Screening a panel of drugs with diverse mechanisms of action yields potential therapeutic agents against neuroblastoma

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Abbreviations: NB, neuroblastoma; RT-CES, real-time cell electronic sensing; EAD, 1-[[3'-(Diethylamino)-2'-hydroxypropyl]amino]-4-[(2''', 3'''-epoxypropyl)-amino-9,10-anthracenedione fumaric acid

Neuroblastoma (NB) is the most common extracranial solid tumor in children. Despite current aggressive therapy, the survival rate for high risk NB remains less than 40%. To identify novel effective chemo-agents against NB, we screened a panel of 96 drugs against two NB cell lines, SK-N-AS and SH-SY5Y. We found 30 compounds that were active against NB cell lines at ≤10 μM concentration. More interestingly, 17 compounds are active at ≤1 μM concentration, and they act through a wide spectrum of diverse mechanisms such as mitotic inhibition, topoisomerase inhibition, targeting various biological pathways, and unknown mechanisms. The majority of these active compounds also induced caspase 3/7 by more than 2-fold. Of these 17 active compounds against NB cell lines at sub-micromolar concentration, eleven compounds are not currently used to treat NB. Among them, nine are FDA approved compounds, and three agents are undergoing clinical trials for various malignancies. Furthermore, we identified four agents active against these NB cell lines that have not yet been tested in the clinical setting. Finally we demonstrated that Cucurbitacin I inhibits neuroblastoma cell growth through inhibition of STAT3 pathway. These drugs thus represent potential novel therapeutic agents for patients with NB, and further validation studies are needed to translate them to the clinic.

Introduction

Neuroblastoma (NB), which accounts for about 8–10% of all pediatric malignancies, is the most common extracranial solid tumor among children. The clinical outcome of NB patients ranges from spontaneous regression to aggressive metastasis depending on various clinical factors such as age of diagnosis, disease stage and MYCN amplification status. Chromosomal aberration also predicts the outcome of NB patients. Favorable tumors are characterized by near-triploid karyotypes with whole chromosome gains. Unfavorable tumors are characterized by structural changes, including deletions of 1p or 11q, unbalanced gain of 17q and/or amplification of the MYCN protooncogene. The cancer genes (e.g., TP53, CDKN2A and RAS) most commonly altered in adult carcinogenesis is rarely aberrant in neuroblastoma. TP53 inactivating mutations are rare in primary neuroblastomas. Identification of homozygous deletion of CDKN2A has been shown in NB cell lines, but there is no consistent evidence in primary tumors. Despite compelling evidence for MYC and RAS cooperation in tumorigenesis, activation of RAS signal transduction does not seem to constitute a preferred secondary pathway in neuroblastomas with MYCN deregulation. Thus, major oncogenic pathways governing human neoplasia do not seem to be deregulated in neuroblastoma with the exception of MYCN in a subset of neuroblastomas. However, improved understanding of neuroblastoma biology might help us to identify the key pathways for development of neuroblastoma therapeutics.

Patients with NB are currently stratified by the Children’s Oncology Group as low, intermediate or high risk. At present, patients with high risk NB are treated with etoposide, doxorubicin, cisplatin, carboplatin, vincristine, topotecan, cyclophosphamide, 13-cis-retinoic acid, anti-GD2 immunotherapy, surgery, radiation and high dose chemotherapy with stem cell rescue. Despite aggressive therapy patients with high-risk disease have less than 40% chance of survival. A high proportion of patients who receive standard agents develop therapy-induced complications including high tone deafness, renal dysfunction, infections...
and a risk of a secondary malignancy. Thus, there is a need to develop novel therapies that are more effective and less toxic. We, therefore, have conducted a drug screen on two different NB cell lines to identify new therapeutic agents for patients with NB.

Results

We conducted a screen using 96 compounds, of which half have been FDA approved for clinical use. The compounds included in our drug screen are known to have diverse mechanisms of action—eight antimetabolites, 12 DNA intercalating agents, 12 topoisomerase inhibitors, five mitotic inhibitors, 44 agents affecting various additional biological pathways, and 15 compounds with unknown mechanisms of action (Suppl. file 1).

Drug screen. In the primary screen, we tested each of the 96 compounds at high (≤10 μM) and low (≤1 μM) concentrations on the SK-N-AS, a cell line derived from a stage 4 neuroblastoma tumor. We considered a compound to be effective if it inhibited cell growth by at least 40% after 72 h of drug treatment at either the high or the low concentrations. Based on this criterion, there are 33 effective compounds at the high concentration (Fig. 1A) and 18 effective compounds at the low concentration (Fig. 1B).

We conducted a secondary screen in which all of the positive hits from the first screen were tested against a different NB cell line SH-SY5Y. In general, SH-SY5Y was more sensitive than SK-N-AS cell line for the most of the agents tested in this study. We were able to confirm the efficacy of all the compounds except dequalinium, valinomycin, and rapamycin at the high concentration (Fig. 2A) and rapamycin at the low concentration (Fig. 2B). Thus, these four compounds were eliminated from further analysis.

At the conclusion of these screens, we identified 30 active compounds in both NB cell lines at ≤10 μM. Of these 30 compounds, 15 are FDA approved; five are currently being used as standard therapy for NB; 14 are under clinical trials for treating different pediatric malignancies including 8 for NB treatment;...
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Daunorubicin caused a greater than 20-fold induction of caspase 3/7 activity (Fig. 3A), while bortezomib, camptothecin, and CDDO-Me caused a greater than 20-fold upregulation of caspase 3/7 activity at the low concentration (Fig. 3B). The majority of compounds stimulated apoptosis more by more than 2-fold at either concentration. A few agents, such as helenalin, perezone, CDDO-Me, arsenic trioxide, PD 0332991, & amonafide at the high concentrations and topotecan & Epoxy anthraquinone derivative (EAD) at the lower concentrations, were considered active on SK-N-AS and SH-SY5Y by the cell viability assay but induced caspase 3/7 activity at a lesser extend (<2-fold) (Fig. 3).

Monitoring growth inhibition profiles in real time. Eight compounds (six at the high and two at the low concentration) showed discordant results where the reduced cell number was evident by cell titer blue but marginal presence or absence of activated caspase 3/7. To measure pro-apoptotic activity of these compounds in NB cell lines, we conducted a Caspase-Glo 3/7® assay on the SK-N-AS cell line. At the high concentration, bortezomib, doxorubicin and daunorubicin caused a greater than 20-fold induction of caspase 3/7 activity (Fig. 3A), while bortezomib, camptothecin, and CDDO-Me caused a greater than 20-fold upregulation of caspase 3/7 activity at the low concentration (Fig. 3B). The majority of compounds stimulated apoptosis more by more than 2-fold at either concentration. A few agents, such as helenalin, perezone, CDDO-Me, arsenic trioxide, PD 0332991, & amonafide at the high concentrations and topotecan & Epoxy anthraquinone derivative (EAD) at the lower concentrations, were considered active on SK-N-AS and SH-SY5Y by the cell viability assay but induced caspase 3/7 activity at a lesser extend (<2-fold) (Fig. 3).

Figure 2. Validating the positive hits from the secondary screen on SH-SY5Y. In the tertiary screen, the 5,000 SH-SY5Y cells were seeded in 96-well plates. The cells were treated with 33 agents at the higher concentration and 18 agents at the lower concentration identified to be active against NB cell lines from the secondary screen, and the cell viability was measured 72 h after the drug treatment. Compounds inhibiting growth by at least 40% were considered effective as indicated by a dashed line on the bar graphs. (A) Percentage of the live SH-SY5Y cells 72 h after the treatment with 33 agents at the high concentration; (B) Percentage of the live SH-SY5Y cells 72 h after the treatment with 18 compounds at the low concentration. The data shown is a mean representative of the three independent measurements.

and 18 are under clinical investigation for the treatment of various adult cancers (Table 1). The majority of the active compounds against NB cell lines were mitotic and topoisomerase inhibitors (Table 1).

Seventeen compounds demonstrated growth inhibitory activities on both NB cell lines at ≤1 μM concentration. Among these 17 agents, there are nine FDA approved compounds. Four compounds are currently used as standard agents for NB therapy, and six drugs are going through clinical trials for NB. Ten of them are going under clinical trials for different pediatric malignancies, and eleven compounds are going under clinical investigation for various adult cancers (Table 1).

Induction of apoptosis. The cause for reduced cell number upon treatment with drug can be due to reduction in cell cycle and induction of cell death or a combination of both. To measure pro-apoptotic activity of these compounds in NB cell lines, we conducted a Caspase-Glo 3/7® assay on the SK-N-AS cell line. At the high concentration, bortezomib, doxorubicin and daunorubicin caused a greater than 20-fold induction of caspase 3/7 activity (Fig. 3A), while bortezomib, camptothecin, and CDDO-Me caused a greater than 20-fold upregulation of caspase 3/7 activity at the low concentration (Fig. 3B). The majority of compounds stimulated apoptosis more by more than 2-fold at either concentration. A few agents, such as helenalin, perezone, CDDO-Me, arsenic trioxide, PD 0332991, & amonafide at the high concentrations and topotecan & Epoxy anthraquinone derivative (EAD) at the lower concentrations, were considered active on SK-N-AS and SH-SY5Y by the cell viability assay but induced caspase 3/7 activity at a lesser extend (<2-fold) (Fig. 3).

Monitoring growth inhibition profiles in real time. Eight compounds (six at the high and two at the low concentration) showed discordant results where the reduced cell number was evident by cell titer blue but marginal presence or absence of activated caspase 3/7. In order to investigate the cause of these discordant results, we therefore used RT-CES method to continuously monitor the cell growth profiles for 72 h following the addition of the...
number within a few hours after the drug addition. Therefore, there probably hardly any viable cells left for the caspase 3/7 assay at 24 h after drug addition (Fig. 4). Arsenic trioxide, amonafide, 30 drugs active against NB cell lines at the high and low concentrations (Suppl. file 2). We observed that helenalin, perezone, and CDDO-Me at the high concentration caused rapid decline in cell number within a few hours after the drug addition. Therefore, there probably hardly any viable cells left for the caspase 3/7 assay at 24 h after drug addition (Fig. 4). Arsenic trioxide, amonafide,
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We next examined if this drug also suppressed cell growth by inhibiting STAT3 activation in neuroblastoma cells. As shown in Figure 5C, Cucurbitacin I reduced STAT3 total protein expression level in SK-N-AS, NBEB and SH-SY5Y cells. More importantly, Cucurbitacin I also inhibited phosphorylation of STAT3 in dose-dependent manner in all four cell lines (SK-N-AS, NBEB, SH-SY5Y and IMR5) (Fig. 5C and D). These results confirmed that Cucurbitacin I inhibited NB cell growth through inhibition of STAT3 activation.

Discussion

Neuroblastoma remains incurable in >60% of patients who present with advanced stage disease. To find novel therapeutic...
Figure 4. Utilizing real time—cell electronic sensing to investigate discordance between the cell viability and the caspase assay measurement. To monitor the effect of the agents active against NB cell lines in real-time, the 5,000 SK-N-AS cells were seeded in 96-well microtiter E-plates. The cells were treated with 30 and 17 agents at higher and lower concentration, respectively, 24 h after the seeding as indicated by the black arrow on the graphs. Following the drug treatment, the cell growth was monitored for an additional 72 h. Cell index, which is proportional to cell number, was induced by (A) Helenalin at 10 μM, (B) Perezone 10 μM, (C) CDDO-Me at 10 μM, (D) Epoxy anthraquinone derivative at 500 nM, (E) Arsenic trioxide at 10 μM, (F) Amonafide at 10 μM, (G) PD 0332991 at 10 μM, and (H) Topotecan at 1 μM on the SK-N-AS cell. The data shown is a mean representative of the three independent measurements.
agents for NB treatment, we conducted a drug screen of compounds with diverse mechanisms of action on two different NB cell lines, SK-N-AS, and SH-SY5Y, both derived from stage 4 tumors. These cell lines are MYCN non-amplified—representative of about 75% of NB patients. NB is a phenotypically heterogeneous tumor, displaying cells of neuronal, melanocytic, or glial/schwannian lineage. This cellular heterogeneity is present in vitro as well, where cells of neuroblastic (N) or stromal (S) type are identified. It has been hypothesized that the sensitivity to drugs may be, at least partially, dependent on the different cell phenotype. Thus in this study we examined the responses of two different types of cell lines (SK-N-AS, S-type and SH-SY5Y, N-type). It seems that N-type cells (SH-SY5Y) are more sensitive to drugs than S-type cells (SK-N-AS) from our current study and also a previous study.

To identify compounds active against NB cell lines, we used a cell viability assay, a caspase 3/7 assay and RT-CES measurements. For the majority of the agents, the results for all three assays were in agreement; however, we observed discordance for a few agents. According to the cell viability assay and RT-CES, helenalin, perpezone and CDDO-Me at the high concentrations were considered highly active against these NB cell lines, but did not stimulate significant caspase 3/7 activity. These incongruous results could be attributed to the fact that these drugs cause a rapid decline in cell number within a few hours after treatment, as detected by RT-CES (Fig. 4A–C), leaving few viable cells remaining from which to measure caspase activity. Furthermore, arsenic trioxide and amonafide at the higher concentration and EAD and topotecan at the lower concentration produced a less degree of induction (<2 fold). In consistence with this observation, the RT-CES data showed that these drugs had gradual activities against NB cells (Fig. 4D–F and H). PD 0332991 is designed to induce cell cycle arrest by CDK 4/6 inhibition, and we observed a less degree of apoptosis (<2 fold) (Fig. 3). Its effectiveness against these NB lines was evident in the cell viability assay as well as the growth inhibition profile obtained from RT-CES illustrated a gradual decline in the cell number (Fig. 4G).

We found that 17 out of the 30 agents were also active against SK-N-AS and SH-SY5Y at lower concentrations (≤1 μM) and is readily achievable in patient serum under physiological condition. Nine of seventeen compounds active against NB cell lines are FDA approved and six are currently under clinical investigation. Among these compounds, one was a DNA intercalating agent, five topoisomerase inhibitors, three mitotic inhibitors, five compounds targeting various biological pathways such as inhibition of Hsp90, proteasome, STAT3, NFκB and AKT and three agents...
Among the agents currently used for NB treatment, doxorubicin, topotecan and vincristine were active against NB cell lines at both the high and the low concentrations. Etoposide was only active in our assay at the high concentration while cisplatin, cyclophosphamide and ifosphamide did not show activity against the two NB cell lines in our assays. Cyclophosphamide and ifosphamide are pro-drugs that require metabolic activation in liver; hence, they should not show activity in our in vitro assay. Cisplatin caused approximately 20% reduction of cell number in SK-N-AS and 97% reduction in SH-SY5Y (data not shown).

In addition, our screen identified bortezomib and one of its structural congeners are more active than those currently used in the standard NB therapies. Bortezomib, a synthetic tripeptide, is a selective proteasome inhibitor currently used in treatment of relapsed multiple myeloma. It has shown to be effective against many malignancies such as lung, breast, pancreatic cancers, glioblastoma multiforme and neuroblastoma in pre-clinical studies; as well as in the murine NB model. Bortezomib and its congener were effective at reducing cell number by greater than 90% at 72 h after drug treatment (Fig. 1). Furthermore, bortezomib and its structural congener were able to induce apoptosis by greater than 15-fold (Fig. 3). These results, along with other pre-clinical and clinical data, suggest that proteasome inhibition represents a novel therapeutic target for NB treatment.

Among the active compounds, CDDO-Me, helenalin and cucurbitacin I belong to a class of compounds known as terpenoids that contain isoprene units. CDDO-Me is a synthetic triterpenoid that has shown efficacy over a wide range of malignancies such as prostate, ovarian and lung cancers, and neuroblastoma. Alabran et al. reported various NB cell lines sensitive to CDDO-Me at concentrations less than 150 nM. Various molecular pathways like p53, STAT and NFκB are linked with the viability, metastasis and chemoresistance in NB. CDDO-Me, which targets multiple molecular pathways like Akt, mTOR, JNK, NFκB, STAT and Notch, may prove to be more effective against NB than targeting a single pathway.

N'-Allynamino-17-demethoxygeldanamycin (17-AAG) showed 70% growth inhibition in both NB cell lines. 17-AAG inhibits the molecular chaperone heat shock protein 90 (Hsp90) that maintains stability and conformation of important proteins like p53, AKT and ERBB2. Therefore, inhibiting Hsp90 leads to blocking of multiple pathways important for the survival of cancer cells. In recent pre-clinical studies, 17-AAG has been reported to inhibit growth of a wide array of malignancies ranging from NB to pancreatic cancer. Recent studies have linked the STAT, p53 and NFκB pathways with advanced stage NB. 17-AAG, which targets a broad spectrum of molecular pathways, may prove to be an effective drug to treat aggressive NB.

In addition to identifying agents that have been previously utilized or currently going under clinical investigation against NB, our study identified a few agents that have not been investigated in NB. Helenalin, a sesquiterpene lactone, has an anti-inflammatory activity. Helenalin has been shown to inhibit NFκB and telomerase. As NFκB expression and telomerase re-expression are crucial for the survival of cancer, helenalin may prove to be an excellent candidate for clinical use; hence, further investigation is warranted.

Cucurbitacin I is a natural product triterpenoid extracted from the fruits of Cucurbita andreana. Pre-clinical data demonstrates that Cucurbitacin I inhibits growth in glioblastoma multiforme and in ALK-positive anaplastic large cell lymphoma cell line by targeting the STAT3 pathway. In this study we also demonstrated that Cucurbitacin I inhibits STAT3 activation and induces apoptosis. This drug may prove to be an effective agent against high stage NB.

In addition, our study identified an epoxy anthraquinone derivative (EAD) as a compound with significant activity in the cell viability assay. Takano et al. has shown EAD inhibits angiogenesis in bovine endothelial cells. Structurally EAD is related to mitoxantrone, which is known to intercalate DNA and inhibit topoisoerase 2. Hence, it is plausible that EAD may also act through a similar mechanism. Although anthraquinones and anthracyclins such as doxorubicin and daunorubicin are among the most effective drugs for treating cancer, they are cardiotoxic at higher cumulative doses; therefore, there is a need to identify new analogs that are more effective and less toxic. In line with this objective, epoxy analogs of anthraquinones have been developed. Future studies will determine its in vivo efficacy, toxicity and suitability for clinical use.

Materials and Methods

Drugs. Drugs and chemical compounds were obtained from the Developmental Therapeutics Program (DTP) of the National Cancer Institute at the US National Institutes of Health (www.dtp.nci.nih.gov). The NCI COMBO plate contained 77 compounds, and in order to diversify the pool of compounds, we added 19 other compounds with varied mechanisms of action. NB cells were tested with drugs at 1 μM (low) and 10 μM (high) concentrations unless noted otherwise (Suppl. file 1). The ≤1 μM drug concentration enabled us to test for in vitro efficacy of a drug on NB cell lines at achievable serum concentrations in patients under physiologically conditions.

Cell culture. Two non MYCN-amplified cell lines, SK-N-AS and SH-SY-5Y, were used in these experiments. These cell lines were procured from the American Type Culture Collection (ATCC). SK-N-AS and SK-SH-5Y were grown in RPMI 1640 and DMEM media respectively, supplemented with 10% FBS (Hyclone, Logan, UT, USA), 1% Glutamine and 1% P/S (Quality Biological, Gaithersburg, MD, USA). Cell culture was maintained as described previously.

Cell viability assay. In the primary drug screen, 5,000 SK-N-AS cells were seeded in each well on 96-well plates; 24 h after seeding, cells were treated with drugs which are diluted with the cell culture medium and DMSO to achieve the desired final drug concentration and 0.1% final concentration of DMSO. We evaluated the efficacy of each compound using the CellTiter Blue cell viability assay (Promega Corporation, Wisconsin, USA).
Madison, WI) at 24, 48 and 72 h after drug treatment as prescribed by the manufacturer's protocol. To confirm all the positive hits from the primary screen, a secondary screen with identical seeding and drug dilution was carried out on SK-N-AS. The hits from the primary screen that we were unable to confirm in the secondary screen were discarded from any further analysis. To reduce cell line specific positive hits, all the hits from the secondary screen were tested on SH-SY5Y cell line with the identical seeding and drug concentration as the previous two screens. For each drug, the cell viability measurement was corrected by cell viability measurement from controls. To calculate the percentage of alive cells, the corrected cell viability measurement for each drug was divided by the corrected DMSO control cell viability measurement.

Apoptosis was measured by Caspase-Glo 3/7® assay (Promega Corporation, Madison, WI) on SK-N-AS cells at 24 h after the drug treatment. The measurement for caspase 3/7 stimulation for each drug was corrected by subtracting the background reading, and the fold induction of caspase 3/7 for each drug was corrected by the positive hits in real-time. 5,000 SK-N-AS cells were seeded in each well of the 96-well microtiter E-plates. Twenty-four hours after the seeding, cells were treated with all positive hits from the primary screen that we were unable to confirm in the secondary screen were tested on SH-SY5Y cell line with the identical seeding and drug dilution was carried out on SK-N-AS. The hits from the primary screen that we were unable to confirm in the secondary screen were discarded from any further analysis. To reduce cell line specific positive hits, all the hits from the secondary screen were tested on SH-SY5Y cell line with the identical seeding and drug concentration as the previous two screens. For each drug, the cell viability measurement was corrected by cell viability measurement from controls. To calculate the percentage of alive cells, the corrected cell viability measurement for each drug was divided by the corrected DMSO control cell viability measurement.

Each treatment was performed in triplicate wells. After normalization to control, the data was reported as a mean of three independent measurements.

**Growth inhibition profile.** Real Time-Cell Electronic Sensing (RT-CES; ACEA Biosciences, Inc., San Diego, CA) technology was used to monitor the growth inhibition induced by the positive hits in real-time. 5,000 SK-N-AS cells were seeded in each well of the 96-well microtiter E-plates. Twenty-four hours after the seeding, cells were treated with all positive hits from the primary screen at the same drug concentration used for viability and apoptosis studies. Growth inhibition caused by these agents was monitored for an additional 72 h after the drug was added. The data was reported as a mean representative of three independent measurements.

**SDS-PAGE and western blotting.** For each cell line, a total of 5 x 10⁶ cells were rinsed twice with ice-cold PBS and added 0.5 ml of the Protein Extraction Solution RIPA (Pierce Biotechnology, Rockford, IL). After incubation for 30 min on ice, cells were scraped and centrifuged. Protein concentrations were measured with the BCA protein assay kit (Pierce Biotechnology). The solubilized extracts containing 50 μg of protein from each cell line were separated by 4–12% NuPAGE® Novex Bis-Tris polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA). After blocking for 1 h, the membranes were probed with primary antibodies diluted 1:1,000 for phospho Stat3 (Tyr705), Stat3 (Cell signaling), and GAPDH (1:5,000, Chemicon International, Temecula, CA) in Tris-buffered saline (TBS)-T containing 5% BSA at 4°C overnight and followed by three washes in TBST. Specific molecules were detected with HRP-labeled anti-rabbit or ant-mouse secondary antibodies (Pierce Biotechnology) and enhanced with SuperSignal Chemiluminescence kit (Pierce Biotechnology). Signal was detected on Kodak Biomax MR X-ray film (Kodak). Quantitative analysis of the western blotting was performed by analysis of the scanned X-ray films with ImageQuant (Ver. 5.2, Molecular Dynamics, Sunnyvale, CA).

**Conclusions**

In conclusion we have identified 17 agents to be active against these NB cell lines at ≤1 μM concentration. Of these compounds, eleven are not currently used to treat NB, nine are FDA approved for clinical use and three are undergoing clinical trials for other indications. Our study has also identified four agents that were highly effective in vitro but have not been tested in human. Therefore, we have identified many active agents for neuroblastoma and provided the impetus for further testing on a larger panel of NB cell lines as well as in vivo validation of these drugs for translation to the clinic.

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**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/GheeyaCBT8-24-Sup.pdf


