Development of a high-throughput screening platform to identify new therapeutic agents for Medulloblastoma Group 3

Inés Fallon, Henar Hernando, Olga Almacellas-Rabaiget, Berta Marti-Fuster, Cesare Spadoni, Darell D Bigner, and Eva Méndez

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1. Introduction

Medulloblastoma (MB) is the most frequently occurring malignant pediatric brain tumor, accounting for 20% of all primary brain tumor cases and responsible for almost 10% of all pediatric cancer deaths [1, 2]. MBs are classified into 4 different molecular subgroups with distinct genetic profiles and outcomes and, despite the heterogeneity among the subgroups, most patients receive similar therapies, consisting of surgery, craniospinal irradiation, and adjuvant multiagent chemotherapy [3]. The most widely used chemotherapeutic agents are cyclophosphamide, lomustine, cisplatin, and vincristine [4]. Although these treatments prolong survival, they are aggressive and lead to treatment resistance followed by tumor relapse. When effective, they lead to a high risk of serious side effects related to toxicity, especially in young children, causing significant long-term impairment of intellectual and neurological functions and having a negative impact on the quality of patients’ life [5].

A great need persists for the development of new therapeutic options that improve patient outcomes and reduce long-term side effects. Particularly pressing is the need for medulloblastoma Group 3 (MB G3) since this subtype alone accounts for 25–30% of all MB cases and has the worst prognosis, especially when associated with MYC amplification present in 17% of patients [6], with only 20% of patients surviving 5 years post-diagnosis [7–9].

The identification of drug candidates for medulloblastoma is hindered by the limitations encountered throughout the drug discovery process. One of the main challenges is that traditional drug discovery uses phenotypic screening on a limited number of cellular models, which do not fully represent the heterogeneity of the tumor [10]. Moreover, despite the ease of handling and low cost of traditional 2-dimension (2D) culture models, the 2D model doesn’t replicate in full the 3-dimension (3D) complexity of an in vivo tumor and the associated effects of the tumor microenvironment, such as that of cell-to-cell contact and the influence of the extracellular matrix (ECM). These complex interactions,
in combination with the unique properties of a 3D structure, affect tumor cell properties and behavior, gene expression, distribution of proteins, and ultimately, response to anticancer drugs [11–13]. There is a growing recognition that 3D cultures reflect more accurately the tumor pathophysiology and recapitulate the in vivo tumor microenvironment in vitro, leading to more effective and targeted treatments for medulloblastoma [11,12,14–16].

Another key factor to highlight is that PBTs differ greatly from their adult counterparts, not only in the incidence but also in histology, molecular pathology, tumor microenvironment and outcome [17]. However, children are largely treated with drugs that are either not brain-specific or were originally developed to treat adult cancers. The direct consequence of this is that they are not effective enough or they exhibit undesired toxicity. In fact, most drug failures in the clinics are due to a lack of efficacy (52%), or a lack of safety (24%) [18]. Thus, the urgent need to perform specific research for children, using translational in vitro models. Finally, anticancer therapies are often associated with potential acute or delayed toxicities, and pediatric patients with brain tumors are at particular risk of neurotoxicity [19]. Acute neurological events during treatment demonstrate the immediate impact of specific chemotherapeutic agents on the brain [20], but delayed CNS issues also have a significant impact in children’s lives. Therefore, the removal of unsafe agents early in the development process, by introducing reliable in vitro neurotoxicity models, is key for success as it is expected to decrease attrition rate later in clinical development and bring new safer therapies to the patients faster. It will also ensure translational efficacy data, which can reduce the substantial costs associated with drug development and streamline the entire process [21–23].

To date, only 7 drugs have been approved for their use in pediatric oncology as the first indication. The treatments currently used with pediatric patients were originally approved only for adult use [24]. This highlights the urgent need to develop and establish translational models specifically designed for pediatric diseases to support the development of more effective and less toxic therapies for these patients. In this paper, we describe PBT-SCREEN, a unique pediatric screening platform that integrates a broad panel of in vitro Medulloblastoma Group 3 human models, specifically designed to identify new therapies for this subgroup of pediatric patients. The most relevant and innovative factor of the platform is that it addresses the main causes of failure during drug development: lack of efficacy, molecular heterogeneity and unacceptable neurotoxicity.

2. Materials & methods

2.1. Cell lines and culture conditions

Medulloblastoma cell lines D341 (HTB-187, ATCC, Manassas, VA) and D384, D425, D458, D487, D556 (obtained from Dr Bigner, Duke University, Durham, NC) were cultured in DMEM media (Gibco, Thermo Fisher, Waltham, MA) complemented with fetal bovine serum (FBS, Trinity Tek, Spain) to a final concentration of 20 %, and supplemented with Penicillin-Streptomycin-Glutamine (P/S/G, Gibco) to 1 % final concentration. D238 (HTB-185, ATCC) and control cell line BJ (CRL-2392, ATCC) cells were cultured in DMEM media complemented with 10 % FBS and supplemented with 1 % P/S/G. HD-MB03 (ACC 740, DMSZ, 2927, ATCC) cells were cultured in DMEM media complemented with 10% human plasma fibronectin (Sigma) for 3 h at 37 °C. Cells were cultured in DMEM/F-12 media (ATCC) supplemented with 1x Neuro-2 Medium Supplement (Sigma) and freshly diluted Fibroblast Growth Factor (Gibco) to a final concentration 40 ng/mL. When confluency was reached, cells were enzymatically dissociated with 0,5x Trypsin (Gibco).

Mycoplasma testing was conducted regularly using MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Walkersville, MD, USA) ensuring no contamination.

2.2. Chemical compounds

Dimethyl sulfoxide (DMSO, Thermo Fisher) was used as a universal solvent to dissolve all chemical compounds.

Tocriscreen 2.0 Micro library (Bio-Techne, Minneapolis, MN, USA) was used for the pilot screening to validate the screening platform. It consists of 1280 biologically active compounds dissolved at 10 mM in DMSO 100 % in 96-well plates. For the primary screening, compounds were diluted to 100 µM 10 % DMSO and reformatted into 384-well plates. For the dose-response curves, new powder compounds were purchased from Tocris.

Medulloblastoma standard inhibitors, used as positive controls, were purchased from MedChemExpress LLC (Princeton, NJ, USA): Vincristine sulfate (Cat. No.: HY-N0488), Cisplatin (Cat. No.: HY-17394), Vorinostat (Cat. No.: HY-10221) and JQ1 (Cat. No.: HY-13030).

Compounds used to validate the neurotoxicity assay can be found in Suppl. Table S1.

All compounds purchased in powder were first dissolved in 100 % DMSO to a final concentration of 10 mM (stock solution). Each time a compound was used, a working solution of 1 mM and 10 % DMSO was prepared by diluting 10 times the 10 mM stock with water. The concentration curves were prepared in 96-well plates by performing two-fold dilutions with 10 % DMSO.

2.3. Cell viability assay

For the viability assays performed in 2D, 384-well plates (Greiner Bio-One España S.A.U., Madrid, Spain) were pre-filled with 3 µL of 10x test compounds, and positive and negative controls. For the test compounds, 10 µM final concentration for the primary screening and different serial dilutions for the secondary screening were prepared at 1 % DMSO final concentration, DMSO 1 % was also added in the positive and negative control wells. 27 µL of cells were seeded using the Multi-drop™ Combi Reagent Dispenser (Model S840300, Thermo Fisher), at a density of 1–3 × 10³ cells/well. Plates were incubated at 37 °C and 5 % CO₂ for 24–48 h and, after each time point, viability was determined using CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) following the manufacturer’s recommendation. Luminescence was measured with the Synergy H1 Hybrid Multi-Mode Reader (Biotek Instruments, Agilent, Winooski, VT, USA).

To assess neurotoxicity with hNSCs, the standard cell viability assay was performed as previously described. For the neurotoxicity assay using neurons differentiated from LUHMES cells, µCLEAR® 384-well plates (Greiner Bio-One) were pre-coated with the reduced growth factor basement membrane matrix Geltrex (Thermo Fisher). Cells were seeded at 2.5 × 10³ cells/well using differentiation media composed of: DMEM/F12 (ATCC) supplemented with 1x N2 neural supplement (Sigma-Aldrich) containing 1 µg/mL tetracycline (Gibco), 1 mM cyclic
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Plates were incubated at 37 °C and 5 % CO2 for 48 h and cell viability was determined as previously described. Cell differentiation was assessed by immunofluorescence staining and western blot for the neuronal marker β-III-Tubulin. The assay was validated analyzing 14 neurotoxic and 14 non-neurotoxic compounds from different therapeutic classes (chemotherapeutic, neurotoxins, antimicrobial, immunomodulators, among others), and gave 71 % sensitivity and 86 % specificity (see Suppl. Material).

For the viability assays in 3D, pre-coated 96-well plates with BioMimesys Brain (HCS Pharma, France), a were used. Briefly, 25 µL of a D341 MB cells suspension at 2.4 × 10^3 cells/µL were seeded into the hydroscotch and incubated for 30 min at 37 °C to let them enter the matrix before adding 175 µL of growth media. Cells were then incubated for 12 days changing spent media every 2 days. After this time, media was removed and 20 µL of 10x test compounds were added at 8 increasing concentrations (1.2 serial dilution, final concentration from 100 to 0.78 µM). Then, 180 µL of media was added and incubated for 48 h. Cell viability assay was performed using CellTiter-Glo 3D Cell Viability Assay (G9683, Promega). This assay is based on the original CellTiter-Glo formulated to have more lytic capacity. Luminescence was measured with the Synergy H1 Hybrid Multi-Mode Reader. For setting up this 3D model, D341 MB G3 cell line was selected.

In all assays, the negative control was used to calculate the background, and its mean value was subtracted from the signal of the test compounds and positive control. The 100 % viability was established from the positive control wells and the % inhibition of the compounds was referred to this value. The compounds’ potency was quantified using the half-maximal inhibitory concentration (IC50), obtained by fitting sigmoidal dose-response models with variable slope (4 parameters) in GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA).

The reproducibility of the assay for each cell line was established by testing positive controls (cells in culture media at 1 % DMSO) and negative controls (culture media at 1 % DMSO) in three different plates and three different days (nine plates in total) and calculating the Z prime factor (Z’ ) as Zhang et al. described [26]. This parameter reflects the robustness of the assay, taking into account the mean and the standard deviation of the maximal and minimal signal in the absence of compounds, as shown in the following formula:

\[
Z' = 1 - \frac{(+3SDC_+ + 3SDC_-)}{|X_C - X_C'|}
\]

where C+ is the positive control (maximal signal); C-, the negative control (minimal signal or background); X+ , the mean and SD, the standard deviation.

Z’ was calculated in each assay to guarantee reproducibility and sensitivity. Any assay with a Z’ < 0.5 was discarded and repeated.

2.4. Apoptosis testing

Cells were seeded in 384-well plates (Greiner Bio-One) pre-filled with 3 µL of 10x test compounds, at eight different concentrations (from 200 µM to 0.01 µM). 27 µL of cell suspension at a density of 1–3 × 10^3 cells/well was added and plates were incubated at 37 °C and 5 % CO2 for 24 h. Apoptosis was measured using the Caspase-Glo 3/7 Assay (Promega). The luminescence signal, proportional to the caspase activity, was recorded with the Synergy H1 Hybrid Multi-Mode Reader. As before, the negative control was used to calculate the background, and its mean value was subtracted from the signal of the test compounds and positive control. Relative luminescence units data was used to calculate the IC50 as the midpoint of the plotted curve.

2.5. Combination synergy calculation

Compound combination assays were performed as previously described in the 2D cell viability assays but adding 1.5 µL of each of the test compounds at 20x.

The synergy, expressed as the Combination Index (CI), was determined by comparing the effect on cell growth of the combination of the two compounds versus the effect of each compound alone.

The synergism of each of the combinations was calculated with two different models:

The Bliss Independence model [27] assumes that compounds act independently, but each contributes to a common result. In this case, the combination index is calculated as:

\[
CI = \frac{(Ea + Eb - EaEb)}{Eab}
\]

where Ea and Eb are the effects of the compounds alone and Eab is the effect of the combination.

For the larger matrices, CompuSyn [28] was used, which uses the median-effect principle of the mass-action law and its combination index theorem of Chou and Talalay [29].

\[
CI = \frac{(Dcomb1)}{(Dalone1) + (Dcomb2)}
\]

where Dcomb is the dose of that compound when combined with the other that reaches 50 % of the effect and Dalone is the dose of the compound alone that reaches 50 % of the effect.

For both models, CI < 1 indicates synergism, CI = 1 additive effect and CI > 1 antagonism.

3. Results

3.1. Establishment of a panel of 2D models for efficacy and toxicity testing

A series of 2D efficacy and toxicity models, based on cell viability, were set up in 384-well plates to enable HTS campaigns. Efficacy models include eight MB G3 cell lines that represent the tumor types from which they were derived [30] and account for tumor heterogeneity: D283, D341, D384, D425, D458, D487, D556 and HD-MB03. They all present MYC amplification or overexpression, representing the patients with the worst of outcome within MB G3. Moreover, the platform incorporates three additional cell lines for assessing toxicity, which is one of the major attrition causes in drug development: normal diploid karyotype fibroblasts BJ, for general toxicity, and hNSC and LUHMES for neurotoxicity. hNSC are thought to be the putative origin of Medulloblastoma Group 3 [31], and LUHMES cells were differentiated into biologically and morphologically active mature neurons. Cell type and characteristics of all the cell lines used are shown in Table 1.

For each of the cell lines, 2D culture conditions were set up (Table 1). The optimal cell number, within the linear range of the luminescence signal, was set up between 1000 and 3000 cells/well for all cell lines (Fig. 1A). The optimal DMSO concentration, that gave 80 % cell viability, was set up at 1 % for all cell lines (Fig. 1B). Z’ was calculated for each plate, and each day, but a global Z’ value for the 3 days was calculated considering all the data points under study, and not as a product of the average of each plates Z’ (Fig. 1C). For all the cell lines daily Z’ values and global Z’ values were above 0.55. Finally, four compounds known to inhibit medulloblastoma growth were used to validate the assay in terms of sensitivity: vincristine (chemotherapy), cisplatin (chemotherapy), vorinostat (HDAC inhibitor), and JQ1 ( BET inhibitor). Results in one representative cell line (D341) are shown in Fig. 1D. JQ1 was selected as the internal control for further experiments due to its potency and reproducibility along with the different cell lines.
active chemotherapeutic agents in the 3D culture and IC
embedded within it and presents a stiffness equivalent to that of the
(Biomimesys [32], and high concordance to primary tumors when RNAseq data is
3.2. Establishment of a 3D model for efficacy testing

To increase the biorelevance of the results of the screening platform, a
drug cell culture for the D341 MB G3 cell line was included. This
representative cell line presents the same molecular characteristic traits
[32], and high concordance to primary tumors when RNAseq data is
assessed [30].

For the development of the 3D cell culture model, 3D HA hydrogel
(Biomimesys®) was used. This scaffold supports growing cells
embedded within it and presents a stiffness equivalent to that of the
brain. The final assay conditions were: 60,000 cells/well, 1 % DMSO,
and Z’ > 0.5. The sensitivity of the assay was evaluated with different
active chemotherapy agents in the 3D culture and IC50 were compared
to 2D culture. The results obtained showed that D341 MB G3
cells grown on 3D conditions could show two different behaviors: 1)
more resistance to chemotherapy drugs than in 2D, like cisplatin,
carboplatin, oxaliplatin or lomustine; 2) equally resistant to chemo-
therapeutic drugs than in 2D, like in the case of JQ1, vincristine,
vinblastine or vinorelbine. We, therefore, concluded that D341 MB G3
cells growing in 3D cultures could be more resistant to drug treatment
compared to 2D or could be similar, but the effect depends largely on
the chemical family of the tested drug (Fig. 2).

3.3. Validation of the PPTB-SCREEN, the high throughput screening
platform

PPT-SCREEN platform was validated by conducting a pilot HTS
campaign (see Suppl. Material for the detailed screening cascade) with 1280 lead-like compounds, tested at 10 µM and 1 % DMSO against the
panel of eight MB and control cell lines. Positive and negative controls
were included as well as JQ1 as an internal standard inhibitor. 94 active
compounds were identified with the following criteria: ≤ XCTRL
- 3SDCTRL cytotoxic activity and ≥ 5 in vitro therapeutic
index (TI) (that is, at least 5 times more activity against the MB cell lines
vs. the BJ control cell line; Fig. 3). The Z’ factor, used to assess the
robustness of all the nine individual HTS assays, ranged from 0.56 to
0.90.

After the primary screening, a series of assays were conducted to
confirm the efficacy of initial hits. We aimed to gain a better under-
standing of the potential of these compounds for the treatment of MB G3
tumors and to identify any further safety concerns that may limit their
clinical use.

The 94 identified hits were re-tested at 4 concentrations (50, 10, 5
and 1 µM), and 14 of the initial hits were confirmed to present a dose-
response effect in MB G3 cell lines but not in control cell line BJ (data
not shown). They were subsequently tested in complete dose-response
curves (14 points) to define their IC50 and confirm in vitro pre-
liminary TI. Finally, eight compounds were selected as hits: LMK 235, PI
828, Pyroxamide, WAY 170523, SAHA, Fusudil hydrochloride, FERβ
033 and (R)-CR8 (Fig. 4).

The efficacy of the 8 confirmed hits was also evaluated in the MB G3
3D model in dose-response curves (from 200 to 1.5 µM). All 8 com-
pounds exhibited activity in this model, with IC50 ≤ 50 µM. Efficacy
results, comparing the IC50 obtained in the 2D and the 3D models after
48 h of treatment are shown in Fig. 5. For all the eight hits, the IC50 in
the 3D model was at least 2-fold the IC50 of the 2D model, except for WAY
170523 where both IC50 were equivalent. Complete dose-response
curves in the MB G3 3D results are shown in Suppl. Figs. S2 and S3.

To assess for neurotoxicity, the 8 hits were tested in dose-response
curves (from 100 to 0.003 µM) in our two models (Fig. 6). In the
hNSC model (Fig. 6A), all compounds, except FERβ 033, exhibited an
IC50 above 100 µM, therefore not considered neurotoxic. The IC50 for
FERβ 033 was estimated at 85 µM, maintaining therefore a preliminary
in vitro TI higher than 5 in this neurotoxicity model. The selected 8 hits
were also tested in our 2D neuronal model. As shown in Fig. 6B, all
compounds except Fusudil, exhibited an IC50 below our 50 µM, thus
neurotoxic and a preliminary in vitro TI below 5. This seems to indicate
that the selected hits could present some neurotoxicity.

Finally, the activation of apoptotic cell death via caspase 3/7 acti-
vation was determined for the 8 selected hits. For that, we tested the 8
hits in dose-response curves (from 200 to 0.024 µM) against our panel
of 2D cultures for the eight MB G3 cell lines. Caspase-3/7 activation
was observed after 24 h in agreement with cell viability loss results,
achieving 50 % of activation at concentrations below the observed
IC50. Only FERβ 033 showed a different mode of action, and the loss of
viability took place at lower concentrations than the activation of
Caspase-3/7. Table 2 shows the concentration at which each compound
is achieving 50 % of Caspase-3/7 activation (effective dose 50).

3.4. Hit combination assays

Combination therapies have become increasingly popular in cancer
treatment, since single agents may predictably fail in clinical patients
due to tumours becoming highly treatment-resistant at relapse. There-
fore, we evaluated the effect of the eight hits when combined in pairs.
We studied the synergistic effect of each combination; thus 28 pairs were
studied combining the IC50 and IC50 concentrations determined as single
agents. These 2 × 2 matrices were assessed in all MB G3 cell models
(Fig. 7A).

We tested 2 × 2 matrices of all the combination pairs (Fig. 7A) for our
8 MB G3 cell models. To study the cell viability inhibition results of the
combinations, the Combination Index (CI) was used, which compares
the effect of each drug alone vs. the effect when combined. The CI was
calculated following the Bliss Independence model for each point in the
2 × 2 matrix (Fig. 7B). We then assigned a score of 0 to 4 to each pair of
compounds, depending on the number of synergistic points that
occurred in each 2 × 2 matrix (CI < 1). For example, in Fig. 7B Fasudil+
(R)-CR8 had a score of 4 (4 out of 4 combinations points showed synergy
in that particular MB G3 cell line), while FERβ 033+(R)-CR8 showed a
score of 0 (no combination exhibited synergy). The average score was
calculated for each combination across the eight cell lines (Fig. 7C),
allowing us to rank them from 0 to 4. We established a cut-off value of

Table 1
Summary table of all the cell lines included in the primary screening with their
characteristics, and screening assay conditions: optimal cell number of cells/well
and Z-prime (Z’).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Characteristic</th>
<th>Cells/well</th>
<th>Z’ factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>D283</td>
<td>Medulloblastoma group 3 (metastatic)</td>
<td>MYC amplification</td>
<td>2000</td>
<td>0.70</td>
</tr>
<tr>
<td>D341</td>
<td>Medulloblastoma group 3 from cerebellum</td>
<td>MYC amplification</td>
<td>3000</td>
<td>0.90</td>
</tr>
<tr>
<td>D384</td>
<td>Medulloblastoma group 3 from cerebellum</td>
<td>MYC amplification</td>
<td>1000</td>
<td>0.64</td>
</tr>
<tr>
<td>D425</td>
<td>Medulloblastoma group 3 from cerebellum</td>
<td>MYC amplification PS3 mutated</td>
<td>1000</td>
<td>0.62</td>
</tr>
<tr>
<td>D458</td>
<td>Medulloblastoma group 3 from cerebellum.</td>
<td>MYC amplification PS3 WT</td>
<td>1000</td>
<td>0.58</td>
</tr>
<tr>
<td>D487</td>
<td>Medulloblastoma group 3 from cerebellum.</td>
<td>Recurrence D425</td>
<td>1000</td>
<td>0.59</td>
</tr>
<tr>
<td>D556</td>
<td>Medulloblastoma group 3 from cerebellum</td>
<td>MYC amplification OTX2 amplification</td>
<td>1000</td>
<td>0.71</td>
</tr>
<tr>
<td>HD03</td>
<td>Medulloblastoma group 3 from cerebellum</td>
<td>MYC amplification</td>
<td>1000</td>
<td>0.83</td>
</tr>
<tr>
<td>BJ Neonatal fibroblasts from foreskin</td>
<td>Normal diploid karyotype</td>
<td>1000</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>LUHIMES Ventral mesencephalon neuron precursor cells</td>
<td>Modified with a MYC-overexpression vector</td>
<td>2500</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>hNSC Human fetal brain-derived neural stem cells</td>
<td>Multipotent stem cells of the nervous system</td>
<td>3000</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>
2 for selecting the best synergistic combinations.


The most synergistic pairs were selected for further studies, yet looking closely at the results we can see that pairs number 1, 3 and 4 are all combinations of an Erβ receptor (FERβ033) inhibitor and an HDAC inhibitor (Pyroxamide, LMK 235 and SAHA). On top of that, pairs 2, 5 and 7 are a combination of the PI3KCA inhibitor (PI 828) with an HDAC inhibitor (Pyroxamide, LMK 235 and SAHA). Therefore, for further studies, one representative HDAC inhibitor, SAHA, was selected since it is a pan-HDAC inhibitor with demonstrated antitumoral activity [33].

Therefore, the 7 selected combinations were reduced to 3 final combinations: FERβ033 + SAHA, PI 828 + SAHA and FERβ033 + (R)-CR8.

To further confirm the synergism in the selected pairs, 5 × 5 combination matrix were performed. Five different concentrations of each compound were combined, based on their IC50 for each cell line (IC50 × 4, IC50 × 2, IC50 × 1, IC50 × 0.5 and IC50 × 0.25).

Results showed an antiproliferative effect of these combinations in MB G3 cells lines, while no effect was observed in the control cell line BJ or hNSC (Fig. 8A). As it was done before, the CI for each point within the matrix was calculated (Fig. 8B), showing a consistent pattern of synergistic points in the three combinations and across all MB G3 cell lines.

4. Discussion

In this paper, we describe a unique screening platform specifically designed to identify new therapeutic agents for Medulloblastoma Group 3. This platform consists of a panel of in vitro cell-based efficacy and toxicity models that account for tumor heterogenicity, translational efficacy and unacceptable toxicity from the very early stage of drug development.

Medulloblastoma G3 tumors are highly heterogeneous, which complicates the process of in vitro tumor modeling. To incorporate this tumor heterogeneity into our screening platform and increase the chances of identifying effective treatments that could be applicable to a larger population of patients, a battery of eight MB Group 3 cell lines were used to develop our 2D efficacy models. This is not typically found in other published studies where medulloblastoma screening models are not subgroup-specific, or if so, the number of cell lines used is much smaller [34–37]. Some screens have been performed in subgroup specific cell lines, but they have not included toxicity assays in the initial steps of the screening [38]. In PBT-SCREEN, the panel of MB G3 cell lines intends to cover clonal diversity from different patients, and every single cell line has been individually set up and optimized to be used in HTS campaigns. For all the assays, Z’ values and global Z’ were above 0.5 (our quality cut-off). This Z’ value warrants the suitability of our assays, in terms of reproducibility and robustness, for their use in full-scale, high-throughput screening campaigns [26]. The sensitivity for each of the cellular models was confirmed with standard

![Fig. 1. Establishment of the 2D culture conditions for the primary screening. A. Representative results of the linear range of the luminescence signal against the increasing number of cells for the D487 cell line. B. Representative results of the DMSO tolerance for the D487 cell line. The dotted line shows 92 % viability at 1 % DMSO, where DMSO concentration was established. C. Representative results of the reproducibility of the assay for the D487 cell line. Positive and negative controls were tested on three different plates and three different days (nine plates in total) and the Z prime factor (Z’) was calculated [36]. The table below shows the Z’ for each day and the global Z’ considering all the data points of the three days. The horizontal line represents the mean luminescence value for the positive controls and the dotted lines the mean value ± 3 standard deviations. D. Representative results of the 4 inhibitors tested in a dose-response curve against D341 cells after 24 h of incubation. IC50 values: 0.08 µM for vincristine, 61.03 µM for cisplatin, 3.18 µM for vorinostat and 0.32 µM for JQ1.](image)
chemotherapeutic agents such vincristine and cisplatin, and with targeted known inhibitors such as vorinostat (HDAC inhibitor) and JQ1 (BET inhibitor) [39–42].

The panel of cell lines included in PBT-SCREEN covers a wide representation of MB tumors and offers a robust HTS system for hit identification. However, 2D cell culture models do not fully replicate the complexity of an in vivo tumor and therefore they may not accurately predict anti-tumor efficacy of investigational new drugs. Primary cells permit a more faithful reproduction of tumors, preserving cell phenotypes and their heterogeneous ability to grow in culture. On the contrary, 3D cell cultures reflect quite accurately the tumor pathophysiology, and recapitulate the in vivo tumor microenvironment [11]. To increase the bio-relevance of the cell culture, instead of growing spheroids, that are the most widely used 3D tumor models [44,45], we have developed a 3D efficacy model that includes a hydrogel that mimics the composition and stiffness of the brain extracellular matrix (ECM). To develop this model, the D341 cell line was selected because it presents high concordance with patient samples and presents molecular characteristic traits of MB G3 [30,32]. Toxicity is another major concern during drug development and represents one of the main causes of clinical drug attrition [18]. For that reason, the PBT-SCREEN platform includes three toxicity models very early in the screening cascade. This approach allows to deselect those compounds that do not exhibit a satisfactory in vitro therapeutic window against a general toxicity model (BJ cell line) in the primary screening during the Hit identification phase. Afterwards, the platform can assess the potential neurotoxicity in two additional models for Hit nomination. The neurotoxicity model based on differentiated neurons that exhibited 71 % sensitivity and 86 % specificity, confirmed its suitability to evaluate the potential risk of neurotoxicity early in drug discovery.

To summarize, in the process of identification and selection of hits, the platform takes into account not only the activity of the test compounds but also their toxicity. And this combination of efficacy and toxicity models very early in the drug discovery process is a unique feature of the PBT-SCREEN platform which makes it an ideal in vitro tool to develop new drugs that are not only effective but safer for children with medulloblastoma.

The validation of the platform was carried out by conducting a pilot HTS with a chemical library of 1280 lead-like compounds. At the end of the screening cascade, 8 active and selective compounds against MB cell lines were detected. All hits were acting via Caspase activation and targeted 6 MB related cancer targets, some of them currently approved or in clinical trials in pediatric patients with malignant brain tumors, including MB: histone deacetylases (HDACs) [46], estrogen receptor beta (ERβ), matrix metalloproteinase-13 (MMP-13), phosphoinositide 3-kinases (PI3Ks) [47], cyclin-dependent kinase 1/2 (CDK-1/2) and

Fig. 2. Drug-response curves of chemotherapeutic drugs in 2D vs. 3D cultures of MB G3 cells. A Cisplatin is less potent in 3D culture, IC50 = 5.3 µM, compared to 2D, IC50 = 0.65 µM, after 48 h treatment. B JQ1 shows a similar pattern of cell inhibition in 2D vs 3D, IC50 0.33 and 0.32 µM, respectively, after 48 h treatment. C Brightfield microscopy images illustrating the morphology of the MB G3 D341 cells growing in 2D and 3D at seeding time and treatment time.
Rho-associated-kinases (ROCKs).

All of them have been considered as potential drug targets for at least one molecular subgroup of MB and some of them, such as PI3K, HDACs and CDKs, included in the molecular pediatric target list of the FDA [48], meaning that there is evidence to suggest their potential relevance to the growth or progression of one or more pediatric cancers. All this confirms the potential of the PBT-SCREEN platform to identify new active compounds against MB G3 cells.

In addition to identifying single agents that could potentially fail in the clinics due to resistance, the screening platform determined the effect of the nominated hits when combined in pairs. Among the three synergistic pairs identified, the combination of

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Fig. 3. Screening campaign results. List of the 94 compounds identified in the primary screening. Cell viability is represented as a heatmap for all MB G3 cell lines and for the control cell line BJ. Yellow means no cell viability inhibition, and the more purple, the greater inhibition of cell viability. The viability values that presented a preliminary TI higher than 5 versus control cell line are boxed in a black dotted line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
HDAC inhibitor SAHA with PI3K inhibitor PI 828 has already been described in the literature in MB assays [49]. More interestingly, a dual compound inhibiting both targets at the same time, Fimepinostat (CUDC-907), is currently being studied in clinical trials for brain tumors, including recurrent MB, Diffuse Intrinsic Pontine Glioma and recurrent High-Grade Glioma) [50]. We further characterized Fimepinostat in our platform (results not shown) and observed a higher potency compared with the combination of the two single inhibitors. Taken together, these results offer supportive evidence for the validity of our screening platform.

Overall, the screening platform provides a comprehensive approach to drug discovery, incorporating models of both efficacy and toxicity to prioritize the identification of promising compounds with a favorable therapeutic window. The inclusion of a broad panel of cell lines and characterization assays, including those for neurotoxicity and 3D efficacy models, ensures the identification of treatments that are effective, safe, and applicable to a larger patient population. Additionally, this screening platform has the potential to identify synergistic drug interactions.

Fig. 4. Results from hit confirmation. Dose-response curves for the selected 8 hits against MB G3 cell lines (warm colors) and control cell line BJ (blue) and for a representative non-selected compound, due to lack of preliminary therapeutic index, after 24 h of treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Comparison of 2D and 3D efficacy results. Efficacy results, comparing the 2D and 3D models for the 8 selected hits after 48 h of treatment.

Fig. 6. Neurotoxicity assays for hit nomination. A Dose-response curves of the 8 hits in the neurotoxicity model using the human Neural Stem Cells after 48 h of treatment. B Dose-response curves of the 8 hits in the neurotoxicity model using differentiated neurons after 48 h of treatment.
Table 2

Apoptosis activation results for the selected 8 hits in the 8 MB G3 cell lines. Relative luminescence units from the Caspase-3/7 assay were used to calculate the IC\(_{50}\) as the midpoint of the plotted curve. For compounds where activation didn’t occur, IC\(_{50}\) couldn’t be calculated, and are represented as non-active (N.A.).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apoptosis IC(_{50}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D283</td>
</tr>
<tr>
<td>(R)-CR8</td>
<td>0.00</td>
</tr>
<tr>
<td>Fasudil</td>
<td>20.46</td>
</tr>
<tr>
<td>FERβ 033</td>
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</tr>
<tr>
<td>LMK 235</td>
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</tr>
<tr>
<td>PI 828</td>
<td>4.10</td>
</tr>
<tr>
<td>Pyroxamide</td>
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<tr>
<td>SAHA</td>
<td>0.38</td>
</tr>
<tr>
<td>WAY 17523</td>
<td>3.12</td>
</tr>
</tbody>
</table>

Fig. 7. Results of the 8 nominated compounds combination matrices using the IC\(_{20}\) and IC\(_{50}\) concentrations. A Representative result of D283 MB G3 cell line, treated with the IC\(_{20}\) and IC\(_{50}\) concentrations of each of the 8 nominated compounds in a 2 × 2 combination matrix. A total of 28 combinations were performed and cell viability inhibition was assessed after 24 h. Yellow means no cell viability inhibition, and the more purple, the greater inhibition of cell viability. B Representative results of D283 MB G3 cell line Combination Indices (CIs). Bliss Independence model was used to calculate the CIs for each point on the 2 × 2 matrices using the cell viability results. Dark red means synergy, light red additivity and white antagonisms. C Average of the synergistic points per pair across all the eight MB G3 cell lines, ranking the pairs from 0 to 4. A cut-off value of >2 was established to select the combinations with the highest overall synergy. Light blue means 0 synergistic average points and dark blue 4 synergistic average points. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
combinations, which can enhance the therapeutic effect of individual drugs and overcome drug resistance. Finally, all the assays included in the PBT-SCREEN are homogeneous and all (except the 3D efficacy model) set up in 384-well plates to allow screening large compound collections. Moreover, the screening platform can be easily extended to other brain tumors, hence its name, making it a versatile tool for drug discovery. With its comprehensive and robust approach, this screening platform holds great promise in the development of effective and safe treatments for MB G3 and has the potential to improve patient outcomes and quality of life.

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Declaration of competing interest

The authors declare that they have no competing interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.j.slasd.2024.100147.

References


