ABSTRACT: Traditional long exposure (24−72 h) cell viability assays for identification of potential drug compounds can fail to identify compounds that are: (a) biologically active but not toxic and (b) inactive without the addition of a synergistic additive. Herein, we report the development of a rapid (1−2 h) compound screening technique using a commercially available cell viability kit (CellTiter-Glo) that has led to the detection of compounds that were not identified as active agents using traditional cytotoxicity screening methods. These compounds, in combination with metabolic inhibitor 2-deoxyglucose, display selectivity toward a pancreatic cancer cell line. An evaluation of 11 mammalian cell lines against 30 novel compounds and two metabolic inhibitors is reported. The inclusion of metabolic inhibitors during an initial screening process, and not simply during mechanistic investigations of a previously identified hit compound, provides a rapid and sensitive tool for identifying drug candidates potentially overlooked by other methods.

INTRODUCTION

Despite significant advances in the detection and clinical management of cancer in recent decades, the disease still remains one of the major causes of death worldwide.1 The discovery and development of novel, small-molecule chemotherapeutic agents that circumvent the unwanted side effects and resistance associated with current well-established anticancer drugs is a pressing need in synthetic chemistry. Following the synthesis of a library of new compounds with anticancer potential, the selection of reliable tools to identify activity rises to the forefront. In vitro assays, performed under controlled environments outside of an organism, are the method of assessment most frequently chosen for initial high-throughput screening of compounds due to the speed, low cost, and wide range of informative assays/techniques available to address key hallmarks of cancer.2−4 Perhaps the most common assays employed are cell-based viability/cytotoxicity and antiproliferative assays.5−4 In a traditional approach, cultured cells are exposed to individual compounds for extended periods of time (24−72 h), followed by a determination of effects on cell viability. A variety of assays have been developed that measure some biological parameters of living or dead cells (e.g., ATP levels, metabolism, enzyme activity, etc.), which are frequently linked to light emission or fluorescence to facilitate high-throughput screening. While these methodologies can rapidly identify compounds targeting some biological process(es) required for viability, they tend to provide little mechanistic information and can fail to identify compounds that are bioactive but not toxic. Furthermore, the outcome can also be dependent on the type of cells utilized, which is why a library is often screened against a panel of different cell lines (e.g., cancer cells derived from different tissues).1 In an effort to develop a rapid method of screening compounds for effects on cell viability, we have developed a new assay technique using a commercially available kit (CellTiter-Glo) that detects anticancer activity
in as short as 1–2 h exposure time with comparable sensitivity to a commonly employed traditional cytotoxicity assay with 24 h incubation.

The production of energy in the form of ATP occurs in living cells over multiple biochemical pathways including glycolysis or mitochondrial oxidative phosphorylation (TCA) pathways. One of the hallmarks of cancer is that cancer cells are known to preferentially utilize glucose to maximize the production of cellular building blocks at the expense of energy production, known as the Warburg effect (displayed in Scheme 1). The prevalent use of the glycolytic metabolic pathway by cancerous cells makes them more susceptible than normal cells to inhibitors of glycolysis. Therefore, the use of metabolic inhibitors is a strategy employed to selectively target cancer cells by exploiting the difference observed between normal and cancerous cells.6

By incorporating a metabolic inhibitor that is known to impact certain pathways, we envisioned the development of a method for screening compounds that would measure the direct effects of a compound on the remaining metabolic pathways available for ATP synthesis in the cell. As a consequence of the pretreatment with a metabolic inhibitor, the cell must utilize the remaining uninhibited pathways to maintain cellular ATP levels. Thus, the ATP levels are determined without killing the cells during the incubation of the cells in the presence of the compound of interest. Any measured reduction in ATP levels corresponds to the inhibition of the metabolic pathways that are uninhibited by the metabolic blocker. Thus, the method provides an approach for screening compounds for the ability to specifically target the uninhibited pathway being used by the cells to generate ATP. Furthermore, because cells pretreated with a metabolic inhibitor targeting a known pathway are forced to use an alternative, uninhibited pathway to maintain ATP levels, the screening method would provide immediate mechanistic information about the active compound mechanism of action.

Herein, we describe the use of a commercially available bioassay kit (CellTiter-Glo) that quantifies ATP levels in a cell culture to detect decreased ATP levels/production after exposure to library compounds in cells pretreated with metabolic inhibitors. This analysis aims to identify bioactive compounds potentially overlooked by a traditional cytotoxicity screen, indicating that modulating cell culture conditions can expand the screening landscape, identify compounds with synergistic activity, and even provide mechanistic insight into compound targets.

## RESULTS AND DISCUSSION

Sulfonamide is a privileged moiety found within a broad range of medicinally relevant molecules. A number of sulfonamide-containing molecules, including indisulam8 and ABT-7519 among others, have shown interesting in vitro and in vivo anticancer activity.10 Recently in our laboratories, the synthesis and cytotoxicity screening of 24 novel heteroaryl N-benzyl sulfonamides was reported.11 The cytotoxicity screening of the compounds (100 μM) was conducted over a 24 h exposure period using a CellTiter-Blue (Promega) assay, which is based upon the conversion of resazurin to the fluorescent resorufin by living cells.12 During the traditional long exposure viability screening of H293 cancer cells, 6 out of the 24 compounds were identified as a hit (compounds 2, 5, 6, 7, 23, and 30), which is defined in our investigation as 50% or lower percent of DMSO control (POC) after exposure to a library compound. Four of the six would be classified as modest hits (POC values of compounds: 2 = 32.9%; 5 = 33.5%; 7 = 50.2%; 23 = 49.8%), and two of the six, compounds 6 and 30 (Figure 1), a strong hit (POC values: 6 = 23.3%; 30 = 18.6%).

To begin our investigation, and to ensure that the reported cell viability screening did not miss potentially interesting compounds that are biologically active but not cytotoxic, we re-evaluated the 24 reported sulfonamides at the same concentration (100 μM) as the reported CellTiter-Blue assay using a CellTiter-Glo (Promega) assay along with six new, related compounds (10, 13, 15, 17, 27, and 28) that were synthesized in our laboratory (shown in Figure 1). CellTiter-Glo has been shown to be a comparable assay to CellTiter-Blue in terms of sensitivity and applicability across a range of cell lines.13,14 CellTiter-Glo is a bioassay that measures cell viability after exposure to compounds (typically 24–72 h) via quantification of ATP released from living cells by measurement of photoluminescent luciferase. The degree of fluorescence that results due to the presence of ATP is directly proportional to the number of living cells in a culture at the time of quantification. We hypothesized that, at 1 h exposure time of the compounds, we are not only quantifying the number of living versus dead cells (by measuring the amount of ATP from the remaining living cells) but also indirectly measuring the ability of cells to produce ATP during proliferation. Cells that have been exposed to a compound that negatively impacts the ability to produce ATP will ultimately die if given time. In other words, the use of a CTG assay at reduced compound exposure times gives a snapshot view of cells that are on a path toward death due to exposure to a library compound.

After screening the 30 compounds using CellTiter-Glo, it was observed that comparable results could be obtained from the assay conducted under reduced compound exposure times (1–2 h) relative to a traditional CellTiter-Blue cytotoxicity assay conducted with 24 h incubation. In a separate control reaction performed based upon literature precedent14,15 in which exogenous ATP was added to media in the absence of cells, it was confirmed that compounds 1–30 (100 μM, 10 min exposure) do not inhibit the CellTiter-Glo luciferase assay itself (see the Supporting Information, Figure S1). Of the 30 compounds screened at 100 μM against H293 cells (kidney cancer), the CellTiter-Blue (CTB) assay with 24 h exposure to library compounds identified seven of the compounds as hits,
while CellTiter-Glo (CTG) at 24 h exposure identified five compounds as hits, and CTG at 1 h exposure identified four of the compounds as hits (Figure 2). Only one of the four, compound 28, would have been identified as a hit by CTG at 1 h but not by CTB.

Cancer cells produce ATP mainly by glycolysis, but also through mitochondrial oxidative phosphorylation (TCA), albeit as a minor pathway. If a compound only affects one of the metabolic pathways for ATP production, it may not be identified as cytotoxic during a traditional cell viability screening assay because the cell can still produce ATP via the alternative pathway (Scheme 1). Therefore, we envisioned the inclusion of a metabolic inhibitor during the initial screening of compounds 1–30 as a means to identify compounds that either display a synergistic or amplified effect, or those that may target one of the metabolic pathways of cancer cells, but may not necessarily be cytotoxic in the absence of a metabolic inhibitor. 2-Deoxyglucose (2DG) was selected as a well-known, FDA-approved inhibitor of the glycolysis pathway, and rotenone was selected as a
representative inhibitor of the oxidative phosphorylation (TCA) pathway.\textsuperscript{17}

A series of control reactions was performed using CTG assay at 1 h exposure to 2DG and/or rotenone against representative cancerous (H293 and BxPC3) and immortalized noncancerous (HDF and MCF10A) cell lines (Figure 3). Although HDF and MCF10A cell lines are noncancerous (not transformed), they still primarily metabolize glucose via glycolysis to allow for continual proliferation. Therefore, they would still be susceptible to treatment with a glycolysis inhibitor such as 2DG. The treatment of cells with 10 mM 2DG had a moderate effect on H293 and BxPC3 cell lines, indicating that they can switch to an alternative metabolic pathway (TCA/ETC) or source of nutrients (i.e., amino acids such as glutamine) in the presence of 2DG. Treatment with rotenone displayed little to no effect, also indicating that the cancerous cells can switch from TCA/ETC to glycolysis. However, when 2DG and rotenone were combined, a significant effect was observed in the cancerous (immortalized and transformed) cell lines, H293 and BxPC3. This indicates that the inhibition of both metabolic pathways that utilize glucose (glycolysis and TCA) in cancerous cells has a synergistic effect. The use of 2DG with nontransformed cells (HDF and MCF10A) also provides a moderate amount of inhibition, whereas rotenone appears to have no effect. The use of 2DG and rotenone together has little additional activity over the effect of 2DG, indicating that immortalized but nontransformed cells such as HDF and MCF10A do not switch metabolic pathways in the presence of 2DG and continue to primarily use the glycolysis pathway and do not display as significant a synergistic effect as the cancerous cell lines. From the data, the $Z'$-factor, which is a measure of the quality of the assay that uses negative (DMSO) and positive (2DG + rotenone) controls, was calculated according to literature precedent to be 0.805 for the 1 h CTG assay.\textsuperscript{18} A $Z'$-factor between 0.5 and 1.0 is considered to be an excellent assay.\textsuperscript{18}

The usage of metabolic inhibitor additives, 2DG and rotenone, was then investigated using a CellTiter-Glo assay with 1 h exposure time of compounds 1–30 at 100 μM (shown in Figure 4). Similar to the CellTiter-Blue viability assay, the most frequently observed hit compounds were comprised of the indole core (compounds 1–7 and 23–30). The addition of a metabolic inhibitor, especially 2DG, appears to increase the sensitivity of the screening to a comparable level to that of a long exposure traditional cytotoxicity assay (Figure 5). Three of the 30 library compounds (24, 28, and 29) displayed a significant increase in activity (>20% POC difference between CTB and CTG assays) with 2DG additive. As shown in Figure 6, 7 of the 30 library compounds were observed as hits in a 24 h CTB assay, 4 were hits in a 1 h CTG, 10 were hits with 2DG additive in a 1 h CTG assay, and 7 were hits with rotenone additive in a 1 h CTG assay (combined assays shown in the Supporting Information, Figure S2).

Having established that a CTG assay performed with 1 h compound exposure time in the presence of 2DG provides comparable results to a 24 h CTB cytotoxicity assay when using H293 cells, we sought to investigate the applicability of the CTG screening method against additional cell lines. A more extensive screening of compounds 1–30 with and without metabolic inhibitors was performed using 10 additional mammalian cell lines (pancreatic—BxPC3; diploid fibroblast—HDF; cervical—HeLa; breast—MCF7, MCF10A,
MDA-MB, SkBr3, and T47D; lung—NCI-H196; and prostate—PC3) using the 1 h CTG assay and the results are shown in the Supporting Information, Table S1. A summary of the results with regard to the number of compound hits observed (out of 30 library compounds) in the absence or presence of 2DG or rotenone against each cell line is shown in Table 1.

In general, cell line T47D was the most susceptible to library compounds 1–30 at 100 μM without any additive (eight hits), and SkBr3 was the least affected (zero hits). With regard to the addition of metabolic inhibitors, the use of 2DG is more effective than rotenone. In 9 of the 11 cell lines, there were a greater number of hits when compounds were combined with 2DG than when the compounds were used without the addition of a metabolic inhibitor. Cell lines such as BxPC3, H293, MCF7, MCF10A, MDA-MB, and NCI-H196 appear to be the most affected by the addition of 2DG to the library compounds. Most notably, BxPC3 cells (pancreatic cancer) had the most pronounced effect between the assay of library compound versus compound + 2DG. Without 2DG, only 3 of the 30 compounds were identified as hits. However, when 2DG was added, 13 compounds registered as hits. Notably, this is more than the 5–7 compound + 2DG hits that were identified when using HDF or MCF10A (noncancerous) cells. This indicates that compounds at 100 μM in the presence of 2DG display evidence of cytotoxic selectivity toward a pancreatic cancer line over noncancerous cells (selectivity calculations of all cell lines are displayed in the Supporting Information, Table S2).

Analysis of the results from the perspective of the library compounds indicates that 14 out of the 30 that were tested registered as a hit in one or more of the assays performed (Table 2). Compounds that were identified as consistent hits (hits in over half of the cell lines tested) expanded from 2 out of 30 when library compound was used alone (compounds 2 and 30), to 5 out of 30 when 2DG was added (compounds 2, 5, 6, 27, and 30). Compound 30 was the most cytotoxic compound, registering as a hit in 10 of the 11 cell lines even without the use of metabolic inhibitors. The addition of 2DG had a substantial impact in combination with compound 5, resulting in a hit in assays of all 11 cell lines. Several compounds only registered as a hit when in combination with
a metabolic inhibitor (1, 22, 23, 24, and 29). Of particular significance are the compounds which register as hits in cancer cell lines, but not in noncancerous (HDF and MCF10A) cells (compounds 1, 3, 4, 7, 22, 23, and 24). Of those seven compounds, four of them are detected as hits only when used in combination with a metabolic inhibitor (compounds 1, 22, 23, and 24).

Compound 5 appeared to benefit most from the addition of 2DG, as displayed in Figure 7. In the absence of a metabolic inhibitor, 5 is only observed as a modest hit in 3 of the 11 cell lines. However, when combined with glycolytic inhibitor 2DG, 5 is detected as a hit in all 11 cell lines, and would be considered strong hits (<25% POC) in 9 of the 11 cell lines. This synergistic effect indicates that compound 5 is likely acting as an inhibitor of the TCA cycle or disrupting mitochondrial production of ATP. However, other possible modes of action exist to explain the observed results. For example, 5 could be serving with 2DG as a synergistic inhibitor of glutamine uptake by cancer cells similar to recent reports.\textsuperscript{18,19} Glutamine, like glucose, is a vital nutrient for cancer cell growth, and the inhibition of glutaminase has been a target for anticancer approaches.\textsuperscript{16,19} An additional explanation of the mode of action of 5 could involve the consumption of ATP in a cellular dead end process, thus resulting in decreased levels of ATP observed by the CTG assay. The exact nature of the synergistic mode of action of 5 in the presence of 2DG is currently under further investigation.

To investigate the enhancement of potency when 2DG was added, IC\textsubscript{50} values of 5 were determined in the presence and absence of 2DG using H293 and BxPC3 cell lines (Table 3). Upon addition of 2DG, compound potency in BxPC3 cells increased approximately 10-fold (from 208 to 18 μM).

Although compound 5 displayed the most enhanced effect against all cell lines when 2DG was added, the observed viability of noncancerous cell lines (HDF and MCF10A) at 100 μM detracts from its potential as an agent for selectivity toward cancerous cells. To identify candidates that discern cancer cells from noncancerous, a selectivity analysis was performed using the CTG screening data (Supporting Information Table S1) in which the POC value of a noncancerous cell line (HDF and MCF10A) is divided by the POC value of a cancer cell line in the absence or presence of metabolic inhibitors and results are displayed in Table S2 of the Supporting Information. In addition, a synergy analysis was performed in which the POC value of a compound without the addition of 2DG was divided by the POC value of the compound with 2DG added. A larger ratio of POC – 2DG/ +2DG indicates a higher selectivity for the cell line in the presence of 2DG. A summary of compounds 1–30 against each cell line is shown in the Supporting Information (Figure S10). It is apparent from the synergy analysis that a number of compounds (1–4, 6, 9, 22, 23, 27) when combined with 2DG display significant activity toward the pancreatic cancer cell line (BxPC3) compared to noncancerous cell lines (Figure 8). It is noteworthy that of those nine compounds, compounds 1, 3, 4, 9, and 22 were not identified as hits using a traditional 24 h cytotoxicity CTB assay with H293 cells.

Further analysis of the compounds that display enhanced synergistic activity toward BxPC3 compared to noncancerous cell lines is shown in Table 4. Although compounds 2, 6, and 27 are generally more potent in the presence of 2DG, they are observed as hits in a wide range of cell lines. Compounds 9 and 22 (an indazole and quinoline, respectively) offer interesting examples outside the scope of indoles that are most consistently observed as bioactive from 1 to 30. Compound

![Figure 6. Venn diagram of hit compounds (product numbers from Figure 1 are displayed) identified by each assay performed at 100 μM using H293 cells. A “hit” is defined as having a POC value <50%. CTG = CellTiter-Glo; CTB = CellTiter-Blue.](image-url)
Table 2. Number of Hits (<50% POC) Observed from 11 Cell Lines for Each Active Library Compound in the Absence or Presence of Metabolic Inhibitors 2DG and Rotenone.

| compound number | −2DG;−rotenone hits (out of 11 cell lines) | +2DG;−rotenone hits (out of 11 cell lines) | −2DG;+rotenone hits (out of 11 cell lines) |
|-----------------|------------------------------------------|-------------------------------------------|------------------------------------------|
| 1               | 0                                        | 3                                         | 0                                        |
| 2               | 6                                        | 10                                        | 4                                        |
| 3               | 1                                        | 3                                         | 0                                        |
| 4               | 1                                        | 3                                         | 1                                        |
| 5               | 3                                        | 11                                        | 8                                        |
| 6               | 3                                        | 8                                         | 3                                        |
| 7               | 1                                        | 1                                         | 0                                        |
| 22              | 0                                        | 1                                         | 0                                        |
| 23              | 0                                        | 3                                         | 0                                        |
| 24              | 0                                        | 5                                         | 4                                        |
| 27              | 3                                        | 8                                         | 6                                        |
| 28              | 3                                        | 5                                         | 2                                        |
| 29              | 0                                        | 1                                         | 1                                        |
| 30              | 10                                       | 10                                        | 8                                        |

All experiments were performed in duplicate using CellTiter-Glo with 1 h compound exposure time at 100 μM of library compound (graphical representation shown in the Supporting Information, Figure S9).

Table 3. IC50 Values for Compound 5 with H293 and BxPC3 Cell Lines.

| compound | cell lines | H293 (μM) | BxPC3 (μM) |
|----------|------------|-----------|------------|
| 5        | H293       | 111.3     | 208.3      |
| 5 + 2DG  | H293       | 19.2      | 18.2       |

Concentration–response curves are provided in the Supporting Information (Figure S25). Values were established using CTG assay with 1 h incubation and determined using nonlinear regression analysis in ED50plus v1.0 software for Microsoft Excel.

9 displays synergistic activity with 2DG toward BxPC3 cells but is not considered a hit at 100 μM. Compound 22 displays synergistic activity and is a moderate hit, but may require a concentration higher than 100 μM to achieve a desirable potency. The most promising of the compounds for further investigation regarding selectivity toward pancreatic cancer are compounds 1, 3, 4, and 23. Each is a hit in the presence of 2DG and displays a 2.5 to 4-fold selectivity toward BxPC3 cells over noncancerous cells. Additionally, the compounds are only observed as moderate hits in 2 of the additional 10 cell lines (H293 and T47D).

The use of a metabolic inhibitor such as 2DG to detect synergistic activity during the initial stages of screening a library of compounds without a preconceived target can provide the opportunity to classify compounds according to their activity. For example, out of compounds 1–30, several were inactive in the absence or presence of metabolic inhibitors against all cell lines in comparison to the positive control of 2DG + rotenone (19 is a representative example of an inactive compound shown in Figure 9). Other compounds displayed effects similar to the positive control (2DG + rotenone) whether in the absence or presence of metabolic inhibitors (compound 30 is shown as a representative example in Figure 9). Compound 5 is an example in which only moderate activities are observed in the absence of 2DG, but significant synergistic effects are observed across all cell lines when combined with 2DG. Finally, compound 4 is displayed as a representative example in which synergistic selectivity is observed with regard to one of the cancer cell lines tested (BxPC3) when combined with 2DG.

**Conclusions**

A new in vitro method for screening library compounds has been described that is performed in a significantly decreased operating time relative to traditional cytotoxicity screening assays (1 h as opposed to 24–72 h). The assay is highly robust, with Z'-factor calculated to be 0.805. The comparison of the newly developed rapid method to a traditional cytotoxicity...
assay has provided evidence of comparable, and in some cases superior, identification of biological activity from a library of 30 synthesized compounds against 11 mammalian cell lines (nine cancerous and two noncancerous). The rapid screening method combines library compounds with metabolic inhibitors to exploit the differences between normal and cancerous cells in the metabolic pathways associated with the cellular production of ATP. As a result of the investigation, it was observed that 2-deoxyglucose (2DG), an inhibitor of glycolysis, generally performed as a more effective synergistic activity for a cell line when 2DG is present.

![Figure 8. Synergy analysis of library compounds 1−30 against noncancerous (HDF and MCF10A) and pancreatic cancer (BxPC3) cell lines. Ratios are obtained by dividing the POC values obtained without 2DG by that of the POC values with 2DG for each cell line and library compound. A higher ratio value indicates a stronger synergistic activity for a cell line when 2DG is present.](image)

**Table 4. Selectivity Analysis of Selected Library Compounds (1, 3, 4, and 23) against Additional Cancerous Cell Lines**

| Compounds (100 μM) | H293 | BxPC3 | HeLa | MCF7 | MDA-MB | NCI-H196 | PC3 | SkBr3 | T47D |
|--------------------|------|-------|------|------|--------|----------|-----|-------|------|
| 1                  | 0.93 | 0.88  | 0.91 | 0.84 | 1.10   | 0.95     | 0.94| 0.88  | 1.62 |
| 1 + 2DG            | **1.78** | **3.13** | 1.30 | 0.99 | 1.39   | 1.25     | 1.50| 1.18  | **1.99** |
| 1 + rotenone       | 1.28 | 1.02  | 1.13 | 1.11 | 1.10   | 1.06     | 1.22| 0.97  | 1.16 |
| 3                  | 0.99 | 0.76  | 0.74 | 0.71 | 0.80   | 0.82     | 0.87| 1.01  | **2.11** |
| 3 + 2DG            | **1.70** | **2.79** | 1.08 | 0.98 | 0.89   | 1.32     | 1.22| 0.92  | **1.96** |
| 3 + rotenone       | 1.46 | 1.20  | 1.18 | 1.04 | 0.99   | 1.14     | 1.56| 1.05  | 1.73 |
| 4                  | 0.83 | 0.84  | 0.82 | 0.68 | 0.78   | 1.02     | 0.79| 0.93  | **1.51** |
| 4 + 2DG            | **1.26** | **3.58** | 1.13 | 0.92 | 1.19   | **1.53** | 1.23| 0.87  | **2.09** |
| 4 + rotenone       | 1.36 | 1.26  | 1.06 | 1.13 | 1.24   | 1.45     | 1.46| 0.93  | **1.95** |
| 23                 | 0.87 | 0.84  | 0.95 | 0.83 | 0.81   | 0.86     | 1.04| 0.93  | 1.48 |
| 23 + 2DG           | **1.83** | **2.49** | 1.00 | 0.93 | 1.01   | 1.04     | 0.94| 1.15  | **1.86** |
| 23 + rotenone      | 1.57 | 1.31  | 1.10 | 0.85 | 1.24   | 1.20     | 1.17| 1.17  | 1.43 |

Values displayed are selectivity ratios obtained using POC values from Table S1. The POC of a noncancerous cell line (HDF or MCF10A) is divided by the POC value of the compound with or without additive and the value displayed in the table is the average of the individual ratios against HDF and MCF10A (see the Supporting Information, Table S2 for additional data). A ratio >1 indicates selectivity toward the cancer cell line over the noncancerous cell line. A ratio >2 is considered to have high selectivity and is highlighted in red. Values in bold are hits (e.g., had POC values <50% against cancerous cell line) in Table S1.

![Figure 9. All values are shown as POC (percent of DMSO control) and error bars represent standard deviation from duplicate experiments. All assays shown are CellTiter-Glo with 1 h incubation time. The zone (moderate and strong) for hit detection is highlighted in green.](image)
partner with the library compounds than rotenone, an inhibitor of mitochondrial oxidative phosphorylation (TCA) pathway. Compound S, observed as a moderate hit using a traditional cytotoxicity assay, displays a significant increase in potency when combined with 2DG, likely due to S serving as a mitochondrial (TCA) inhibitor. In addition, four compounds (1, 3, 4, and 23) were identified using the rapid screening method as compounds possessing synergistic activity with 2DG that are highly selective for BxPC3, a pancreatic cancer cell line. We believe that the screening method described within will provide a powerful tool for applications that involve high-throughput, initial screenings of compounds in the field of drug discovery, and development due to: (1) drastic reduction in operational time; (2) detection of synergistic activity using an FDA-approved metabolic inhibitor such as 2DG; (3) ability to screen across various cell lines; and (4) acquisition of mechanistic information during the initial screening. A potential limitation of the screening assay is that a definitive cause for an observed decrease in cellular ATP levels by library compounds may not be provided without further investigation into the mode of action of individual hits. In addition, it is possible that a cytotoxic compound may not be detected as a hit if it functions through a mode of action that does not involve ATP production. Expansion of the synthesized library of heteroaromatic N-benzyl sulfonamides with regard to structural modification of the indole core, further inquiry into the mode of action of the compounds identified as hits in this study, and exploration into the selectivity toward pancreatic cancer cell lines are all lines of investigation currently underway in our laboratories.

**METHODS**

**Materials and Methods (Bioactivity Assays).** Cell-based Glo kits, such as CellTiter-Blue and CellTiter-Glo, were obtained from Promega (Madison, WI). All cell cultures were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All other materials and supplies were purchased from commercial sources and used without additional purification. Cell cultures were maintained in DMEM (Fisher Scientific) supplemented with 10% fetal bovine serum and Penn/Strep. Cultures were maintained in a 37 °C water-jacketed incubator with 5% CO₂. For assays that include 2-DG, a 30 mM stock solution in 1H2O was added directly to the well containing 100 μL of this dilute stock is added to the well containing 100 μL of this dilute stock in M. Fluorescence was measured either on a TECAN Safire plate reader (ex=560/em=590) or using a Promega GloMax Multi+ detection system. IC₅₀ values were determined using nonlinear regression analysis in Graph-Prism software from library compound doses of 0, 6.25, 12.5, 25, 50, and 100 μM (CellTiter-Glo assay, 1 h exposure).

**Materials and Methods (Synthesis).** All solvents and reagents were purchased from commercial sources and used without further purification. Iₐ (＞99.99%, metal basis) was purchased from Alfa Aesar. 1H and 13C NMR spectra were recorded on a Varian 400 MHz spectrometer in deuterated chloroform (CDCl₃) or deuterated acetone ((CD₃)₂CO). The solvent residual peak was used as an internal reference (CDCl₃: 1H = 7.26 ppm, 13C = 77.02 ppm; (CD₃)₂CO: 1H = 2.05 ppm, 13C = 29.84 ppm). Data are reported in the following order: chemical shifts (δ) are reported in ppm, and spin–spin coupling constants (J) are reported in Hz, while multiplicities are abbreviated by s (singlet), bs (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), bt (broad triplet), dt (doublet of triplets), and m (multiplet). A Nicolet i50 FT-IR spectrometer with peaks reported in reciprocal centimeters (cm⁻¹) was used for recording infrared spectra. Melting points (uncorrected) were recorded using a Mel-Temp II (Laboratory Devices). Accurate mass spectrum (HRMS—high-resolution mass spectrometry) was performed using a Thermo Scientific Exacta spectrometer (Waltham, MA) operating in positive and negative mode (ESI—electrospray ionization).

**Compound Characterization.** The synthesis and characterization of compounds 1–9, 11, 12, 14, 16, 18–26, 29, and 30 has been previously reported by our laboratory, and the compounds were used without additional modification.11 Compounds 13, 15, 17, 27, and 28 were synthesized following the reported procedure.11

4-Chloro-N-[(1-methyl-1H-indazol-5-yl)methyl]-benzenesulfonamide (10). White solid (12 mg, 28%). mp 148–150 °C. Purification (hexanes/EtOAc, 60:40). Rₙ = 0.19.

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**Compound Characterization.** The synthesis and characterization of compounds 1–9, 11, 12, 14, 16, 18–26, 29, and 30 has been previously reported by our laboratory, and the compounds were used without additional modification.11 Compounds 13, 15, 17, 27, and 28 were synthesized following the reported procedure.11

N-[(2-Methoxy-pyridin-3-yl)methyl]-benzenesulfonamide (13). White solid (12 mg, 32%). mp 93–97 °C. Purification (hexanes/EtOAc, 50:50). Rₙ = 0.47. 1H NMR (400 MHz, CDCl₃): δ = 7.97 (d, 1H = 5.1 Hz, 1H), 7.75 (m, 2H), 7.48 (m, 1H), 7.42–7.34 (m, 3H), 6.71 (dd, J₁ = 7.0 Hz, J₂ = 5.1 Hz, 1H), 5.40 (bs, 1H), 4.12 (d, J = 6.3 Hz, 2H), and 3.84 (s, 3H) ppm. 13C NMR (100 MHz, CDCl₃): δ 139.5, 139.2, 138.6, 132.7, 129.4, 128.6, 128.0, 126.5, 132.9, 120.5, 109.5, 47.6, and 35.7 ppm. IR (neat): ν = 3300, 2923, 2851, 1514, 1326, 1150, 1091, 817, 750, and 620 cm⁻¹. HRMS (ESI): calculated for C₁₃H₁₂ClN₂O₂S [M + H]⁺ requires m/z 336.0573, found m/z 336.0564.

N-(2-Methoxy-pyridin-3-yl)methyl]-benzenesulfonamide (13). White solid (12 mg, 32%). mp 93–97 °C. Purification (hexanes/EtOAc, 50:50). Rₙ = 0.47. 1H NMR (400 MHz, CDCl₃): δ = 7.97 (d, 1H = 5.1 Hz, 1H), 7.75 (m, 2H), 7.48 (m, 1H), 7.42–7.34 (m, 3H), 6.71 (dd, J₁ = 7.0 Hz, J₂ = 5.1 Hz, 1H), 5.40 (bs, 1H), 4.12 (d, J = 6.3 Hz, 2H), and 3.84 (s, 3H) ppm. 13C NMR (100 MHz, CDCl₃): δ 161.3, 146.2, 140.1, 137.7, 132.5, 128.8, 126.8, 118.7, 116.7, 53.3, and 43.1 ppm. IR (neat): ν = 3057, 2917, 2850, 1601, 1589, 1468, 1461, 1336, 1334, 1250, 1158, 1080, 1012, 105, 749, 693, 579, and 528 cm⁻¹. HRMS (ESI): calculated for C₁₃H₁₂ClN₂O₂S [M + H]⁺ requires m/z 279.08034, found m/z 279.0814.

N-(Pyrimidin-5-ylmethyl)benzenesulfonamide (15). White solid (6 mg, 19%). mp 54–60 °C. Purification (100%
EtOAc). Rf = 0.34. 1H NMR (400 MHz, CDCl3): δ = 9.11 (s, 1H), 8.61 (s, 2H), 7.86 (m, 2H), 7.61 (m, 1H), 7.53 (m, 2H), 5.10 (bs, 1H), and 4.21 (s, 2H) ppm. 13C NMR (100 MHz, CDCl3): δ 158.3, 156.4, 139.5, 133.2, 130.1, 129.4, 127.0, and 42.5 ppm. IR (neat): ν = 3084, 2876, 1675, 1569, 1445, 1410, 1313, 1154, 1074, 1043, and 688 cm−1. HRMS (ESI): calculated for C16H14ClN2O2S [M + H]+ requires m/z 269.04185, found m/z 269.04183.

4-Methyl-N-(1,3-thiazol-4-ylmethyl)-benzenesulfonamide (28). Tan solid (17 mg, 42%). mp 250.06502, found 250.06349. IR (neat): ν = 3084, 2876, 1675, 1569, 1445, 1410, 1313, 1154, 1074, 1043, and 662 cm−1. HRMS (ESI): calculated for C11H13N2O2S2 [M + H]+ requires m/z 250.06502, found m/z 250.06349.

N-(4-Bromo-1H-indol-3-ylmethyl)-4-chlorobenzenesulfonamide (27). White solid (7 mg, 25%). mp 186–188 °C. Purification (hexanes/EtOAc, 60:40). Rf = 0.18. 1H NMR (400 MHz, CD3CO2): δ = 10.48 (bs, 1H), 7.83 (dt, J1 = 8.6 Hz, J2 = 2.3 Hz, 2H), 7.51 (dt, J1 = 8.2 Hz, J2 = 2.3 Hz, 2H), 7.37 (dd, J1 = 8.2 Hz, J2 = 0.8 Hz, 1H), 7.33 (m, 1H), 7.16 (dd, J1 = 7.4 Hz, J2 = 0.8 Hz, 1H), 6.98 (t, J1 = 7.4 × (2) Hz, 1H), 6.61 (bt, J1 = 5.1 Hz, 1H), and 4.55 (d, J1 = 5.1 Hz, 2H) ppm. 13C NMR (100 MHz, (CD3)2CO): δ 141.1, 139.0, 138.4, 129.7, 129.6, 127.8, 127.6, 124.1, 123.4, 113.8, 112.03, 111.98, and 40.0 ppm. IR (neat): ν = 3355, 1704, 1334, 1189, 1092, and 747 cm−1. HRMS (ESI): calculated for C16H13BrClN2O2S [M − H]− requires m/z 396.94131, found m/z 396.94183.

N-(1H-Iodo-3-indol-1-ylmethyl)-benzenesulfonamide (28). Tan solid (17 mg, 42%). mp 204–206 °C. Purification (hexanes/EtOAc, 50:50). Rf = 0.59. 1H NMR (400 MHz, CD3CO2): δ = 10.06 (bs, 1H), 7.80 (dt, J1 = 9.0 Hz, J2 = 2.3 Hz, 2H), 7.49 (dt, J1 = 8.6 Hz, J2 = 2.0 Hz, 2H), 7.34 (t, J1 = 4.5 × (2) Hz, 1H), 7.16 (dd, J1 = 1.6 Hz, J2 = 0.8 Hz, 1H), 6.99 (d, J1 = 5.5 Hz, 2H), 6.75 (bt, J1 = 5.9 Hz, 1H), 4.32 (d, J1 = 5.9 Hz, 2H), and 2.44 (s, 3H) ppm. 13C NMR (100 MHz, CD3CO2): δ 141.0, 138.3, 137.1, 129.6, 129.5, 127.2, 124.7, 123.0, 121.4, 117.2, 111.9, 39.8, and 16.8 ppm. IR (neat): ν = 3402, 2863, 1600, 1458, 1412, 1314, 1258, and 1146 cm−1. HRMS (ESI): calculated for C16H12N2O2S1 [M + H]+ requires m/z 269.04131, found m/z 269.04183.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02203.

Experimental details regarding the synthesis of compounds, full results of screening assays, additional graphical depictions and Venn diagrams of results, and 1H and 13C NMR of compounds 10, 13, 15, 17, 27, and 28 (PDF)

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The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research results discussed in this publication were made possible in part by funding through the award for project number HR18-013, from the Oklahoma Center for the Advancement of Science and Technology. A.A.L. is grateful for the financial support provided by the lab start-up contribution from The University of Tulsa. The authors would also like to thank the Tulsa Undergraduate Research Challenge (TURC) and Chemistry Summer Undergraduate Research Program (CSURP) for support.

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