulin and/or actin cytoskeleton (20). Despite the fact that the actin and tubulin cytoskeletons are often viewed as distinct entities, recent studies have highlighted a number of structural and regulatory interactions shared by these proteins. Building on our earlier study, we demonstrated that in a clinically relevant human acute lymphoblastic leukaemia (ALL) xenograft model, alterations in the actin and tubulin cytoskeleton are involved in intrinsic and acquired vincristine resistance. Interestingly, of the 19 proteins displaying altered expression levels in ALL xenografts with intrinsic or acquired vincristine resistance, 11 were associated with the actin cytoskeleton (18). In particular, the major cytoskeletal protein, γ-actin, was found to be downregulated in vincristine-resistant leukaemia xenografts (18). Our most recent studies identified distinct mutations in γ-actin for the first time in TBA-resistant cancer cell lines, with consequent loss of wild-type protein (21). Interestingly, both exogenous expression of mutant γ-actin and silencing of γ-actin expression induced significant resistance to TBAs (21). Together, these results demonstrated for the first time a novel functional role for γ-actin in drug action and resistance. Studies are ongoing to solve the mechanistic basis for the interaction of MTs and γ-actin in TBA resistance.

Targeting Drug Resistant Cancer

To circumvent intrinsic and acquired resistance to TBAs, new compounds, such as the epothilones, that can act on MDR cancer cells are currently being developed and evaluated in clinical trials. Improved understanding of the role of MT alterations in the TBA drug response is enabling researchers to devise ways to exploit this knowledge to improve drug targeting, drug selection and tailoring therapy. Elsewhere, the development of new therapeutic strategies using TBAs at low concentrations and administered more frequently has aroused considerable interest (22). These new therapeutics, also termed as metronomic chemotherapies, are expected to enhance the use of existing anti-microtubule agents and better target drug-refractory disease.

Conclusion

Drugs that target the tubulin/microtubule system are potent mitotic poisons. Resistance to these agents can be multifactorial and the clinical relevance of various mechanisms is still unknown. Studies in cell-free and in vitro models of resistance have contributed to our understanding of various MT components in the presence of TBAs. The strong association between clinical resistance to TBAs and specific tubulin and MT alterations has highlighted the need to focus on the mechanistic basis for differential drug sensitivity. Improved knowledge of the role and impact of MT composition may enable future profiling of tumours and tailored therapy for patients based on the differential expression of MT proteins. Opportunities to exploit this new knowledge of tubulin/microtubule changes in tumours will enhance the use of existing anti-microtubule agents and better target drug-refractory disease.

References