Published in final edited form as:

Cancer Immunol Immunother. 2011 January ; 60(1): 133–144. doi:10.1007/s00262-010-0926-x.

Topoisomerase Inhibitors Modulate Expression of Melanocytic Antigens and Enhance T Cell Recognition of Tumor Cells

Timothy J. Haggerty^{1,2,*}, Ian S. Dunn^{1,2}, Lenora B. Rose¹, Estelle E. Newton¹, Sunil Martin³, James L. Riley³, and James T. Kurnick^{1,2}

¹CytoCure LLC, Suite 430C, 100 Cummings Center, Beverly, MA 01915

²Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

³Abramson Family Cancer Research Institute and Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

Abstract

While there are many obstacles to immune destruction of autologous tumors, there is mounting evidence that tumor antigen recognition does occur. Unfortunately, immune recognition rarely controls clinically significant tumors. Even the most effective immune response will fail if tumors fail to express target antigens. Importantly, reduced tumor antigen expression often results from changes in gene regulation rather than irrevocable loss of genetic information. Such perturbations are often reversible by specific compounds or biological mediators, prompting a search for agents with improved antigen-enhancing properties. Some recent findings have suggested that certain conventional chemotherapeutic agents may have beneficial properties for cancer treatment beyond their direct cytotoxicities against tumor cells. Accordingly, we screened an important subset of these agents, topoisomerase inhibitors, for their effects on antigen levels in tumor cells. Our analyses demonstrate up-regulation of antigen expression in a variety of melanoma cell lines and gliomas in response to nanomolar levels of certain specific topoisomerase inhibitors. To demonstrate the ability of CD8+T cells to recognize tumors, we assayed cytokine secretion in T cells transfected with T cell receptors directed against Melan-A/MART-1 antigen. Three days of daunorubicin treatment resulted in enhanced antigen expression by tumor cells, in turn inducing co-cultured antigen-specific T cells to secrete Interleukin-2 and Interferon- γ . These results demonstrate that specific topoisomerase inhibitors can augment melanoma antigen production, suggesting that a combination of chemotherapy and immunotherapy may be of potential value in the treatment of otherwise insensitive cancers.

Keywords

topoisomerase inhibitors; immunotherapy; melanoma; Melan-A/MART-1

INTRODUCTION

Immunotherapy continues to hold great promise as a clinical anti-tumor strategy. Yet by its nature as a process contingent upon specific molecular recognition events, immunological destruction of tumor cells requires the maintenance of antigenic structures on the transformed tumor targets. Thus, widespread loss of such antigens will inevitably be accompanied by immunotherapeutic failure. This logical conclusion has been supported by

*corresponding author thaggerty@cytocure.com, phone: 978-232-1243, fax: 978-232-1243.

abundant experimental data [1,2]. It is now recognized that the immune system itself can shape the growth of tumor populations by providing a powerful negative selection ('immunoediting') in favor of antigen-loss variants [3]. Although immune targeting against a spectrum of tumor antigens can overcome antigen-loss of individual antigens, unfortunately co-ordinate expression loss for an entire suite of antigens can occur if they share regulatory control mechanisms, as has been observed for many melanocyte differentiation antigens [4,5]. Yet at the same time, this observation underscores a vitally important issue we have previously demonstrated: loss of antigen expression need not arise from irreversible processes (such as gene mutation or deletion), but can (and often does) result from alterations in gene regulatory pathways [6,2]. Such changes leave the relevant coding sequences intact, and are in principle reversible.

In response to this challenge, we have initiated screening programs for agents capable of reversing the 'antigen-silencing' effect which enables tumor escape from immune surveillance. From such studies, we have identified both small molecule drugs (principally kinase inhibitors; [7]), and biological mediators (especially interferon-beta; [8]) which actively up-regulate tumor antigen expression. To be effective as drugs, any such agents should show potency and appropriate pharmacokinetics *in vivo*. Historically, many anti-cancer drugs have been inherently highly toxic, and their therapeutic benefits are founded upon the balance between maximizing their anti-tumor effects and minimizing non-specific toxicities on bystander cells. Recent observations have added new factors to consider, since some chemotherapeutic agents may have additional properties of therapeutic relevance, beyond direct tumor toxicity. For example, there are reported anti-angiogenic effects through targeting of endothelial cells [9,10] and beneficial effects on the regulatory arm of the immune system [11–13].

Within the entire set of cytotoxic drugs used for cancer treatments, inhibitors of both topoisomerases I and II represent important and intensively studied drugs, and these compounds can also manifest anti-angiogenic or immune-enhancing effects in suitable dosages [10,11]. Topoisomerases are known to have roles in specific gene expression [14,15]. Even more cogently, DNA damage (as induced by UV radiation or other processes) has been implicated in the activation of melanocytic genes [16–18].

Drugs targeting topoisomerases are divided into two broad classes. The first class, which includes most of the clinically active agents, including doxorubicin, lead to increases in the levels of topoisomerase–DNA covalent complexes. Because these agents generate lesions that include DNA strand breaks and protein covalently bound to DNA, they have been termed topoisomerase "poisons." A second class of compounds inhibits the catalytic activity of topoisomerases but does not generate covalent complexes. Agents in this second class are thought to kill cells through the elimination of the essential enzymatic activity of topoisomerases and are therefore termed "catalytic inhibitors." Both topoisomerase types I and II cause extensive genomic damage when their normal catalytic cycles are disrupted by drugs acting as poisons [19–21].

Given these intriguing observations, we assessed whether topoisomerase inhibitors might have the added benefit of the up-regulation of tumor antigens. We therefore initially tested melanoma cell lines with daunorubicin (an anthracycline, in common with doxorubicin), and indeed found significant antigen-enhancing effects. Subsequent investigations showed that several topoisomerase I inhibitors can augment T cell recognition of tumor targets. These studies demonstrate the functional relationship between tumor cell antigen expression and immune function in responding T cells. The results with low dose topoisomerase inhibitors parallel our previous findings with IFN-beta and MAP-kinase inhibitors, which also reconstitute T cell recognition of tumor cells otherwise escaping immune destruction [8,7].

MATERIALS AND METHODS

Cell culture, and topoisomerase inhibitors

General culture conditions for cell propagation and the origins of most of the melanoma cell lines have been previously described [6,2]. Melanoma cells were cultured in DMEM with 10% FBS. The T cell receptor (TCR)-negative Jurkat T cell line derivative J.RT3-T3.5 was obtained from ATCC. T cells were cultured in RPMI with 10% FBS. IFN-beta-1a (Avonex) was obtained from Biogen-Idec (Cambridge, MA) and reconstituted according to the manufacturer's recommendations. The topoisomerase inhibitors daunorubicin, doxorubicin, epirubicin, etoposide, camptothecin, kaempferol, AG1387, and topotecan were purchased from CalBiochem (La Jolla, CA); 10-hydroxycamptothecin, β -lapachone, ellipticine, and novobiocin from BioMol (Plymouth Meeting, PA); and apigenin, idarubicin, XK469, rubitecan, sobuzoxane, ATA, and merbarone from Sigma (St. Louis, MA).

EGFP reporter cell line

The generation and application of EGFP reporter cells has been previously described [8]. Briefly, a 1200 base pair human genomic DNA segment encompassing the Melan-A/MART-1 promoter was used to generate a construct driving expression of the EGFP reporter gene. Stable transfectants with this construct were generated in the low-antigen cell line A375 and the high-antigen line MM96L+. The EGFP expression response patterns of these cells to IFN-beta and MAP kinase inhibitors recapitulates the antigen up-regulation of endogenous Melan-A/MART-1 induced by these agents [8].

Toxicity measurements

The toxicity of compounds was measured using the WST reagent system from Roche (Indianapolis, IN) [22]. Cells were plated in a 96-well culture plate. The time zero absorbance (A_0) was measured at 450nm 24 hours after plating, and inhibitors were added at this time. Cells were allowed to grow for an additional 48 hrs. WST was added to each well and after 1 hr the hydrolysis of the WST was read in a plate reader at 450nm. Wells were normalized by subtracting a 655nm background reading. Triplicates for each sample were determined. A control for medium only (A_{Con}) was subtracted from each reading.

Treatment of cells with topoisomerase inhibitors

Cells were plated at a density of 1×10^5 in 1 ml of media in a 24-well plate and cultured for varying times as indicated in results. Typically, 3 days of culture in the presence of topoisomerase inhibitors was optimal for the functional studies performed. After incubation cells were collected by trypsinization and then evaluated by flow cytometry.

Flow cytometry

Staining and flow cytometric analyses of cytoplasmic Melan-A/MART-1, and gp100 expression were performed as described previously [6]. Cells were fixed with 1% formaldehyde, permeabilized with 0.1% saponin, stained with monoclonal antibodies to melanocyte antigens (MART-1, Vector Labs; gp100 HMB45, DakoCytomation) and then visualized with goat anti-mouse FITC-conjugated secondary antibody (Zymed). The level of EGFP was determined by flow cytometry of unfixed cells washed and resuspended in 1X PBS.

Generation of TCR-encoding lentiviral particles

The previously published codon-optimized sequence (1D3[23,24]) was synthesized by GeneArt (Burlingame, CA) and placed into a third generation lentiviral transfer vector [25]. The TCR α and TCR β sequences were separated by a P2A sequence as previously described

to facilitate coordinate expression under control of the EF-1 α promoter [26]. Lentiviral vector containing supernatants were prepared as previously described [27]. Briefly, the ID3 transfer vector was co-transfected with expression vectors encoding HIV-1_{REV}, HIV-1_{GAG.POL}, and VSV-G into 293 T cells. After 48 hr, media supernatants containing the virus particles were collected and these were concentrated using a 100,000 molecular weight cut-off filter. Virus titers were determined by infecting J.RT3 cells and staining for CD3. A pool of transduced J.RT3 cells stably expressing the exogenous Melan-A/MART-1 specific TCR at >95% efficiency was expanded for further use.

Assays of CD8+ T lymphocytes

Primary CD8+ T lymphocytes were obtained using heparin-treated blood incubated with a negative selection cocktail RosettaSep from STEMCELL Technologies (Vancouver, BC, Canada) to generate 95% pure CD8 T cells. These cells were stimulated for 24 hours with CD3 / CD28 beads (InVitrogen Dynal AS, Oslo, Norway), and cultured with 200 U/ml recombinant IL-2 (Proleukin) from Cetus (Emeryville, CA) supplemented media. For transduction of the Melan-A/MART-1 specific TCR, 1×10^5 stimulated primary CD8 cells were incubated with lentiviral vector at a MOI of 10. Cells were grown for two days after lentiviral infection and then stained with tetramer as described below. The CD8+ T cells were further propagated for 2 weeks in medium containing 200U/ml recombinant IL-2 prior to use in cellular assays described below.

Tetramer staining

Tetramers were prepared by assembly of denatured recombinant major histocompatibility complex (MHC) Class I HLA-A2 plus peptide followed by dialysis [28]. Cells were incubated with MHC class I tetramers conjugated to phycoerythrin (PE) (kindly provided by Pedro Romero of Ludwig Institute for Cancer Research, Lausanne) with the Melan-A/MART-1 peptide ELAGIGILTV. Staining buffer contained 0.1% sodium azide and 0.1% BSA. Staining was carried out at room temperature. Cells were washed, stained and then rewashed before evaluation by flow cytometry.

Assay for T cell activation by tumor cells

Melanoma cells were treated with antigen-modulating agents (daunorubicin or IFN- β) for three days prior to counting and plating for the co-culture assay (cells were washed and resuspended in fresh medium that no longer contained the treatment agents during the co-culture). 100 μ l of 5×10^5 cells/ml tumor cells were mixed with 50 μ l of 5×10^5 cells/ml T cells in a 96-well V-bottom plate and incubated overnight (16–24 hours). Plates were centrifuged to pellet the cells and 120 μ l of supernatant fluid was removed from each well for cytokine assay. To assess antigen-specific T cell responses, 3.33 μ g/ml Melan-A/MART-1 peptide was added to the co-culture. The sequence of the Melan-A/MART-1 peptide was ₂₆ELAGIGILTV₃₅ (A27L from wild type) and the NY-ESO-1 peptide ₁₅₇SLLMWITQV₁₆₅ (C165V from wild type).

Cytokine ELISA

The protocols for evaluation of either IL-2 or IFN-gamma were similar, following the manufacturer's recommendations. A standard curve using known concentrations of each cytokine was included in each ELISA experiment. ELISA plates were coated with capture antibody and then washed with 1X PBS 0.5% Tween-20. Wells were blocked with 1X PBS 1% FBS for 1 hour and rewashed. Supernatants and standards were added to the wells and incubated for 2 hr at room temperature and wells were washed again. The detection antibody and HRP secondary were added together and incubated for 1 hr. The HRP color reaction proceeded for 15min before being stopped with 2N H₂SO₄. The absorbance 450nm was read

in a BioRad 3550 plate reader. IL-2 levels for experimental samples (pgs/ml) were calculated from the standard curve.

RESULTS

Enhanced Antigen Expression on Melanomas and Gliomas by Daunorubicin

We utilized a series of melanomas and gliomas to demonstrate augmentation of antigen expression after a 3-day exposure to the anthracycline topoisomerase II inhibitor daunorubicin. Table 1 lists the resulting increases in antigen expression when cells were treated with 100 nM daunorubicin for three days. An example of the flow results for antigen expression is provided in the bottom panels of Fig. 1 (further examples are provided in Supplementary Data; Fig. SD1). These demonstrations included enhancement of Melan-A/MART-1 on melanomas with both high (MU89, MM96L+, MO, 136.2) and low (MUX, MM96L-, A375 and H59-44T) initial antigen expression. In addition, daunorubicin augmented levels of gp100 (HMB-45) on these melanomas, and also boosted gp100 expression in two gliomas (U-87 and U-118).

Since previous work demonstrated that cellular responses to other antigen up-regulating agents (IFN-beta and MAP kinase inhibitors) were recapitulated using an extended Melan-A/MART-1 promoter / EGFP reporter system [8], we tested the same reporter cell lines with topoisomerase inhibitor assays. We accordingly showed that these cell lines produced a similar augmentation of EGFP fluorescence in response to daunorubicin as for endogenous Melan-A/MART-1 itself (Fig. 1). The Melan-A/MART-1 promoter / EGFP reporter system was then used for dose-response studies. As shown in Fig. 2, this dose of daunorubicin is optimal on the MU89 cell line, and similar doses are effective on the other cell lines tested. It is noteworthy that daunorubicin was quite toxic at the 100 nM dosage (Supplementary Data; Fig. SD2), and at 200 nM (and above), the vast majority of cultured tumor cells cannot survive the 3-day culture period of this drug. Notably, the surviving daunorubicin-treated tumor cells undergo significant size and morphological changes, becoming much larger and more irregularly shaped than controls (These cellular alterations can be quantified by flow cytometry (Supplementary Data; Fig. SD2), by determining the increases in forward scatter (as an index of cell size), and side scatter (reflecting increased intracellular complexity and granularity).

While the initial studies were performed at 3 days of culture, two types of kinetic observations were then made, by both varying the culture period, and determining how long the daunorubicin must be present in order to be effective. With our validated Melan-A/MART-1 promoter EGFP reporter system [8], we showed that treated cells take more than 2 days to manifest significant increases in Melan-A/MART-1 reporter activity (Fig. 3A), but this increase continues beyond 3 days of culture and plateaus between 4 and 6 days. The cumulative effects of the toxicity and culture crowding conditions lead to a decrease in antigen expression at the two higher doses of daunorubicin at day 7 (Fig. 3A).

Transient high concentrations of daunorubicin (≥ 200 nM) induce maximal Melan-A/MART-1 promoter up-regulation with short exposures (2–6 hr). At these higher doses, daunorubicin toxicity limits the feasible exposure time, but cells can survive for several days if the high drug levels are removed after treatments of ≤ 6 hr duration. While it takes several days for the antigen expression to manifest in daunorubicin-treated cells, it is notable that the tumor cells need only be in the presence of the drug for a few hours in order to manifest antigen-enhancing activity (Fig. 3B). At the highest doses, 500 and 1000 nM, the level of EGFP has reached a maximum plateau at the earliest time point tested (2 hours) and does not increase at 4- and 6-hour time points. Thus, the highest doses of daunorubicin are already optimally active if the drug is present for as little as 2 hours. It is noteworthy that

because of the short exposure time, between 2 and 6 hours, high doses of daunorubicin, 200, 500, and 1000 nM, can be tolerated, longer exposure to these high doses is too toxic to allow data collection at the 3 day time point. Thus, the decreased time of exposure to higher doses reduced toxicity enough to allow cells to survive under these conditions. The lower doses of daunorubicin take longer to fully optimize activity, although again they are quite active in only short exposure cultures. At lower doses, 25 and 50 nM, two phases of the curve are observed, a slow increase until 12 hours and then a sharper increase. In contrast, an intermediate dose (100nM, the dose at which most assays were carried out) has a straight-line increase in EGFP levels, the EGFP level increases with increasing time of exposure. Daunorubicin and many other topoisomerase inhibitors can intercalate into DNA duplexes and remain bound [29–31], consistent with changes induced by high doses, even after excess drug is removed from culture. At lower doses of daunorubicin (25 and 50 nM) exposure for at least 24 hr is required to induce a 2-fold increase in Melan-A/MART-1 reporter activity (Fig. 3B).

As the functional relevance of antigen up-regulation for immunotherapy is intimately linked with recognition of tumor targets by CD8+ cytotoxic T lymphocytes (CTLs), we also tested the effect of daunorubicin on Class I MHC expression. We found that expression of MHC Class I was reproducibly elevated by daunorubicin treatment of four melanoma cell lines (Table SD1). Since T Cell recognition of tumors depends on both nominal antigen and the presenting MHC molecule, the fact that the daunorubicin enhances expression of both of these proteins would provide an advantage to the cellular immune response against treated tumor cells.

Enhanced T Cell Recognition of Treated Tumor Cells

The enhancement of intracellular expression of specific antigenic proteins by daunorubicin is highly reproducible, but the ability of T cells to recognize treated target cells is the best correlate with an immunologically significant enhancement of differentiation antigen expression. In order to measure T cell recognition of melanoma cells, we used a lentiviral vector encoding a cloned T cell receptor (TCR), whose expression in recipient transduced T cells confers the ability to recognize a Melan-A/MART-1 peptide (AAGIGILTV) in the context of HLA-A2. This TCR was transduced into both a T cell tumor line (TCR-deficient Jurkat J.RT3-T3.5 cells) and normal CD8+ Peripheral Blood Leukocyte (PBL)-derived T cells. Recognition by the transduced TCR of its cognate Melan-A/MART-1 peptide: HLA-A2 complex (expressed in target cells) results in activation of the transduced cells, leading in turn to IL-2 secretion. In the absence of TCR expression, the initial J.RT3 cells cannot express surface CD3 as part of the normal antigen receptor and signal transduction complex [32]. As shown in Fig. 4A, transduction of the specific T cell receptor allows J.RT3 cells to express both surface CD3 (Fig. 4Ai), and bind Melan-A/MART-1 peptide / HLA-A2 tetramers (Fig. 4Aii). Transduction of the same T cell receptor into primary activated CD8+ T cells also resulted in binding of the same specific tetramers (Fig. 4Aiii).

More importantly, as shown in Fig. 4B, when these same TCR-transduced T cells are presented with antigen, they produce readily detectable levels of specific cytokines. Engagement of the transduced TCRs results in production of IL-2 (shown with J.RT3 Jurkat cells; Fig. 4Bi), or IFN- γ (shown with primary CD8+ T cells; Fig. 4Bii). As can be readily appreciated, the J.RT3 cells in Fig. 4Bi produce low levels of IL-2 in response to control HLA-A2+ MU89 cells. Daunorubicin treatment induced nearly 5-fold enhanced IL-2 production. This very significant induction constituted ~50% of the experimental maximum observed when all available surface HLA-A2 molecules on the target cells are artificially saturated through pulsing with excess specific Melan-A/MART-1 peptide (Fig. 4Bi). An unrelated HLA-A2-restricted peptide (NY-ESO-1) failed to induce IL-2, demonstrating the specificity of this antigen-recognition process.

Similarly, primary CD8⁺ T cells expressing the same TCRs with Melan-A/MART-1 peptide / HLA-A2 specificity respond to daunorubicin-treated tumor cells with production of IFN- γ over 4-fold the background activity seen with untreated MU89 cells. This high level of response was similar to that resulting from IFN- β stimulation of MU89 cells, or these tumor cells pulsed with Melan-A/MART-1 peptide (Fig. 4Bii). Both the untransduced J.RT3 cells and the untransduced PBL produce no cytokine when confronted with the same antigen-presenting cells, further confirming the recognition specificity and sensitivity of the lentiviral-infected cells expressing the specific TCR.

Effects of Different Topoisomerases on Melan-A/MART-1 expression

We tested a series of both topoisomerase I and II inhibitory compounds with different reported effects and mechanisms. The pharmacological activity of topoisomerase inhibitors has been historically grouped into the categories of poisons (compounds stabilizing otherwise transient topoisomerase-DNA covalent complexes [20,21]), or catalytic inhibitors that block catalysis by direct interaction with topoisomerase protein itself [33]. Fig. 5 summarizes a series of flow studies with a range of topoisomerase inhibitors (further specific examples shown in Supplementary Data; Fig. SD3). The strongest antigen enhancement activity was shown by topoisomerase poisons, irrespective of specificity towards topoisomerase I or II. In contrast, the catalytic inhibitors were generally impotent at enhancing antigen. However, topoisomerase poisons themselves varied in activity. The structurally related anthracyclines daunorubicin, doxorubicin, epirubicin and idarubicin are all topoisomerase II inhibitors with potent antigen enhancing capacity in the nanomolar range (Fig. 5). The non-anthracycline topoisomerase II poisons etoposide [30] and ellipticine [34] showed significant activity, although requiring a full order of magnitude higher concentration of drug than the anthracycline set (Fig SD3). Among the topoisomerase I inhibitors, the related poisons topotecan and rubitecan are highly active, and camptothecin and hydroxy-camptothecin are also very potent, while kaempferol has detectable but far more modest activity, in common with the catalytic topoisomerase I inhibitor AG1387. Apigenin and β -lapachone were essentially inactive, despite the recorded activity of β -lapachone towards both topoisomerases I and II [35,36].

Effects on Cell Size and Morphology in Relation to Antigen Expression

Flow analyses of anthracycline-treated cells typically showed increases in cell volume (Supplementary Data; Figs. SD1 and SD4). It might be thought that an increased cell volume will only magnify the net amounts of all proteins expressed per cell unit, without an essential increase in the density of proteins per unit volume. All inhibitors that enhanced antigen expression indeed caused measurable morphological changes as well, but there was no observable correlation between cell size and shape perturbations and antigen levels (Supplementary Data; Fig. SD4). Moreover, some drugs that induced increases in cell size and granularity did not increase specific antigen expression, suggesting that the enhanced antigen expression is not merely a reflection of increased cell size and complexity. Irrespective of cell size alterations, increased antigen expression is clearly dependent on protein synthesis, as demonstrated through the effects of the inhibitor cycloheximide (Supplementary Data; Fig. SD5).

DISCUSSION

Combination therapies against tumors have a higher inherent probability of success than treatments with single agents, given the need to counter a wide variety of possible resistance mechanisms. In particular, certain combination therapies for leukemias and lymphomas have resulted in very positive outcomes [37,38]. As we have previously noted, one of the obstacles to immunotherapy is the emergence of antigen-negative escape variants, and we

have sought and identified agents that can enhance and restore antigen expression to augment immune recognition and destruction of the tumors [8,7]. We were also motivated to test conventional chemotherapeutic drugs for their effects on melanoma cell antigen expression. At high prescribed doses, these drugs are designed for anti-tumor cytotoxicity, yet in lower doses may also have utility in metronomic therapy against neoangiogenesis [9,10] or by useful immune modulation [11]. In practice, standard chemotherapy has had very limited efficacy for treatment of melanoma [39], but up-regulation of antigen levels by the same drugs might provide an additional mechanism for immune-mediated cancer therapy.

Several additional factors prompted us to specifically examine topoisomerase inhibitors for their potential effects on tumor antigen expression. The cytotoxic mechanisms of many of the major current anticancer drugs is attributable to blockade of topoisomerase function [20,40,21], which in turn can have varied effects on gene expression [41–44]. Most importantly, the association of DNA damage with melanocytic gene regulation [16–18] provided a potential link between topoisomerases and melanocytic tumor antigen expression, which we elected to pursue in this study.

Our evaluation of a variety of inhibitors of both Type I and II topoisomerases showed clear-cut effects on antigen expression, but these activities showed significant differences among the different agents. Inhibition of topoisomerases is clearly associated with up-regulation of antigen expression, but this effect is clearly not simply related to inhibition of enzyme catalysis. In fact, catalytic inhibitors showed at best very modest activities, and were mostly ineffectual (Fig. 5). On the other hand, certain topoisomerase poisons profoundly modulated antigen levels, and moreover improved the recognition of treated tumor cells by specific T cells (Fig. 4).

In considering these data, it is important to keep in mind the imperfect specificities of topoisomerase II inhibitors. The widely-used anthracycline inhibitors daunorubicin, doxorubicin and their derivatives are generally ascribed as directed towards topoisomerase II [45,40], but nevertheless have been shown to also significantly target topoisomerase I, at least under some circumstances [46,47]. Yet in contrast, camptothecin appears to be remarkably selective towards topoisomerase I [20]. Based solely on the known selectivity of camptothecin and the measurable promiscuity of anthracyclines, at present we cannot exclude the possibility that the topoisomerase target relevant to melanoma antigen expression is largely or even uniquely topoisomerase I. Even so, additional evidence is necessary to confirm the link between topoisomerase I inhibition by camptothecin and the up-regulation of antigen expression.

Comparison of the general effectiveness of topoisomerase poisons to the poor activity of catalytic inhibitors (Fig. 5) suggests that the increased antigen levels are not simply due to ablation of topoisomerase activity in itself. Irrespective of their mechanistic details, poisons of topoisomerases are defined by their ability to convert these normal essential enzymes into agents of destruction, by inducing the accumulation of double-stranded or single-stranded DNA breaks [48,49]. Past a certain threshold of such induced genomic damage, the end result is cell death (a desirable therapeutic outcome, of course, for tumor cells). Prior to this occurring, though, the combination of cell cycle arrest and differential gene expression increase may result in increased cell volumes, which we typically observe by flow analysis of anthracycline-treated cells (Results and Supplementary Data; Figs. SD1 and SD4). While our data show no necessary correlation between increased cell volume and antigen expression levels, the poisoning of topoisomerases appears to produce the most potent antigen responses, as opposed to direct enzymatic inhibition. The DNA damage response, which is a direct consequence of topoisomerase poisoning [50,51], may consequently be the

central stimulus for antigen up-regulation [16–18], rather than topoisomerase inhibition *per se*. Such a proposal is consistent with our results, but further work will be required to dissect the components of the topoisomerase-induced DNA damage pathway directly impinging on antigen expression.

Importantly, the ability of antigen-specific T cells to recognize the treated tumor cells provides a functional correlate with enhanced protein expression. Of note, our results indicate that genes for both differentiation antigens and MHC Class I are included among those up-regulated by specific inhibitors directed against either topoisomerases I or II. Since both antigen and MHC are enhanced, T Cell recognition of the tumors benefits from the increase of both of these elements needed for TCR engagement of tumor cells [52]. Increased T cell activation following topoisomerase inhibitor treatment of target cells then is a good correlate of improved T cell recognition of target, and in turn improved antigen presentation. Therefore, our T cell system provides a biologically meaningful read-out for the success of antigen up-regulation, and in a therapeutic context, it is the most relevant *in vitro* assay we have available. This system unequivocally reports that the tumor antigen augmentation mediated by specific topoisomerase inhibitors allows greatly improved T cell recognition of melanoma targets, of crucial importance for immunotherapy.

It is becoming increasingly clear that the outcome of chemotherapy is influenced by critical factors beyond the direct killing of target tumor cells, including the control of angiogenesis and the successful deployment of the host immune system. Accordingly, chemotherapy and immunotherapy should not be considered in isolation from each other [53]. The importance of these observations is emphasized by remarkable recent findings concerning the unanticipated and therapeutically relevant effects (beyond direct cytotoxicity) of many common chemotherapeutic agents. In suitable dose ranges, anthracyclines and other drugs have thus been shown to block angiogenesis [10,9], suppress Treg-mediated immune down-modulation [12,11], increase the efficiency of immune killing of both target and bystander tumor cells [54], improve antigen-presentation by dendritic cells [55], and stimulate the anticancer immune response [56,57].

Here we add an additional dimension to this diverse therapeutic picture, where these agents can also potentially boost specific immune recognition of tumor targets and help marshal the host immune system towards anti-tumor cytotoxicity. In terms of the choice of drug doses for effective responses, our own experience with the T cell assay suggests that high concentrations of anthracyclines can block T cell activation despite concomitant tumor antigen up-regulation (unpublished observations). This finding is consistent with the known lymphocytic toxicity of high doses of anthracycline drugs [58]. A reduced dose *in vivo* which preserves antigen up-regulation without ablating the effector arm of T cell immunity is therefore highly desirable, although simultaneous dampening of the regulatory T cell arm [13,12,11] would be a welcome side-benefit.

Indeed, the potential role of anti-tumor immune responses in contributing to the overall success of chemotherapy in general has become a topic of recent interest [53,59]. It is also a reasonable prospect that immunological or anti-angiogenic suppression of tumor cells surviving a primary chemical cytotoxic challenge would significantly forestall the development of drug resistance in residual tumor populations. Any combination therapy against multiple independent targets in a pathogen or tumor can reduce the frequency of selection for resistant variants, since the probability of the occurrence of multiple genetic resistance mutations is accordingly lower than for mutations countering any single agent in isolation. Treatment with appropriate topoisomerase inhibitors combined with immunotherapy therefore has the potential for a greater initial impact, since pre-existing cells which are independently either drug-resistant or low expressors of antigen are still

susceptible to the combined therapeutic challenge. Following the first round of treatment, survivors with drug-induced antigen augmentation are then enhanced targets for ongoing immunotherapy.

An important factor determining the future utility of topoisomerase poisoning in tumor antigen modulation is the achievable levels of circulating drugs in human cancer patients, for which numerous and long-standing pharmacokinetic studies can be resourced. In the case of anthracyclines, many different dosage regimens have been used for cancer therapy, the most conservative approach is to consider the pharmacokinetic outcome of single-dose treatments. Studies with doxorubicin (whose antigen-up-regulation effects were comparable to the closely related daunorubicin (Fig. 5)) using single treatments of 50–60 mg.m⁻² have shown patient plasma levels in the 100 nM range after 6 hr, persisting to at least 30 nM after 24 hr [60,61]. These doses are well above those required *in vitro* for prolonged antigen up-regulation (Fig. 3), even if the drug treatments were to be immediately discontinued.

Even so, beneficial immune responses in patients might be significantly blunted, or even ablated, if the chemotherapeutic treatment proved toxic to sensitive immune effector cells. The conundrum of drugs useful for antitumor activity but with concurrent negative effects on the immune system has been recently highlighted [62,53,59]. A powerful way to side-step this is adoptive immunotherapy, where TCRs against antigens known to be up-regulated by initial treatments are transduced *in vitro* into autologous CD8+ T cells from patient PBLs (such as the CD8+ T cells that express TCRs specific for Melan-A/MART-1 used in our investigations), expanded, and re-infused back into the autologous recipient [63] after the primary chemotherapeutic treatment.

Design of a truly optimal oncological treatment regimen with designated topoisomerase inhibitors must investigate *in vivo* pharmacokinetics and dose responses for multiple biological effects: direct tumor cytotoxicity, endothelial cell cytotoxicity, antigen stimulation on tumor cells, compatibility with the T cell effector arm, and suppression of Treg cells. While a single drug may be unlikely to satisfy all these criteria, the cost-effectiveness of repositioning existing drugs in new roles has a high current profile in the pharmaceutical industry [64]. Our data support the contention that conventional cytotoxic topoisomerase inhibitors may find new applications in therapies for melanomas and possibly other tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Carl June for advice in use of the lentiviral T cell receptor system and helpful commentary on the manuscript. We also acknowledge Pedro Romero for provision of tetramers and assistance in improving the manuscript. This work was supported in part by grants from the NIH: R43-CA86153, R43-CA96271, R43-CA94700, R43-CA128309 and from the International Cancer Research Scholarship.

References

1. Pandolfi F, Cianci R, Lolli S, Dunn IS, Newton EE, Haggerty TJ, Boyle LA, Kurnick JT. Strategies to overcome obstacles to successful immunotherapy of melanoma. *Int J Immunopathol Pharmacol*. 2008; 21:493–500. [PubMed: 18831916]
2. Durda PJ, Dunn IS, Rose LB, Butera D, Benson EM, Pandolfi F, Kurnick JT. Induction of "Antigen silencing" in melanomas by oncostatin m: Down-modulation of melanocyte antigen expression. *Mol Cancer Res*. 2003; 1:411–419. [PubMed: 12692260]

3. Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, Diamond MS, Koebel CM, Arthur C, White JM, Schreiber RD. A critical function for type I interferons in cancer immunoeediting. *Nat Immunol.* 2005; 6:722–729. [PubMed: 15951814]
4. Levy C, Khaled M, Fisher DE. Mitf: Master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med.* 2006; 12:406–414. [PubMed: 16899407]
5. Cheli Y, Ohanna M, Ballotti R, Bertolotto C. Fifteen-year quest for microphthalmia-associated transcription factor target genes. *Pigment Cell Melanoma Res.* 2010; 23:27–40. [PubMed: 19995375]
6. Kurnick JT, Ramirez-Montagut T, Boyle LA, Andrews DM, Pandolfi F, Durda PJ, Butera D, Dunn IS, Benson EM, Gobin SJ, van den Elsen PJ. A novel autocrine pathway of tumor escape from immune recognition: Melanoma cell lines produce a soluble protein that diminishes expression of the gene encoding the melanocyte lineage melan-a/mart-1 antigen through down-modulation of its promoter. *J Immunol.* 2001; 167:1204–1211. [PubMed: 11466335]
7. Kono M, Dunn I, Durda P, Butera D, Rose LB, Haggerty T, Benson E, Kurnick J. Role of the map kinase signaling pathway in the regulation of human melanocytic antigen expression. *Mol Cancer Res.* 2006; 4:779–792. [PubMed: 17050671]
8. Dunn IS, Haggerty TJ, Kono M, Durda PJ, Butera D, Macdonald DB, Benson EM, Rose LB, Kurnick JT. Enhancement of human melanoma antigen expression by IFN- β . *J Immunol.* 2007; 179:2134–2142. [PubMed: 17675472]
9. Scharovsky OG, Mainetti LE, Rozados VR. Metronomic chemotherapy: Changing the paradigm that more is better. *Curr Oncol.* 2009; 16:7–15. [PubMed: 19370174]
10. Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer.* 2004; 4:423–436. [PubMed: 15170445]
11. Menard C, Martin F, Apetoh L, Bouyer F, Ghiringhelli F. Cancer chemotherapy: Not only a direct cytotoxic effect, but also an adjuvant for antitumor immunity. *Cancer Immunol Immunother.* 2008; 57:1579–1587. [PubMed: 18369619]
12. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, Solary E, Le Cesne A, Zitvogel L, Chauffert B. Metronomic cyclophosphamide regimen selectively depletes CD4⁺CD25⁺ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother.* 2007; 56:641–648. [PubMed: 16960692]
13. Banissi C, Ghiringhelli F, Chen L, Carpentier AF. Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model. *Cancer Immunol Immunother.* 2009; 58:1627–1634. [PubMed: 19221744]
14. Ju BG, Lunyak VV, Perissi V, Garcia-Bassets I, Rose DW, Glass CK, Rosenfeld MG. A topoisomerase II β -mediated DNA break required for regulated transcription. *Science.* 2006; 312:1798–1802. [PubMed: 16794079]
15. Lotito L, Russo A, Chillemi G, Bueno S, Cavalieri D, Capranico G. Global transcription regulation by DNA topoisomerase II in exponentially growing *Saccharomyces cerevisiae* cells: Activation of telomere-proximal genes by TOP1 deletion. *J Mol Biol.* 2008; 377:311–322. [PubMed: 18272174]
16. Eller MS, Ostrom K, Gilchrist BA. DNA damage enhances melanogenesis. *Proc Natl Acad Sci U S A.* 1996; 93:1087–1092. [PubMed: 8577719]
17. Park HY, Kosmadaki M, Yaar M, Gilchrist BA. Cellular mechanisms regulating human melanogenesis. *Cell Mol Life Sci.* 2009; 66:1493–1506. [PubMed: 19153661]
18. Inomata K, Aoto T, Binh NT, Okamoto N, Tanimura S, Wakayama T, Iseki S, Hara E, Masunaga T, Shimizu H, Nishimura EK. Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. *Cell.* 2009; 137:1088–1099. [PubMed: 19524511]
19. Wilstermann AM, Osheroff N. Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes. *Curr Top Med Chem.* 2003; 3:321–338. [PubMed: 12570766]
20. Pommier Y. Topoisomerase II inhibitors: Camptothecins and beyond. *Nat Rev Cancer.* 2006; 6:789–802. [PubMed: 16990856]
21. Deweese JE, Osheroff N. The DNA cleavage reaction of topoisomerase II: Wolf in sheep's clothing. *Nucleic Acids Res.* 2009; 37:738–748. [PubMed: 19042970]
22. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods.* 1983; 65:55–63. [PubMed: 6606682]

23. Dietrich PY, Le Gal FA, Dutoit V, Pittet MJ, Trautman L, Zippelius A, Cognet I, Widmer V, Walker PR, Michielin O, Guillaume P, Connerotte T, Jotereau F, Coulie PG, Romero P, Cerottini JC, Bonneville M, Valmori D. Prevalent role of tcr alpha-chain in the selection of the preimmune repertoire specific for a human tumor-associated self-antigen. *J Immunol.* 2003; 170:5103–5109. [PubMed: 12734356]
24. Jorritsma A, Gomez-Eerland R, Dokter M, van de Kastelee W, Zoet YM, Doxiadis II, Rufer N, Romero P, Morgan RA, Schumacher TN, Haanen JB. Selecting highly affine and well-expressed tcrs for gene therapy of melanoma. *Blood.* 2007; 110:3564–3572. [PubMed: 17660381]
25. Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, Naldini L. A third-generation lentivirus vector with a conditional packaging system. *J Virol.* 1998; 72:8463–8471. [PubMed: 9765382]
26. Varela-Rohena A, Molloy PE, Dunn SM, Li Y, Suhoski MM, Carroll RG, Milicic A, Mahon T, Sutton DH, Laugel B, Moysey R, Cameron BJ, Vuidepot A, Purbhoo MA, Cole DK, Phillips RE, June CH, Jakobsen BK, Sewell AK, Riley JL. Control of hiv-1 immune escape by cd8 t cells expressing enhanced t-cell receptor. *Nat Med.* 2008; 14:1390–1395. [PubMed: 18997777]
27. Parry RV, Rumbley CA, Vandenberghe LH, June CH, Riley JL. Cd28 and inducible costimulatory protein src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, bcl-xl, and il-2 expression in primary human cd4 t lymphocytes. *J Immunol.* 2003; 171:166–174. [PubMed: 12816995]
28. Romero P, Dunbar PR, Valmori D, Pittet M, Ogg GS, Rimoldi D, Chen JL, Lienard D, Cerottini JC, Cerundolo V. Ex vivo staining of metastatic lymph nodes by class i major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic t lymphocytes. *J Exp Med.* 1998; 188:1641–1650. [PubMed: 9802976]
29. Cutts SM, Swift LP, Rephaeli A, Nudelman A, Phillips DR. Sequence specificity of adriamycin-DNA adducts in human tumor cells. *Mol Cancer Ther.* 2003; 2:661–670. [PubMed: 12883039]
30. Strumberg D, Nitiss JL, Dong J, Kohn KW, Pommier Y. Molecular analysis of yeast and human type ii topoisomerases. Enzyme-DNA and drug interactions. *J Biol Chem.* 1999; 274:28246–28255. [PubMed: 10497180]
31. Zhang H, Gao YG, van der Marel GA, van Boom JH, Wang AH. Simultaneous incorporations of two anticancer drugs into DNA. The structures of formaldehyde-cross-linked adducts of daunorubicin-d(CG(arac)gcg) and doxorubicin-d(CA(arac)gtg) complexes at high resolution. *J Biol Chem.* 1993; 268:10095–10101. [PubMed: 8486678]
32. Kuhns MS, Davis MM, Garcia KC. Deconstructing the form and function of the tcr/cd3 complex. *Immunity.* 2006; 24:133–139. [PubMed: 16473826]
33. Larsen AK, Escargueil AE, Skladanowski A. Catalytic topoisomerase ii inhibitors in cancer therapy. *Pharmacol Ther.* 2003; 99:167–181. [PubMed: 12888111]
34. Stiborova M, Sejbál J, Borek-Dohalska L, Aimova D, Poljakova J, Forsterova K, Rupertova M, Wiesner J, Hudecek J, Wiessler M, Frei E. The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes p450, through metabolism to 13-hydroxyellipticine and ellipticine n2-oxide. *Cancer Res.* 2004; 64:8374–8380. [PubMed: 15548707]
35. Krishnan P, Bastow KF. Novel mechanisms of DNA topoisomerase ii inhibition by pyranonaphthoquinone derivatives-eleutherin, alpha lapachone, and beta lapachone. *Biochem Pharmacol.* 2000; 60:1367–1379. [PubMed: 11008131]
36. Li CJ, Averboukh L, Pardee AB. Beta-lapachone, a novel DNA topoisomerase i inhibitor with a mode of action different from camptothecin. *J Biol Chem.* 1993; 268:22463–22468. [PubMed: 8226754]
37. Morschhauser F, Dreyling M, Rohatiner A, Hagemeister F, Bischof Delaloye A. Rationale for consolidation to improve progression-free survival in patients with non-hodgkin's lymphoma: A review of the evidence. *Oncologist.* 2009; 14 Suppl 2:17–29. [PubMed: 19819921]
38. Sebban C, Mounier N, Brousse N, Belanger C, Brice P, Haioun C, Tilly H, Feugier P, Bouabdallah R, Doyen C, Salles G, Coiffier B. Standard chemotherapy with interferon compared with chop followed by high-dose therapy with autologous stem cell transplantation in untreated patients with advanced follicular lymphoma: The gelf-94 randomized study from the groupe d'etude des lymphomes de l'adulte (gela). *Blood.* 2006; 108:2540–2544. [PubMed: 16835383]

39. Tawbi HA, Kirkwood JM. Management of metastatic melanoma. *Semin Oncol.* 2007; 34:532–545. [PubMed: 18083377]
40. Guano F, Pourquier P, Tinelli S, Binaschi M, Bigioni M, Animati F, Manzini S, Zunino F, Kohlhaagen G, Pommier Y, Capranico G. Topoisomerase poisoning activity of novel disaccharide anthracyclines. *Mol Pharmacol.* 1999; 56:77–84. [PubMed: 10385686]
41. Collins I, Weber A, Levens D. Transcriptional consequences of topoisomerase inhibition. *Mol Cell Biol.* 2001; 21:8437–8451. [PubMed: 11713279]
42. Daigeler A, Klein-Hitpass L, Chromik MA, Muller O, Hauser J, Homann HH, Steinau HU, Lehnhardt M. Heterogeneous in vitro effects of doxorubicin on gene expression in primary human liposarcoma cultures. *BMC Cancer.* 2008; 8:313. [PubMed: 18959781]
43. Folgueira MA, Carraro DM, Brentani H, Patrao DF, Barbosa EM, Netto MM, Caldeira JR, Katayama ML, Soares FA, Oliveira CT, Reis LF, Kaiano JH, Camargo LP, Vencio RZ, Snitcovsky IM, Makdissi FB, e Silva PJ, Goes JC, Brentani MM. Gene expression profile associated with response to doxorubicin-based therapy in breast cancer. *Clin Cancer Res.* 2005; 11:7434–7443. [PubMed: 16243817]
44. Reymann S, Borlak J. Topoisomerase ii inhibition involves characteristic chromosomal expression patterns. *BMC Genomics.* 2008; 9:324. [PubMed: 18611269]
45. Zunino F, Capranico G. DNA topoisomerase ii as the primary target of anti-tumor anthracyclines. *Anticancer Drug Des.* 1990; 5:307–317. [PubMed: 1963303]
46. Foglesong PD, Reckord C, Swink S. Doxorubicin inhibits human DNA topoisomerase i. *Cancer Chemother Pharmacol.* 1992; 30:123–125. [PubMed: 1318169]
47. Crow RT, Crothers DM. Inhibition of topoisomerase i by anthracycline antibiotics: Evidence for general inhibition of topoisomerase i by DNA-binding agents. *J Med Chem.* 1994; 37:3191–3194. [PubMed: 7932544]
48. Nitiss JL. DNA topoisomerase ii and its growing repertoire of biological functions. *Nat Rev Cancer.* 2009; 9:327–337. [PubMed: 19377505]
49. Leppard JB, Champoux JJ. Human DNA topoisomerase i: Relaxation, roles, and damage control. *Chromosoma.* 2005; 114:75–85. [PubMed: 15830206]
50. Shah R, El-Deiry WS. P53-dependent activation of a molecular beacon in tumor cells following exposure to doxorubicin chemotherapy. *Cancer Biol Ther.* 2004; 3:871–875. [PubMed: 15254427]
51. Montecucco A, Biamonti G. Cellular response to etoposide treatment. *Cancer Lett.* 2007; 252:9–18. [PubMed: 17166655]
52. Gonzalez PA, Carreno LJ, Coombs D, Mora JE, Palmieri E, Goldstein B, Nathenson SG, Kalergis AM. T cell receptor binding kinetics required for t cell activation depend on the density of cognate ligand on the antigen-presenting cell. *Proc Natl Acad Sci U S A.* 2005; 102:4824–4829. [PubMed: 15772168]
53. Zitvogel L, Apetoh L, Ghiringhelli F, Andre F, Tesniere A, Kroemer G. The anticancer immune response: Indispensable for therapeutic success? *J Clin Invest.* 2008; 118:1991–2001. [PubMed: 18523649]
54. Ramakrishnan R, Assudani D, Nagaraj S, Hunter T, Cho HI, Antonia S, Altiock S, Celis E, Gabrilovich DI. Chemotherapy enhances tumor cell susceptibility to ctl-mediated killing during cancer immunotherapy in mice. *J Clin Invest.* 2010; 120:1111–1124. [PubMed: 20234093]
55. Shurin GV, Tourkova IL, Kaneno R, Shurin MR. Chemotherapeutic agents in noncytotoxic concentrations increase antigen presentation by dendritic cells via an il-12-dependent mechanism. *J Immunol.* 2009; 183:137–144. [PubMed: 19535620]
56. Ewens A, Luo L, Berleth E, Alderfer J, Wollman R, Hafeez BB, Kanter P, Mihich E, Ehrke MJ. Doxorubicin plus interleukin-2 chemoimmunotherapy against breast cancer in mice. *Cancer Res.* 2006; 66:5419–5426. [PubMed: 16707470]
57. Ujhazy P, Zaleskis G, Mihich E, Ehrke MJ, Berleth ES. Doxorubicin induces specific immune functions and cytokine expression in peritoneal cells. *Cancer Immunol Immunother.* 2003; 52:463–472. [PubMed: 12698271]
58. Ferraro C, Quemeneur L, Prigent AF, Taverne C, Revillard JP, Bonnefoy-Berard N. Anthracyclines trigger apoptosis of both g0–g1 and cycling peripheral blood lymphocytes and

- induce massive deletion of mature t and b cells. *Cancer Res.* 2000; 60:1901–1907. [PubMed: 10766178]
59. Zitvogel L, Kroemer G. The dilemma of anticancer therapy: Tumor-specific versus immune effects. *Blood.* 2008; 112:4364–4365. [PubMed: 19029450]
60. Creasey WA, McIntosh LS, Brescia T, Odujinrin O, Aspnes GT, Murray E, Marsh JC. Clinical effects and pharmacokinetics of different dosage schedules of adriamycin. *Cancer Res.* 1976; 36:216–221. [PubMed: 942583]
61. Reich SD, Steinberg F, Bachur NR, Riggs CE Jr, Goebel R, Berman M. Mathematical model for adriamycin (doxorubicin) pharmacokinetics. *Cancer Chemother Pharmacol.* 1979; 3:125–131. [PubMed: 509697]
62. Zebedin E, Simma O, Schuster C, Putz EM, Fajmann S, Warsch W, Eckelhart E, Stoiber D, Weisz E, Schmid JA, Pickl WF, Baumgartner C, Valent P, Piekorz RP, Freissmuth M, Sexl V. Leukemic challenge unmasks a requirement for pi3kdelta in nk cell-mediated tumor surveillance. *Blood.* 2008; 112:4655–4664. [PubMed: 18684865]
63. June CH, Blazar BR, Riley JL. Engineering lymphocyte subsets: Tools, trials and tribulations. *Nat Rev Immunol.* 2009; 9:704–716. [PubMed: 19859065]
64. Ashburn TT, Thor KB. Drug repositioning: Identifying and developing new uses for existing drugs. *Nat Rev Drug Discov.* 2004; 3:673–683. [PubMed: 15286734]

Fig. 1.

Comparison of daunorubicin responses of cells expressing EGFP under the control of the Melan-A/MART-1 promoter, with endogenous Melan-A/MART-1. Top panels; MM96L+ and A375 with Melan-A/MART-1 promoter EGFP reporters with and without daunorubicin as shown. Bottom panels; intracellular antibody staining of endogenous Melan-A/MART-1 expression in the same cell lines with and without daunorubicin.

Fig. 2.
Dose response of EGFP fluorescence induced by daunorubicin in A375 MART::EGFP cells (squares and solid line) and left Y-axis. Toxicity as measured by growth inhibition (circles and dashed line) was assessed by the WST assay (Methods) and graphed using right Y-axis.

Fig. 3.

A. Kinetics of daunorubicin response for antigen expression levels. The A375 MART::EGFP reporter melanoma tumor cells were exposed to various amounts of daunorubicin for various periods of time. **B.** Effects of transient daunorubicin treatments on Melan-A/MART-1 expression. The A375 MART::EGFP reporter cell line was exposed to various concentrations of daunorubicin for the indicated times, after which cells were provided with drug-free media. The level of EGFP was determined 72 hr after daunorubicin exposure. Note the x-axis is discontinuous, as noted by breaks in the x-axis and graphs, and the first data point represents the untreated control level of EGFP indicated by zero. The geometric mean of flow histograms is plotted.

Fig. 4.

A. Surface staining of T cells transduced with Melan-A/MART-1 specific TCR. TCR-negative Jurkats Panels (i) shows TCR-negative Jurkats stained with antibody to CD3. Panel (ii) shows TCR-negative Jurkats stained with Melan-A/MART-1 tetramer. Panel (iii) shows staining of primary CD8 T cells with Melan-A/MART-1 tetramer. **B.** Cytokine induction by T cells exposed to melanoma tumors. (i) Melan-A/MART-1 TCR-transduced TCR minus Jurkat T cells (J.RT3) and IL-2, the average of three replicates is shown with standard deviations indicated by the error bars. (ii). Control untransfected primary CD8 T cells and primary CD8 T cells transduced with Melan-A/MART-1 TCR were co-cultured with the

melanoma cell line MU89 for 24 hours and the IFN-gamma in the culture media was measured.

Fig. 5.

Effective concentration ranges of topoisomerase inhibitors for achieving doubling of Melan-A/MART-1 reporter activity (Methods) under the same conditions. These windows of activity (black bands) cover active concentrations spanning lower limits (loss of activity by dilution), up to maximal practical levels determined by toxicity, where all are assessed under the same exposure times in culture (72 hour assay). By these criteria, sets of inhibitors were grouped through similarities in their activity patterns as high, medium, or low potency; or as having no significant effect. Inhibitors are categorized according to their major target (topoisomerase I or II), and as poisons or catalytic inhibitors in each case. Adjacent sets of inhibitors in gray shading are structurally related.

Table 1

Effects of daunorubicin on expression of melanocytic antigens in melanomas and gliomas.

Cell Line	Antigen Baseline	Test Antigen	Geometric Mean ^a	
			Control	Daunorubicin ^b
MU89	high	Mart-1	66.1	155.9
		Hmb45	82.1	189.2
MUX	low	Mart-1	9.9	18.7
		Hmb45	15.7	38.9
MM96L+	high	Mart-1	124.0	283.9
		Hmb45	165.9	298.0
MM96L-	low	Mart-1	10.7	26.6
		Hmb45	18.8	70.2
A375	low	Mart-1	6.5	18.3
		Hmb45	14.8	45.9
MO	high	Mart-1	42.8	153.2
		Hmb45	55.9	240.3
136.2	high	Mart-1	76.8	123.7
		Hmb45	136.5	219.2
H59-44T	low	Mart-1	4.7	12.4
		Hmb45	9.3	30.8
Glioma-87	low	Mart-1	6.3	15.3
		Hmb45	13.2	33.2
Glioma-118	low	Mart-1	10.6	16.6
		Hmb45	22.3	42.5

^aGeometric mean refers to fluorescence determinations by means of flow analysis.^bAll determinations were performed after a 3 day incubation of the tumor cells with daunorubicin at 100 nM.