Selective Targeting of Tumorigenic Cancer Cell Lines by Microtubule Inhibitors

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Abstract

For anticancer drug therapy, it is critical to kill those cells with highest tumorigenic potential, even when they comprise a relatively small fraction of the overall tumor cell population. We have used the established NCI/DTP 60 cell line growth inhibition assay as a platform for exploring the relationship between chemical structure and growth inhibition in both tumorigenic and non-tumorigenic cancer cell lines. Using experimental measurements of “take rate” in ectopic implants as a proxy for tumorigenic potential, we identified eight chemical agents that appear to strongly and selectively inhibit the growth of the most tumorigenic cell lines. Biochemical assay data and structure-activity relationships indicate that these compounds act by inhibiting tubulin polymerization. Yet, their activity against tumorigenic cell lines is more selective than that of the other microtubule inhibitors in clinical use. Biochemical differences in the tubulin subunits that make up microtubules, or differences in the function of microtubules in mitotic spindle assembly or cell division may be associated with the selectivity of these compounds.

Introduction

The aggressiveness of different kinds of tumor cells derived from human patients can be assessed in terms of their tumorigenic potential in mouse xenograft models. For example, tumorigenic potential in mouse xenografts has recently been used to define the cancer “stem cells”, which presumably correspond to the subpopulation of malignant cells that drive the formation and growth of the tumor [1]. Accordingly, it has been postulated that some cancers are composed of a heterogeneous collection of cells, only a minority of which are capable of forming new tumors [2]. These cells can be enriched from heterogenous tumor cell populations on the basis of their expression of cell-surface markers. In breast tumors, for example, cells co-expressing high levels of CD44 and epithelial specific antigen (ESA) and low levels of CD24 are the tumor initiating cells [2]. Likewise, in colon and brain cancer, subpopulations of cells expressing high levels of CD133 (PROML1) initiate the tumors [3,4]. Most importantly, upon transplantation into immunocompromised mice, tumor-initiating cells can fully reconstitute a tumor with heterogeneity reminiscent of the original tumor [2-4]. Although the concept of a cancer “stem cell” is still controversial, from a therapeutic standpoint, anticancer agents directed against tumorigenic cancer cells may be the most effective at eradicating tumors.

The drug discovery and development sector of National Cancer Institute (NCI), the Developmental Therapeutics Program (DTP), has utilized a panel of 60 human tumor-derived cell lines to screen the chemotherapeutic potential of more than 75000 compounds [5,6]. This panel of 60 cell lines is commonly known as “NCI60 cell lines.” The cell lines represent various leukemias, melanomas and cancers of the lung, colon, brain, ovary, breast, prostate and kidney [5]. Apart from their use in drug screening, the tumorigenic potential of these cell lines has been measured by xenotransplanting these cells into immunocompromised mice and assessing their ability to form new tumors [6]. Different cell lines in the NCI60 panel display a range of tumorigenic potentials upon transplantation into immunocompromised mice. The tumorigenic potential has been recorded as each cell line’s “take-rate.”

As a hypothesis, differences in tumorigenic potential among the NCI cancer cell lines may reflect variations in proliferative activity and tumor-initiating characteristics of the actual cancer cells as they exist in the tumors of cancer patients. Thus, NCI60 cell lines demonstrating high take rate may be more representative of tumor-initiating cancer cells found in situ. Here, we identify compounds from the DTP database that are most active against cell lines with the highest take rate, and proceed to establish a putative mechanism of action for these compounds by performing structure-activity relationship studies, and comparing them to standard anticancer agents whose mechanism of action is known. In addition, differences in tumorigenic potential and responsiveness to these agents are shown to be related to differences in gene expression between NCI60 cell lines with high and low tumorigenic potentials, as well as to gene expression markers of tumorigenic cancer cells.

Results

Identification of selectively cytotoxic compounds

Growth inhibitory activity in the DTP collection of chemical agents as represented by $-\log GI50$ can be compared to the four
categories of take-rate using Pearson correlation coefficients. Using this approach, nine compounds having correlation coefficient greater than 0.5 in magnitude were identified out of 34,909 compounds tested (Figure 1). All nine correlation coefficients were positive, indicating that these agents were more active at inhibiting cell growth in the most tumorigenic cell lines (Figure 2). Because the expected number of compounds out of 34,909 having a correlation coefficient exceeding 0.5 in magnitude by chance is 0.7 with a 95th percentile of two compounds, it is very unlikely that two or more of these nine compounds are false positives.

None of the standard anticancer agents in the DTP database surpass these nine compounds in terms of selective cytotoxic activity against the most tumorigenic cell lines. The greatest correlation coefficient observed among the standard anticancer agents is 0.47 for vinblastine, which is an antimitotic agent. In fact, antimitotic agents are the only mechanistic class showing consistent non-negligible positive correlation with take-rate. Despite their positive correlation coefficients, none of the antimitotic standard anticancer agents show correlation coefficient greater than 0.5, suggesting that the nine compounds identified in our correlation analysis may be uniquely selective against the most tumorigenic cell lines. Several of these nine compounds exhibit a wide selectivity window with difference in $\log_{10}$GI50 between tumorigenic and non-tumorigenic cell lines of two or more. Compounds 384634, 385177, 5468780, 361500 and 379512 are comparable to all of the standard antimitotic agents in regards to their cytotoxicity; however, their selectivity window is much wider (Figure 3).

Inhibition of tubulin polymerization as possible mechanism of action

The compounds identified point to a major structure-activity relationship class: four of the compounds identified share a core naphthyridin structure (see Figure 1). Three of these compounds (385177, 5468780, 5468781) are structurally related, through the presence of a naphthelene group at position R2. These structures differ from each other based only on the positioning of one or two methyl group on the A ring: compounds 385177 and 5468780 contain a methyl group at positions R3 and R2, respectively, while compound 5468781 contains two methyl groups at positions R3 and R2. The other compound (384634) differs from the three previously mentioned compounds because the group 3'-methoxy substituted benzene ring substitutes the naphthalene group at position R2. This compound also contains a methyl group at position R3 on ring A. The presence of the core structure common to all the compounds in this group suggests that it may play a cornerstone role in the mechanism of action for this cohort of compounds.

In order to identify a possible mechanism of action, the nine compounds were clustered together with the 168 standard anticancer agents using the 881 key CACTVS fingerprints. Cutting the dendrogram at a Tanimoto coefficient of 0.7, five of the nine compounds are clustered with nine standard anticancer agents including various antitubulin agents such as vinblastine and vincristine. Subsequent analysis of the scientific literature revealed that many of our compounds do indeed inhibit polymerization of tubulin in vitro. Compound 384634 has been synthesized and has
shown to demonstrate antitubulin activity in a tubulin polymerization assay [11]. Likewise, isosteres of compound 385177, 5468780 and 5468781 potently inhibit tubulin polymerization [12]. It is highly plausible that compound 379512 is an antitubulin agent as well, because a number of compounds containing the 2-phenylquinolone ring structure have been synthesized and exhibit tubulin polymerization [13–17]. Compound 5388755 is almost structurally identical to Combretastatin A-4, which is a very potent antitubulin agent [18].

COMPARE analysis [19] was performed to further characterize the mechanism of action of the compounds. In COMPARE, a correlation coefficient of 0.6 is generally taken to indicate evidence for similar mechanisms of action between the tested and reference compounds. The higher the correlation coefficient, the more likely it is that the compounds share the same intracellular target [9]. The correlation coefficient of the COMPARE computations for the eight most potent compounds and the antimitotic standard anticancer agents reveals several compounds showing high correlations with microtubule inhibitors colchicine, maytansine, vinblastine and vincristine (Table 1). None of these compounds show similarity to any of the agents from other mechanistic classes such as topoisomerase inhibitors, alkylating agents and DNA/
RNA antimetabolites (data not shown). None of the compounds exhibit strong correlation with taxol, which is an antimitotic agent that acts by stabilizing microtubules.

**Antitubulin activity parallels selective cytotoxicity**

In order to identify the role of antitubulin activity in generating selective cytotoxicity, we identified twelve additional DTP compounds (Figure 4) that are structurally related to some of the nine compounds we identified in our correlation analysis but that lack antitubulin activity [11–15]. If antitubulin activity confers selective cytotoxicity, these compounds with no antitubulin activity should demonstrate no selective cytotoxicity. The scatterplot comparing the association between cytotoxicity and take-rate for these twelve compounds indicates that none of these compounds show selective cytotoxicity (Figure 5), and they are largely inactive in the cell growth inhibition assay.

**Gene expression analysis**

A number of previous research studies have identified CD44, CD24, and CD133 (PROML1) as being markers for tumorigenic potential or stem-cell-like characteristics, with CD44 and CD133 being relatively highly expressed in tumorigenic lines, and CD24 being expressed at low levels. Thus, we searched for specific genes whose expression may be related to the selective cytotoxic activity of the compounds identified. For this purpose, transcriptional profiling data was mined for genes whose expression across the cell lines correlates with tumorigenic potential. In this data set, we found that take rate is independent of PROML1, CD44, and the

**Table 1. COMPARE analysis of eight compounds to various agents from the antimitotic activity class.**

| Seed     | Colchicine | Maytansine | Rhizoxin | Taxol | Vinblastine | Vincristine |
|----------|------------|------------|----------|-------|-------------|-------------|
| 384634   | 0.69       | 0.73       | 0.51     | 0.36  | 0.85        | 0.70        |
| 385177   | 0.50       | 0.42       | 0.41     | 0.32  | 0.51        | 0.36        |
| 5468780  | 0.60       | 0.62       | 0.22     | 0.32  | 0.63        | 0.58        |
| 5468781  | 0.55       | 0.59       | 0.31     | 0.38  | 0.59        | 0.46        |
| 319428   | 0.27       | 0.37       | 0.24     | 0.27  | 0.53        | 0.35        |
| 361500   | 0.75       | 0.72       | 0.58     | 0.34  | 0.77        | 0.56        |
| 5388755  | 0.67       | 0.70       | 0.60     | 0.44  | 0.63        | 0.60        |
| 379512   | 0.41       | 0.43       | 0.32     | 0.28  | 0.29        | 0.65        |

![Figure 3. Selectivity windows for eight compounds identified in our virtual screen (G–N) and several standard anticancer agents having antimicrotubule activity (A–F).](doi:10.1371/journal.pone.0004470.g003)

doi:10.1371/journal.pone.0004470.t001
log ratio CD44-CD24 in the NCI60 cell lines. We also analyzed the expression of twenty or so different tubulin isotypes (alpha, beta, and gamma) and found no correlation with take rate.

Although candidate tumorigenicity marker genes PROM1, CD44, and CD44-CD24 were not associated with take-rate, we did identify genes expressed at substantially higher levels in the more tumorigenic cell lines. On the U95A array platform, a transcription factor (DBP), an integrin (ITGA6, two probe sets), and a membrane skeletal protein (ADD3, two probe sets) followed this pattern of expression. Six named genes and two unnamed genes are expressed at substantially higher levels in the less tumorigenic as compared to the more tumorigenic cell lines on the U95A array platform: PTGIS, JAK1, MGC5560, XPC, NRG1, and SULF1. On the U133A/B platform TMEM18, ACACB, and GMCL1 were positively associated with tumorigenic potential, along with two unnamed probesets (229930_at and 230312_at). No negative associations meeting our selection criteria were identified on the U133A/B array platform. The functional significance of putative stem cell marker genes in relation to tumorigenesis or increased sensitivity to microtubule inhibitors is not clear.

Discussion

By data mining the DTP archive, we are able to identify compounds that are preferentially toxic against the most tumorigenic of the NCI60 cell lines, based on the take rate of the cell lines in a mouse xenograft model. We also established that the activity of these compounds was not correlated to the expression of cell surface stem cell markers reported in the literature. Nevertheless, tumorigenic potential is the most important functional relationship between the most aggressive tumor cells and in vitro model for drug screening. Therefore, the anticancer agents identified based on their activity against the most tumorigenic cell lines may be considered as candidate anticancer agents that are specifically directed against subpopulations of cancer cells that drive the growth of tumors.

One of these agents (384634) has been found to inhibit microtubule polymerization. Likewise, isosteres of three of our agents (385177, 5468780, 5468781) have also been shown to inhibit microtubule polymerization, suggesting a single mechanism of action. Interestingly, Compound 5388755 is structurally related to the potent antitubulin agent Combretastatin A-4. It is also possible that compound 379512 acts by inhibiting tubulin polymerization because several different agents containing the quinolone ring structure have demonstrated antitubulin activity. COMPARE analysis corroborates the similarities between the anticancer agents identified here and various different microtubule inhibitors. With the exception of compound 319428, all of our compounds show strong similarity with colchicine, maytansine, vinblastine and vincristine. None of our compounds show significant relationship to taxol, which acts by stabilizing microtubules.

From our analysis, antitubulin activity is likely to be responsible for selective cytotoxicity against tumorigenic cell lines. A select number of structurally related compounds with no antitubulin activity were analyzed for their pattern of cytotoxicity toward NCI60 cell lines. None of these compounds demonstrated selective toxicity.
cytotoxicity. In fact, most of these compounds were inactive. Together with their antitubulin activity, the selectivity of our compounds toward highly tumorigenic cell lines suggests that microtubules of tumorigenic and non-tumorigenic cell lines may differ. Interestingly, no difference in tubulin gene expression level was observed between highly tumorigenic and non-tumorigenic cell lines. It is plausible that observed selective cytotoxicity is not due to difference in tubulin gene expression but rather a result of differences in post-translational modifications (PTMs) [20]. Recently, various experimental results have supported the notion that tubulin PTMs lead to the functional diversity of microtubules. Many tubulin PTMs have been identified including deetyrosination, glutamylation, glycylation, acetylation phosphorylation and palmitoylation [20–22]. Differences in tubulin isotype expression and PTMs have been associated with cell differentiation and developmental transitions [23–25]. Because microtubules are key to mitotic spindle assembly and cell division, differences in mitotic spindle structure and function between tumorigenic and non-tumorigenic cell lines may be associated with the selectivity of these compounds.

In conclusion, we have identified a family of microtubule inhibitors that are mostly toxic against tumorigenic cell lines.
Established cancer cell lines demonstrating high tumorigenicity in xenograft models may capture some properties of cancer cell subpopulations that are responsible for initiating and spreading the tumors. Therefore, we propose that this family of microtubule inhibitors, or related compounds with similar selectivity characteristics, should be considered as prime candidates for further evaluation as anticancer agents.

Materials and Methods

Primary data
The compound growth inhibition data was obtained from the NCI 60 cell line antitumor screen. The growth inhibitory activity of each compound corresponds to the molar drug concentration required to cause 50% growth inhibition (GI50). Most assays use a maximum concentration of 0.0001 M (the cell line screen and GI50 parameter are described in [7]). For microarray gene expression analysis, we used five publicly-available data sets for the NCI60 cell lines: triplicate experiments using the Affymetrix U95A platform provided by Novartis, a single U95A data set provided by Gene Logic, and a single Affymetrix U133A/B data set provided by Gene Logic. The GI50 data were obtained from http://dtp.nci.nih.gov/docs/cancer/cancer_data.html and all gene expression data were obtained from http://dtp.nci.nih.gov/mtargets/download.html. All gene expression and GI50 assay data are analyzed on the log scale.

Rating of tumorigenic potential
The NCI60 cell lines have been experimentally evaluated for tumorigenic potential by transplantation of the cell lines into immuno-compromised mice. The experiments and results are provided in the Anticancer Drug Development Guide [6]. For different cell lines, these data are given either quantitatively or qualitatively, or sometimes as ranges, as the “take rate,” or proportion of attempted implants that yielded a tumor. We converted the take rate data into four ordered categories for analysis: 0 for no growth, 1 for 1–60% take rate, 2 for 60–80% take rate and 3 for 80–100% take rate. Cell lines that overlap two categories are rated at the lower category. For instance, a cell line with 70–90% take rate is rated as category 3. These ratings of tumorigenic potential are denoted “TP.”

Compound selection
Compounds active against high take-rate cell lines were identified by comparing the growth inhibition measurement (−log GI50) to the four-level rating of take-rate, using Pearson correlation. Thresholds of 0.4 and 0.5 were used to define moderate and strong correlations. Statistical significance was assessed by calculating the expected number of compounds out of all compounds tested that would be expected to have a correlation exceeding a given threshold by chance (based on applying Fisher’s Z-transformation and using a standard normal reference distribution).

Gene expression analysis
Compounds active against cell lines that express relatively high levels of PROM1 or CD44-CD24 were identified using Pearson correlation coefficients between −log GI50 and either log scale expression of PROM1, or the difference between log scale expression levels of CD44 and CD24. PROM1 is represented by a unique probeset on both platforms, CD44 is represented by two U95A probesets and by six U133A/B probesets, and CD24 is represented by one U95A probeset and by six U133A/B probesets. When multiple probesets are available, all are analyzed separately, and differences among all pairs of CD44/CD24 probesets are analyzed separately. For all analyses, compounds for which fewer than 50 cell lines had a GI50 value, or which had no variability in their GI50 values, were excluded from our analysis.

Standard anticancer agents
A set of 168 compounds with anticancer activity was compiled, and a subset of 121 of them was annotated according to their presumed mechanism of action [8–10]. The data we used were obtained from http://dtp.nci.nih.gov/docs/cancer/searches/standard_mechanism_list.html, and include the following mechanism of action classes and numbers of unique structures: alkylating agents (35), antimetabolites (13), topoisomerase 1 inhibitors (24), topoisomerase II inhibitors (15), DNA anti-metabolites (16), and RNA/DNA anti-metabolites (18).

Chemical structure comparisons
All compounds discussed here are part of PubChem, and all reported structural comparisons are based on Tanimoto coefficients using the 881 key CACTVS fingerprints. Calculations of Tanimoto coefficients and hierarchical clustering of chemical structures based on Tanimoto coefficients was done using the NCBI portal to PubChem (http://pubchem.ncbi.nlm.nih.gov). We converted all compound identifiers from the DTP’s NSC identifier to PubChem’s CID identifier for structural analysis.

COMPARE analysis
COMPARE computations for all of the potent compounds against standard anticancer agents from various mechanistic classes are performed. Pearson correlation coefficients corresponding to high concentration of 0.0001 M are reported for a majority of the compounds. For compounds that are tested with an alternative high concentration, the Pearson correlation coefficients are obtained from pairs with the closest high concentration.

Selectivity window
The selectivity window was calculated by taking the difference between the average −log GI50 of the most tumorigenic cell lines (take-rate category 3) and the least tumorigenic cell lines (take-rate category 0).

Author Contributions
Conceived and designed the experiments: GR. Analyzed the data: NMA. Wrote the paper: GR KS.

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