Published in final edited form as:
Expert Rev Anticancer Ther. 2015 March ; 15(3): 277–281. doi:10.1586/14737140.2015.1016425.

Die-hard survivors: heterogeneity in apoptotic thresholds may underlie chemoresistance

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Abstract

The unmatched efficacy of microtubule-targeting agents (MTAs) as chemotherapeutics was once assumed to originate from their impact on mitotic processes; however, this misconception is being eroded by amassing data that MTAs instead target interphase functions in patients' tumors. What remains murky is how MTAs target malignant cells over non-malignant ones if proliferation rates do not distinguish them. In many instances, malignant cells are actually more 'primed' for apoptosis than non-malignant ones. Nevertheless, even if most cells within the tumor are more apoptosis-susceptible than those in healthy tissues, there likely exist small subpopulations of apoptosis-resistant clones that engender incomplete responses to MTAs and relapse. Therefore, intratumor heterogeneity in terms of proximity to the apoptotic threshold must be better understood to facilitate the design of chemotherapeutic regimens, which may benefit from including drugs like BH3 mimetics that help in lowering the apoptotic threshold of tumor cells within these chemoresistant subpopulations.

Keywords

apoptotic threshold; chemoresistance; intratumor heterogeneity; mitochondrial priming; tubulin inhibitors

A persistent misperception

The luster of mitosis as a chemotherapeutic target has begun to dim in the face of accumulating evidence that, in most cases, a minority of cells in patients' tumors are mitotic [1]. Nevertheless, the clinical glory of MTAs, whose efficacy was originally believed to derive from anti-mitotic actions, persists owing to the virtually unparalleled effectiveness of these drugs compared to novel targeted ones. Indeed, MTAs constitute a cornerstone of

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Financial & competing interests disclosure
The article was funded by NIH. The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

cancer treatment today despite the passage of about half a century since their introduction to the oncology clinic, which was heralded by the discovery of the Vinca alkaloids [2]. The long-standing theory regarding the mechanism of action of MTAs is ‘mitosis-focused’ because these drugs at microtubule dynamicity-suppressing concentrations potently disrupt the mitotic spindle apparatus, thereby inducing cell death [3]. These observations were originally made *in vitro* using continuously cultured cells that have high mitotic indices, resulting in doubling times of only a day or two; however, the doubling times of most solid tumors and some hematologic malignancies (e.g., chronic lymphoblastic leukemia) are measured in hundreds of days [1,4,5]. In fact, the mitotic indices of patients’ tumors are often <1%; therefore, mitosis is an improbable MTA target in many patients [4]. It is thus unsurprising that there has been only marginal clinical success for drugs designed to specifically target mitosis [1,4–6].

On the brink of success: thresholds as the basis for MTAs’ tumor selectivity

Now that the principal mechanism of action of MTAs in patients’ tumors is coming into focus after decades of research – specifically, modulation of interphase functions, as depicted in Figure 1 – a critical question materializes: if the proliferation rates of most malignant and non-malignant cells are not very different, then how do MTAs target the malignant ones? Insight into this conundrum may be gained from the observation that cancers susceptible to one type of cytotoxic drug frequently also respond to others with very different mechanisms, whereas cancers that resist one kind of chemotherapy tend to resist them all [7]. A particularly intriguing explanation for this phenomenon is that certain malignancies are more chemosensitive because they exist closer to the apoptotic threshold. For instance, patients whose tumors are ‘primed’ (i.e., that have mitochondria that more readily depolarize in response to proapoptotic Bcl-2 family members) demonstrate more favorable clinical outcomes, such as improved response to therapy and enhanced progression-free survival [7]. Moreover, out of all normal cells and tissues, the most chemo-sensitive ones – peripheral blood mononuclear cells and bone marrow, respectively – exhibit the strongest priming [7]. Further evidence of enhanced priming as a determinant of MTA specificity comes from the finding that antiapoptotic factors are overexpressed in a diversity of cancers relative to normal tissues (especially Mcl-1 and Bcl-x_L in solid tumors, Bcl-2 in hematological malignancies and Bfl-1 melanoma) and are associated with chemoresistance [8–10]. Similarly, colon cancer stem cells are resistant to conventional chemotherapeutics due to decreased mitochondrial priming; however, chemosensitivity can be induced by small-molecule inhibitors of antiapoptotic Bcl-2 family members [11] (called BH3 mimetics, due to their mimicry of proapoptotic BH3-only Bcl-2 family members [12]). Intensifying mitochondrial priming with a BH3 mimetic augments chemosensitivity to various agents (including the MTAs paclitaxel, docetaxel and vincristine) in various continuous and primary cancer cell lines *in vitro* and *in vivo* [7,10,13–16]. Along similar lines, expression of proapoptotic proteins is often a prerequisite for chemosensitivity. For instance, the BH3-only protein BIM must be expressed for chemosensitivity to a multiplicity of agents, including paclitaxel [12]. Altogether, the selectivity of MTAs for tumor cells compared with normal ones is more likely to stem from the closer proximity of tumor cells to the apoptotic threshold than an enhanced proliferation rate in many cases. The side effects of MTAs may arise because certain non-malignant cells (e.g., myeloid, gastrointestinal, and epidermal cells) exist at a similar proximity to the apoptotic threshold as malignant cells, resulting in a narrow therapeutic window.

It has often been assumed that an intrinsic feature of cancer is apoptosis resistance, mediated by the gain of proto-oncogenes or loss of tumor suppressors, although the reality is not necessarily so straightforward. For instance, the oncoprotein c-Myc fuels unchecked proliferation while also enhancing susceptibility to apoptosis [17]. Similarly, loss of the function of the tumor-suppressor retinoblastoma protein drives unrestrained proliferation but concomitantly antagonizes prosurvival mechanisms (e.g., the antiapoptotic function of Bag-1) [18]. The oncoprotein Ras may have either anti- or proapoptotic effects depending on which Ras effector pathway is activated (e.g., anti-apoptotic Ras-PI3K or proapoptotic Ras-Raf-MAPKK-MAPK) and the status of Ras-regulated factors (e.g., promoter hypermethylation of proapoptotic *RASSF1*) [19]. To counter any proapoptotic program of such oncoproteins, cancer cells must execute antiapoptotic mechanisms (e.g., upregulation of Bcl-X_L) or else be susceptible to chemotherapeutics like MTAs. Thus, the cancer cells that are more susceptible to MTAs than normal cells are those that lie closer to the apoptotic threshold (Figure 2). Proximity to the apoptotic threshold is clearly not an adaptive mechanism for cancer cells but rather an ‘unintended’ consequence of certain pro-proliferative or pro-metastatic factors (e.g., c-Myc). The proapoptotic effects are beneficial, however, to rapidly proliferating, non-malignant tissues, which often avail themselves of similar mechanisms to compel cell division (e.g., inactivation of retinoblastoma protein via hyperphosphorylation [20]). Because programs that promote proliferation may also stimulate proapoptotic pathways, it can seem as though proliferation itself engenders sensitivity to MTAs. Instead, cancer cells that are susceptible to MTAs may be those that have not successfully deployed antiapoptotic mechanisms (or which do not rely on oncoproteins with inherent anti-apoptotic effects, such as Bcr-Abl [17]), not necessarily those that have a higher mitotic index. Perhaps it would be more accurate to posit that apoptosis resistance is an intrinsic feature of *chemoresistant* cancer cells. Altogether, it seems that the multifarious perturbations that ensue from disruption of inter-phase microtubule dynamics precipitate death in sufficiently primed cancer cells. As a result, the key to optimizing the efficacy of MTAs (or other chemotherapeutics for that matter) may lie in combining these agents with novel drugs (e.g., BH3 mimetics) that exacerbate this proapoptotic vulnerability in cancer cells.

The road forward: studying & combating heterogeneity in apoptotic thresholds

Key obstacles in the treatment of many cancer patients with MTAs are chemoresistance and relapse. For most patients undergoing chemotherapy, certain clones within the tumor are resistant to treatment, resulting in a failure to achieve pathologic complete response or an apparent complete response followed by relapse sometime later [21]. Thus, intratumor heterogeneity is a leading cause of chemoresistance. Within a tumor, there may be great variability among cells in the levels of pro- and anti-apoptotic factors, with certain clones being more apoptosis reluctant than others. In support of this notion, it was recently discovered that extensive intratumor heterogeneity exists in Bcl-2 transcript levels in follicular lymphoma as assessed at the single-cell level [22]. Patient-derived lymphoma cells that expressed higher levels of Bcl-2 were more resistant to the cytotoxin doxorubicin. Although MTAs and apoptotic thresholds *per se* were not tested in this study, the findings lend credence to the notion that a driver of chemoresistance is intratumor heterogeneity in apoptotic thresholds. Heterogeneity in Bcl-2 expression has also been detected at the protein level in t(14;18)-positive follicular lymphomas [23], lung adenocarcinomas [24] and mucosa-associated lymphoid tissue lymphoma [25]. Moreover, cell-to-cell heterogeneity in mitochondrial outer membrane permeabilization was observed in HT-29 colon adenocarcinoma cells following treatment with staurosporine, a pan-kinase inhibitor that induces apoptosis [26]. However, the impact of cell-to-cell heterogeneity in mitochondrial priming on MTA responsiveness remains to be tested. The extant data nonetheless suggest that variation exists within cancer cell populations in terms of proximity to the apoptotic threshold and imply that it may be beneficial to employ combination chemotherapy with BH3 mimetics or other drugs to enhance apoptosis susceptibility in the subpopulations of chemoresistant clones, such as those overexpressing Bcl-2 and thus potentially lying farther from the apoptotic threshold. Even if such subpopulations are relatively small within the tumor, it has been found that it may be more beneficial to choose chemotherapeutic combinations that take many subpopulations into account rather than merely targeting the most predominant single population [27]. It will be critical to determine how to selectively target apoptosis-reluctant subpopulations to avoid systemic toxicity.

As potential chemotherapeutic strategies such as this are tested, the use of non-traditional culture systems will be indispensable. Conventional continuous cell lines exist as rather homogenous monolayers without vasculatures or immune systems. These cultures are often hyperoxic, reliably receive nutrients and have been 'bred' for a high proliferation rate [28]. Solid tumors, by contrast, are highly heterogeneous, 3D structures that associate with stromal cells in a complex, potentially noxious microenvironment [29]. They possess a gradient of vital substrates such that the cortex is typically well nourished and the core starved. Other stressors include various leukocytes (such as natural killer cells, macrophages and cytotoxic T cells [30]) and, potentially, periodic anticancer therapies. Cancer cells *in vitro* can respond altogether differently to MTAs than the same cells *in vivo*. For instance, Janssen *et al*. recently uncovered that most docetaxel-treated colorectal cancer cells in isografts and xenografts, unlike their culture dish counterparts, initiated apoptosis *prior* to entering mitosis [31]. These findings underscore how vital the choice of a model is for research on MTAs and urge caution in the interpretation of findings based on experiments using continuous cell lines. Implanting continuous cell lines into Xenografting cultered cells provides a more realistic setting for them, although their microenvironment remains non-human and immunodeficient and, perhaps consequently, their doubling times (about 1–12 days) are still accelerated relative to those in most patients' tumors [1]. Indeed, the murine tumor microenvironment is in some way less pro-apoptotic than the culture dish [32]. As we further explore the interphase actions of MTAs and work toward developing superior agents, it will be crucial to supplement experiments using cell lines with those implementing other models, such as 3D culture systems [33], to bolster the clinical success of novel chemotherapeutics.

Expert commentary & five-year view

MTAs will likely persist as mainstays of clinical treatment in the near future. For many, if not most, cancers MTAs are unlikely to be supplanted by targeted anti-mitotics, which have mostly failed in clinical trials given that patients' tumors are not as highly proliferative as previously assumed. Combination chemotherapy with agents that target interphase processes, on the contrary, holds great promise and merits further study. Clinical trials testing chemotherapeutic regimens that combine MTAs with drugs that selectively induce or exacerbate pro-apoptotic vulnerability in cancer cells, such as BH3 mimetics, may prove particularly fruitful in combating the chemoresistance-causing, intratumoral subpopulations that exhibit decreased mitochondrial priming. It will be imperative to consider intratumor heterogeneity in terms of proximity to the apoptotic threshold in the design of novel agents and treatment regimens to decrease the probability of incomplete drug responses and relapse. Little research currently exists on the topic of the contribution of intratumor heterogeneity in apoptotic thresholds to clinical MTA resistance, although based on strong circumstantial evidence the topic unmistakably warrants further study.

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Key issues

- Interphase processes are likely the chief targets of microtubule-targeting agents (MTAs) in many patients' tumors.
- Thus, the notion that MTAs selectively target tumor cells because they are (seemingly) more highly proliferative than non-malignant cells is misguided.
- A more probable explanation for the degree of selectivity MTAs have for chemosensitive tumor cells is that these cells exist closer to the apoptotic threshold than non-malignant cells, an idea that is empirically supported even if it runs counter to the timeworn dictum that a defining feature of cancer is apoptosis reluctance.
- Many types of tumors exhibit profound genotypic and phenotypic cell-to-cell heterogeneity, with certain subpopulations of tumor cells more staunchly resisting the actions of MTAs.
- MTA-resistant clones may be less primed for apoptosis than MTA-sensitive ones.
- Cell-to-cell heterogeneity in Bcl-2 levels within tumors, which correlates with chemoresistance, supports the hypothesis that intratumor heterogeneity in proximity to the apoptotic threshold likely exists intratumorally and drives chemoresistance, although little research on the topic has been conducted.
- Chemotherapeutic regimens that incorporate drugs to selectively augment mitochondrial priming or to otherwise antagonize apoptosis reluctance (such as BH3 mimetics) in these clones may combat treatment failure and relapse.
- Given the reality of intratumor heterogeneity, it will be necessary to include heterogeneous culture systems and animal models in addition to the typically homogenous ones employed to generate translationally relevant conclusions that can improve chemotherapeutic regimens.

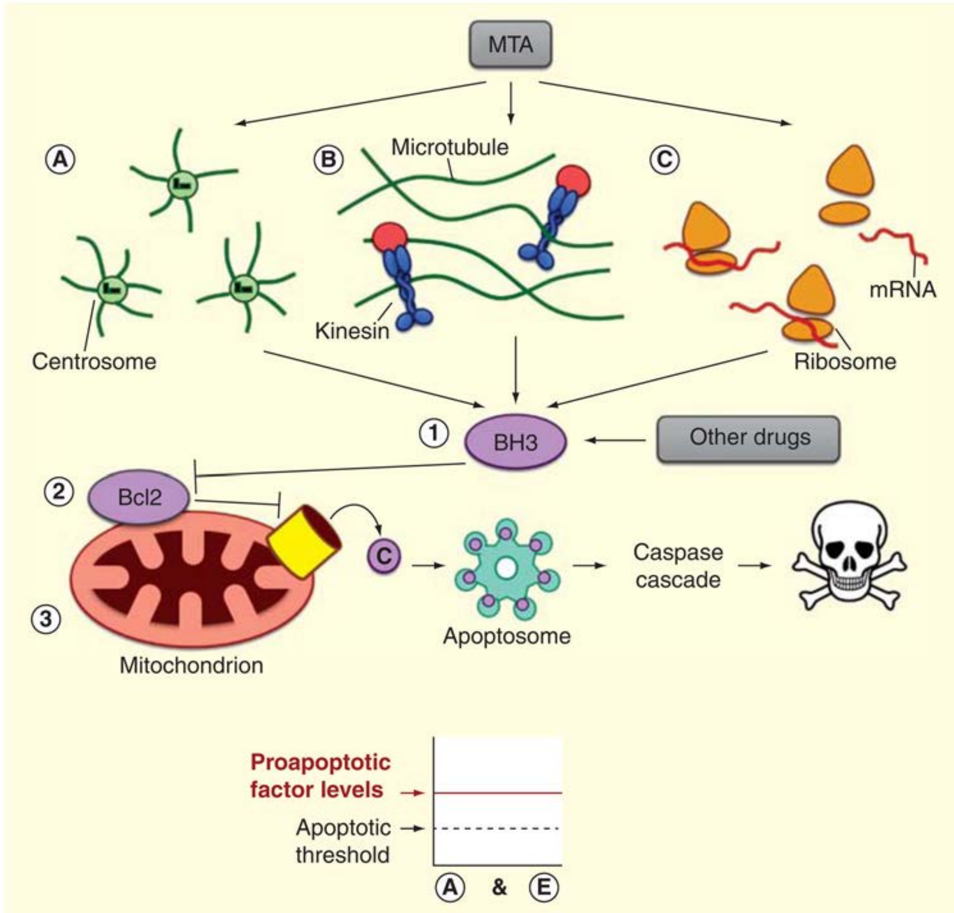

Figure 1. Inherent differences in the balance of BH3-only proteins, anti-apoptotic proteins, and mitochondrial priming determine the sensitivity of cancer cells to MTAs and other chemotherapeutic agents

MTAs disrupt a variety of interphase processes causing **(A)** centrosome declustering (which impairs directional migration), **(B)** inhibition of cargo transport, and **(C)** repression of translation, resulting in the expression or activation of proapoptotic BH3-only proteins. Other chemotherapeutic drugs with diverse mechanisms also prompt expression or activation of these proteins. Breaching the apoptotic threshold to cause an irreversible commitment of the cell to apoptosis can be achieved by sufficiently (1) inducing expression of or activating BH3-only proteins, (2) diminishing expression of or inhibiting antiapoptotic Bcl-2 family proteins, or (3) priming mitochondria to drive mitochondrial depolarization and the release of cyctochrome c. Cytochrome c joins Apaf-1 to form the apoptosome, which stimulates the caspase cascade and ultimately results in apoptotic cell death.

MTAs: Microtubule-targeting agents.

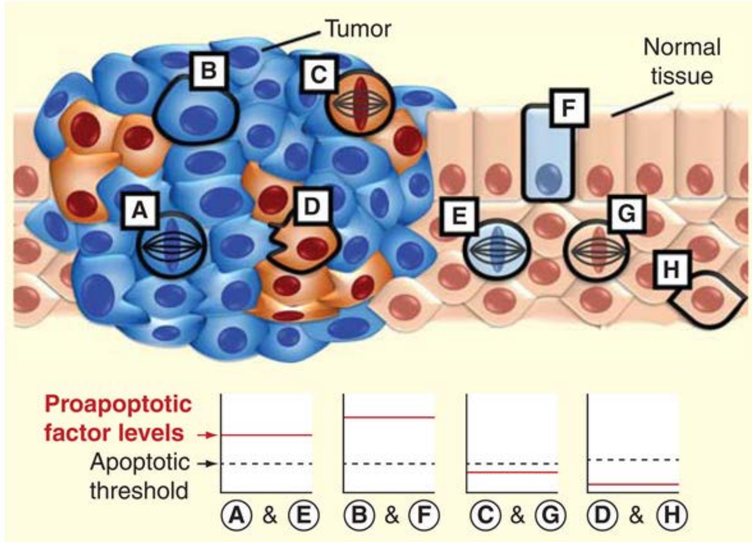

Figure 2. Differential sensitivity of malignant and non-malignant cells to MTAs

(A) Chemosensitive malignant mitotic cell: most malignant mitotic cells exceed their relatively low apoptotic thresholds, although there are few mitotic cells in the tumor. **(B)** Chemosensitive malignant interphase cell: many malignant interphase cells have a highly proapoptotic milieu resulting in their low apoptotic thresholds that can be easily breached by MTAs, thus making such cells highly sensitive to MTA action. **(C)** Chemoresistant malignant mitotic cell: a minority of mitotic malignant cells are chemoresistant, although they may lie closer to the apoptotic threshold than their interphase counterparts. **(D)** Chemoresistant malignant interphase cell: some malignant interphase cells do not exceed the apoptotic threshold, resulting in residual disease after chemotherapy or recurrence after an apparent complete response. **(E)** Chemosensitive non-malignant mitotic cell: many non-malignant mitotic cells exceed their apoptotic thresholds, resulting in side effects in proliferating tissues. **(F)** Chemosensitive non-malignant inter-phase cell: some non-malignant interphase cells exceed their apoptotic thresholds depending on the tissue type, resulting in side effects irrespective of proliferation rate. **(G)** Chemoresistant non-malignant mitotic cell: unlike most mitotic non-malignant cells, a minority are chemoresistant, although they may lie closer to the apoptotic threshold that their interphase counterparts. **(H)** Chemoresistant non-malignant interphase cell: most non-malignant interphase cells do not exceed their apoptotic thresholds, affording a therapeutic index to MTAs.

MTAs: Microtubule-targeting agents.