Therapeutic Discovery

Adenosine A2A and Beta-2 Adrenergic Receptor Agonists: Novel Selective and Synergistic Multiple Myeloma Targets Discovered through Systematic Combination Screening

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Abstract

The use of combination drug regimens has dramatically improved the clinical outcome for patients with multiple myeloma. However, to date, combination treatments have been limited to approved drugs and a small number of emerging agents. Using a systematic approach to identify synergistic drug combinations, combination high-throughput screening (cHTS) technology, adenosine A2A and β-2 adrenergic receptor (β2AR) agonists were shown to be highly synergistic, selective, and novel agents that enhance glucocorticoid activity in B-cell malignancies. Unexpectedly, A2A and β2AR agonists also synergize with melphalan, lenalidomide, bortezomib, and doxorubicin. An analysis of agonists, in combination with dexamethasone or melphalan in 83 cell lines, reveals substantial activity in multiple myeloma and diffuse large B-cell lymphoma cell lines. Combination effects are also observed with dexamethasone as well as bortezomib, using multiple myeloma patient samples and mouse multiple myeloma xenograft assays. Our results provide compelling evidence in support of development of A2A and β2AR agonists for use in multi-drug combination therapy for multiple myeloma. Furthermore, use of cHTS for the discovery and evaluation of new targets and combination therapies has the potential to improve cancer treatment paradigms and patient outcomes. Mol Cancer Ther; 1–11. ©2012 AACR.

Introduction

The identification of novel synergistic drug combinations is an important endeavor that undoubtedly will improve the outcome of cancer treatments. Unfortunately, to date, combination treatments have mainly been the result of using 2 (or more) agents already validated in the clinic or the pairing of approved with emerging drugs. As a result, the diversity of combination therapies is limited as synergistic drug pairings have been relatively few. Although there is clear evidence that cancer combinations can work better than monotherapies (1–3), to date, combination activities have not been systematically explored.

We developed a combination high-throughput screening (cHTS) approach that allows us to explore the activity for thousands of compounds to identify paired agents with synergistic activity (4, 5). During screening, a dose matrix is generated that defines single-agent and combination activity over a wide concentration range. Such a detailed analysis can help identify target-relevant effects and guide testing of lead drug combinations in vivo, as the dose matrix provides the concentration ratios where the drugs are synergistic and highlights the individual agent concentration ranges required to see combination activity. Our screening platform allows a deep exploration of drug combination space and is robust enough for analysis in many cell lines. The use of many cell lines is important, as knowledge about drug sensitivity/combination activities should be examined within the context of various genetic backgrounds and expression profiling to direct the use of drug combinations to patients who are most likely to respond to treatment.

Glucocorticoids are standard of care for a number of hematologic malignancies including multiple myeloma (MM), non–Hodgkin lymphoma, and acute lymphoblastic leukemia and are usually used in combination with other drugs for the treatment of these diseases. With multiple myeloma, the first drug used in combination was the glucocorticoid prednisone, initially paired with...
melphalan (in 1969; ref. 6). In the past decade, thalidomide, lenalidomide, bortezomib, and doxorubicin have achieved U.S. Food and Drug Administration (FDA) approval and along with melphalan, are frequently deployed as part of multicomination therapies that include either prednisone or dexamethasone (7). Preclinical studies suggested these drugs could provide benefit in combination as enhanced activity (additive or synergistic) was observed for combinations versus single-agents (8–10). The successful translation of these preclinical studies points to the value of using current multiple myeloma models for combination drug discovery.

MM.1S is a glucocorticoid-sensitive multiple myeloma cell line used frequently along with MM.1R, a derivative glucocorticoid-insensitive line, to identify and characterize compounds that kill multiple myeloma cells via glucocorticoid-dependent and -independent pathways (11). The issue of glucocorticoid sensitivity is important as this class of drug is frequently less efficacious with long-term use, requiring a switch to different drug regimens or use of additional drugs that might increase glucocorticoid sensitivity. We have used the MM.1S cell line to identify 2 classes of drugs that synergize with dexamethasone to kill multiple myeloma cells. These agents are novel, have good breadth of activity for multiple myeloma and possible use for a wider variety of B-cell malignancies. We show that combination activity is not restricted to glucocorticoids, the novel agents are synergistic with other standard-of-care drugs, suggesting benefit when used with currently deployed drug regimens.

Materials and Methods

Cell lines and reagents

All cell lines were obtained from American Type Culture Collection, DSMZ, or Lonza and subject to their authentication methods. The exceptions are MM.1S (Dr. Stephen Rosen, Northwestern University, Evanston, IL) and SU-DHL-7, OCI-ly1, OCI-ly3, OCI-ly4, OCI-ly10, and OCI-ly19 (Dr. Margaret Shipp, Dana-Farber Cancer Institute, Boston, MA). Cell identity for noncommercial lines is supported by morphology, known drug sensitivities, and gene expression studies. All compounds used in this study were obtained from commercial sources. See Supplementary Fig. S1 for information about multiple myeloma drugs and the adenosine and β2-adrenergic receptor (β2AR) agonists used in this study.

Combination high-throughput screening

Drug combination studies were conducted by plating MM.1S cells in 384-well plates at 1,000 to 1,500 cells per well, cultured for 18 to 24 hours, and treated with increasing concentrations of each drug to generate a dose matrix (4, 5). After 72 hours, cell viability was assessed by ATPlite luminescence using an EnVision 2103 according to the manufacturer’s instructions (PerkinElmer Life and Analytical Sciences, Inc.).

Analysis of combination activity

A “dose matrix” was used to evaluate the combination effects between compounds, where all possible mixtures of serially diluted single agents at the indicated concentrations were evaluated including zero (12–14). Synergy was determined by the Loewe additivity model, a generally accepted reference for synergy (15–17). The isobologram (18) shows how much less drug is required in combination to achieve a desired effect level, when compared with the single-agent doses needed to reach that effect. The combination index (CI) is the ratio of the total effective drug dose of the combination compared with that of the single-agents required to achieve a given effect level (16). A more detailed discussion of combination activity analysis can be found in the Supplementary Fig. S2.

Patient samples

Samples collected from informed patients who had provided informed consent according to Dana-Farber Cancer Institute Institutional Review Board–approved protocol. Tumor cells were isolated using CD138 microbeads (Miltenyi Biotec) and viability measurements conducted as described (19).

Animal studies

Male severe combined immunodeficient (SCID) mice 6 to 8 weeks of age (Charles River Laboratories) were used for MM.1S and H929 xenograft studies. Female SCID mice 5 to 6 weeks of age (Harlan, Inc.) were used for the RPMI-8226 studies. Animal-shaven flanks were subcutaneously injected with $1.5 \times 10^7$ logarithmically growing MM.1S and H929 cells or $1 \times 10^7$ RPMI-8226 cells. Once tumors grew to 130 to 250 mm$^3$ in size, animals were pair-matched by tumor size into treatment and control groups. Drug stocks were prepared in EtOH and diluted with PBS to generate a 10% EtOH:90% PBS solution for injections. Dexamethasone, CGS-21680, and salmeterol were delivered by subcutaneous injection daily, whereas bortezomib was injected intravenously via the tail vein. Control animals received 10% EtOH:90% PBS s.c. daily. Animals were removed from the study if there was overall poor body condition, the tumor volume was above 3,000 mm$^3$, body weight loss was greater than or equal to 20%, or if the tumors became ulcerated. Mean change in body weight and tumor volume was calculated on the last day, when there was not more than one animal removed from each study group. Statistical differences were analyzed using an ANOVA. Normal distribution was determined by the goodness of fit for normality test. When warranted by the omnibus test, a Tukey–Kramer honestly significant difference (HSD) post hoc analysis was used to determine differences between individual groups.

Microarray analysis

MM.1S cells were not treated or treated for 6 hours with CGS-21680 (12.5 nmol/L), salmeterol (1 nmol/L), dexamethasone (25 nmol/L or 2 μmol/L), CGS-21680 (12.5 nmol/L), and H929 cells or 107 RPMI-8226 cells. Once tumors grew to 130 to 250 mm$^3$ in size, animals were pair-matched by tumor size into treatment and control groups. Drug stocks were prepared in EtOH and diluted with PBS to generate a 10% EtOH:90% PBS solution for injections. Dexamethasone, CGS-21680, and salmeterol were delivered by subcutaneous injection daily, whereas bortezomib was injected intravenously via the tail vein. Control animals received 10% EtOH:90% PBS s.c. daily. Animals were removed from the study if there was overall poor body condition, the tumor volume was above 3,000 mm$^3$, body weight loss was greater than or equal to 20%, or if the tumors became ulcerated. Mean change in body weight and tumor volume was calculated on the last day, when there was not more than one animal removed from each study group. Statistical differences were analyzed using an ANOVA. Normal distribution was determined by the goodness of fit for normality test. When warranted by the omnibus test, a Tukey–Kramer honestly significant difference (HSD) post hoc analysis was used to determine differences between individual groups.
Results

The identification of adenosine receptor and β2AR agonists as potent enhancers of multiple myeloma drugs

The MM.1S multiple myeloma cell line was used to screen approved drugs and molecular probes to identify targets that synergize with dexamethasone to inhibit proliferation. A total of 1,601 unique combinations were evaluated using a 384-well dose matrix format, which facilitates the identification of synergistic activity through analysis of drug combination activity over a wide concentration range (4, 5). Two classes of compounds were found that are potently synergistic with dexamethasone, exemplified by CGS-21680, an adenosine receptor (AdR) agonist (Fig. 1A), and salmeterol (Fig. 1D), a β2AR agonist. Both agents, when combined with dexamethasone, increase the maximal effect and potency of dexamethasone (Fig. 1B and E) and are strongly synergistic as revealed by isobologram analysis (Fig. 1C and F) and the calculated excess inhibition over the predicted Loewe additivity model (Supplementary Fig. S2A). Other AdR agonists and β2AR agonists are also strongly synergistic in combination with dexamethasone (Supplementary Fig. S2B and S2C), suggesting that activation of these receptors is important for activity. As combination activity is observed broadly in many multiple myeloma cell lines (Supplementary Fig. S3A and S3B and see below), AdR and β2AR agonists may represent novel multiple myeloma drug targets.

Glucocorticoids are invariably used in the clinic in combination with one or more drugs. As AdR or β2AR agonists might be added to existing multiple myeloma combination therapies, understanding how these agonists affect the activities of other approved multiple myeloma drugs is important. We therefore examined the antiproliferative activity of melphalan, lenalidomide, bortezomib, and doxorubicin in combination with either an AdR agonist or β2AR agonist using the MM.1S cell line. Surprisingly, synergy was observed with agonists that target either receptor class (CGS-21680, an AdR agonist, and salmeterol, a β2AR agonist) when in combination with melphalan, lenalidomide, bortezomib, or doxorubicin (Tables 1 and 2; Supplementary Fig. S4A and S4B). To determine whether the combination activity is cytostatic or cytotoxic, MM.1S cells were treated with individual agents or drug combinations, and at various times, cells were analyzed by flow cytometry to determine the percentage of cells that express the apoptotic marker Annexin V. As shown in Supplementary Fig. S5A, treatment of cells with 20 nmol/L CGS-21680 (AdR agonist), 0.13 nmol/L salmeterol (β2AR agonist), or 20 nmol/L dexamethasone for 24 or 48 hours had minimal effects on cell viability. In contrast, substantially greater induction of apoptosis was observed when either of these agonists was used in combination with dexamethasone, with most of the apoptosis occurring within 24 hours of drug exposure. The combinations containing CGS-21680 or salmeterol with lenalidomide (Supplementary Fig. S5B) or bortezomib (Supplementary Fig. S5C) also showed greater induction of apoptosis by the combination as compared with the single agents. As a more stringent test of drug treatment on cell viability, we examined the extent to which salmeterol and bortezomib affect the ability of RPMI-8226 multiple myeloma cells to form colonies when used as single agents or in combination. Cells were exposed to drugs for 5 hours, washed, plated at low density in Methocult, and incubated for 2 weeks to allow for colony formation. Salmeterol and bortezomib each individually had some effect, with enhanced activity observed when the drugs were used together (Supplementary Fig. S5D), consistent with proliferation and apoptosis assay results.

Validation of the A2A adenosine and β2AR subtypes as multiple myeloma drug targets

We observe AdR agonist combination activity with CGS-21680 (an AdR A2A subtype agonist), Chloro-IB-MECA (A3 receptor agonist), ADAC (A1 receptor agonist), HE-NECA (an AdR pan-agonist), and other AdR agonists (Supplementary Fig. S2B and data not shown). All are active in our assays. Although some agonists are preferentially selective for a given AdR subtype, selectivity is lost when the compounds are tested at increasing concentrations due to the relatedness of the receptor subtypes and the cross-reactive nature of the compounds. We have used AdR subtype selective antagonists and siRNA to show that the A2A receptor (A2AR) subtype is singularly responsible for AdR agonist antiproliferative effects (19).

We took the same approach to unequivocally show that β2AR is important for activity and synergy. Using MM.1S cells, 0.25 μmol/L lenalidomide was combined with 5 nmol/L clenbuterol (a potent β2AR agonist) and combination activity observed. Addition of the β2AR antagonist 0.9 μmol/L butoxamine did not affect lenalidomide activity but reduced clenbuterol single-agent and combination activity (Supplementary Fig. S6A and S6B). Increasing the butoxamine concentration to 9 μmol/L abolished clenbuterol activity without reducing the lenalidomide effect. Similar results were obtained with a second selective β2AR antagonist, ICI 118551 (Supplementary Fig. S6C). As additional validation, 2 different siRNAs targeting the β2AR receptor reduce the antiproliferative activity of the β2AR agonist levamlbuterol, but not an siRNA targeting A2AR (Supplementary Fig. S6D). Finally, neither the β-1 agonist dobutamine nor the β-3 agonist BRL-37344 has antiproliferative activity as single agents or in combination with dexamethasone at target-relevant concentrations, consistent with the β2AR subtype as the target of interest (data not shown).
Breadth of activity and selectivity

To further examine A2AR and β2AR agonist selectivity and breadth, we evaluated combination activity in a panel of 83 cell lines including solid tumor types and hematologic malignancies. Single agents and combinations with dexamethasone and melphalan were systematically studied at multiple ratios of clinically relevant concentrations. Using a quantitative synergy score based on the Loewe model (13, 15), we observe that combination activity for A2AR or β2AR agonists is highly selective for hematologic malignancies with synergy observed most frequently in multiple myeloma and diffuse large B-cell lymphoma (DLBCL) cell lines (Fig. 2; Supplementary Fig. S7). With A2A agonists, synergy is also observed with the B-cell lines JM-1 [pre-B-acute lymphocytic leukemia (ALL)] and GA-10 (Burkitt lymphoma). Using a relative synergy cutoff (synergy score > 1), we find that 13 of the 18 multiple myeloma cell lines tested show a synergistic interaction between the A2AR agonist CGS-21680 and dexamethasone (Fig. 2A) and 11 of 18 show a synergistic interaction between CGS-21680 and melphalan (Supplementary Fig. S7A). Using this same measure, 9 of 18 multiple myeloma cell lines show synergy with combinations of the β2AR agonist salmeterol with either dexamethasone or melphalan (Fig. 2B; Supplementary Fig. S7B). Eight and 6 of the cell lines in this multiple myeloma cell panel were insensitive or responded weakly to dexamethasone (<50% inhibition at 0.225 μmol/L) and melphalan (<50% inhibition at 10 μmol/L), respectively, when assayed as single agents. All cell lines were treated with the same concentrations of dexamethasone or melphalan, pointing to A2AR agonists having a higher breadth of activity across the multiple myeloma cell line panel than β2AR agonists. An intriguing observation is the strong synergy observed for A2AR or β2AR agonists with dexamethasone in the glucocorticoid-insensitive cell lines EJM and ANBL-6, which suggests that these agents may help restore steroid sensitivity in refractory patients (Fig. 2; Supplementary Fig. S3).

Figure 1. Adenosine receptor and β2AR agonists strongly synergize with dexamethasone and other standard-of-care drugs to inhibit multiple myeloma cell proliferation. A and D, a 9 × 9 dose matrix was generated for AdR agonist CGS-21680 × dexamethasone (A) and β2AR agonist salmeterol × dexamethasone (D). The matrix samples all mixtures of 2 serially diluted single-agent concentrations. Inhibition is visualized using a color scale. The data in A are the average of 6 dose matrix whereas data in D are the average of 2 dose matrix. Control wells (no drug addition) are not part of the 9 × 9 dose matrix whereas data in D are the average of 2 dose matrix. Control wells (no drug addition) are not part of the 9 × 9 dose matrix whereas data in D are the average of 2 dose matrix. Percent inhibition at 50% inhibition at 10 μmol/L, respectively, when assayed as single agents. All cell lines were treated with the same concentrations of dexamethasone or melphalan, pointing to A2AR agonists having a higher breadth of activity across the multiple myeloma cell line panel than β2AR agonists. An intriguing observation is the strong synergy observed for A2AR or β2AR agonists with dexamethasone in the glucocorticoid-insensitive cell lines EJM and ANBL-6, which suggests that these agents may help restore steroid sensitivity in refractory patients (Fig. 2; Supplementary Fig. S3).
Combination effects were not observed when CGS-21680 or salmeterol was used in combination with dexamethasone or bortezomib in nontransformed cells (Supplementary Fig. S8).

Analysis of A2AR and β2AR agonist activity in antitumor models ex vivo and in vivo

We examined the combination activity of the A2AR agonist CGS-21680 and β2AR agonist salmeterol in combination with dexamethasone using CD138+ multiple myeloma cells isolated from the bone marrow of 4 patients. Tumor cells were incubated with clinically relevant concentrations of single agents or combinations for 48 hours and viability determined (Fig. 3). Tumor cells from all 4 patients were less viable when treated with A2A or β2AR agonists and dexamethasone versus the single agents. Patients 1 and 3 had the best response. Cells from patient 4 had the worst response. Interestingly, single-agent or combination activity was examined in 3 different multiple myeloma cell lines (Fig. 4 and Table 3). In MM.1S cells, combination of CGS-21680 and dexamethasone showed expected levels of single-agent activity in these models. CGS-21680 and salmeterol alone showed modest activity at best. Importantly, the addition of either CGS-21680 or bortezomib resulted in no additional toxicity in these models as measured by body weight change.

Transcriptome analysis

As a first step toward understanding the multitarget mechanisms important for combination drug synergy, we generated gene expression profiles for MM.1S cells either not treated or after 6-hour incubation with single agents or combinations. Supervised hierarchical analysis was conducted to identify genes upregulated or downregulated by combination drug treatment (Supplementary Fig. S9). Some potential mechanism-relevant drug combination–specific gene expression changes are highlighted in Fig. 5. The transcription factors MAF, IRF4, and MYC are all downregulated upon combination treatment. The rate-limiting metabolic enzymes HK2, PDK1, CYCS, SCD, and FASN are also downregulated, as is CDK6, important for cell-cycle progression. Upregulation of the proapoptotic gene BCL2L11 (BIM) and reduction of antiapoptotic BCL2L1 (BCL-XL) is observed. TSC22D3 (GILZ) a leucine zipper transcription factor is upregulated by combination drug treatment.

Table 1. Combination activities for AdR agonist CGS-21680 with multiple myeloma standard-of-care drugs

<table>
<thead>
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<th>AdR combination (CGS-21680)</th>
<th>CI</th>
<th>CI %I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib</td>
<td>0.75</td>
<td>55</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.26</td>
<td>60</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.45</td>
<td>75</td>
</tr>
<tr>
<td>Lenalidomide</td>
<td>0.21</td>
<td>55</td>
</tr>
<tr>
<td>Melphalan</td>
<td>0.41</td>
<td>65</td>
</tr>
</tbody>
</table>

NOTE: CGS-21680 was crossed with multiple myeloma standard-of-care drugs bortezomib, dexamethasone, doxorubicin, lenalidomide, and melphalan in MM.1S cells using the 9 × 9 dose matrix format (see Supplementary Fig. S3A and S3B for representative data). The CI was determined at the effect levels listed (CI%I). The CI is an estimate of how much drug was needed in combination relative to the single-agent doses required to achieve the chosen effect level and CI values <1 are synergistic. See Supplementary Fig. S1 legend for additional information.

Table 2. Combination activities for β2AR agonist salmeterol with multiple myeloma standard-of-care drugs

<table>
<thead>
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<th>β2AR combination (salmeterol)</th>
<th>CI</th>
<th>CI %I</th>
</tr>
</thead>
<tbody>
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<td>Bortezomib</td>
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<td>40</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.23</td>
<td>50</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.37</td>
<td>50</td>
</tr>
<tr>
<td>Lenalidomide</td>
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</tr>
<tr>
<td>Melphalan</td>
<td>0.43</td>
<td>55</td>
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</table>

NOTE: β2AR was crossed with multiple myeloma standard-of-care drugs bortezomib, dexamethasone, doxorubicin, lenalidomide, and melphalan in MM.1S cells using the 9 × 9 dose matrix format (see Supplementary Fig. S3A and S3B for representative data). The CI was determined at the effect levels listed (CI%I). The CI is an estimate of how much drug was needed in combination relative to the single-agent doses required to achieve the chosen effect level and CI values <1 are synergistic. See Supplementary Fig. S1 legend for additional information.
Discussion

Using cHTS, we have discovered that agonists of either the A2A or β2AR have potent synergistic antiproliferative activity when used in combination with dexamethasone (Fig. 1 and Tables 1 and 2; Supplementary Figs. S2B, S2C, S3A, and S3B). Induction of apoptosis is rapid and occurs within 24 hours of drug treatment (Supplementary Fig. S5). Synergistic combination activities are also observed when A2AR or β2AR agonists are crossed with the multiple myeloma drugs melphalan, lenalidomide, bortezomib, and doxorubicin at clinically relevant concentrations (Tables 1 and 2; Supplementary Fig. S4A and S4B). The A2AR and β2AR are essential for activity as antagonists or siRNA specific for the A2AR or β2AR block agonist effects (ref. 19; Supplementary Fig. S6). Greater than additive drug combination activities are observed with cell lines, patient tumor samples cultured ex vivo (Fig. 3), and multiple myeloma xenografts (Fig. 4 and Table 3).

We screened 83 cell lines and observed that A2AR and β2AR agonists combination activities are highly selective for hematologic malignancies with synergy observed most frequently in multiple myeloma and diffuse large B-cell lymphoma cell lines (Fig. 2; Supplementary Fig. S7). The analysis of additional cell lines and combinations may point to wider use of these agents for cancer.
treatment. In our analysis, A2AR agonists had a highest breadth of activity across the 18 multiple myeloma cell line panel. The multiple myeloma cell lines differ with respect to sensitivity to agonists as single agents, which may help explain the lack of combination activity in some cell lines.

Multiple myeloma is currently an incurable disease. While many patients respond to one or more existing

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**Figure 3.** A2AR and β2AR agonist combination activity in patient tumor cells cultured ex vivo. A, sensitivity of primary multiple myeloma tumor cells to CGS-21680 and dexamethasone (patients 1–3) and bortezomib (patient 4) as single agents and in combination. B, sensitivity to salmeterol and dexamethasone (patients 1–3) or salmeterol and bortezomib (patient 4) as single agents and in combination. The results for the agonists and dexamethasone single agent and combination are the mean and SD from 3 to 6 replicate measurements whereas with bortezomib/dexamethasone, 2 replicate measurements. Bort, bortezomib; Combo, combination; Dex, dexamethasone; Sal, salmeterol.

**Figure 4.** In vivo activities of the A2A AdR agonist CGS-21680 and the β2AR agonists salmeterol in combination with dexamethasone using an MM.1S xenograft model. Mice (6–7 per group; see Table 3) were challenged subcutaneously with MM.1S cells and treated with the various single agents and combinations indicated. See Materials and Methods for additional details. Dex, dexamethasone.
Another interesting property of A2AR and β2AR agonists is their ability to increase the maximal efficacy and potency of multiple myeloma drugs. Potency shifts can be particularly important for drugs that have significant adverse effects that limit their use. Bortezomib is one such drug, which is difficult to dose near the maximum tolerated dose for long durations due to peripheral neuropathy, and dose reductions are frequent in older more fragile patients. The use of bortezomib in combination with A2AR or β2AR agonists may allow a reduction in dose (and adverse effects) without loss of efficacy. This hypothesis is supported by the observation in MM.1S xenografts, the addition of CGS-21680 to a β2AR agonist with significant adverse effects that limit their use.

<table>
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<tr>
<th>Cell line</th>
<th>Group</th>
<th>n</th>
<th>Frequency</th>
<th>mg/kg</th>
<th>Route</th>
<th>Mean change (%)</th>
<th>Mean change (%)</th>
<th>Mean, d</th>
<th>P &lt; 0.05 vs. parts (ANOVA)</th>
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<td>s.c.</td>
<td></td>
<td>11 ± 2.7</td>
<td>1,636.5 ± 446.7</td>
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<td>29.9 ± 2.9</td>
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<td>Dexamethasone</td>
<td>7</td>
<td>Daily</td>
<td>1, s.c.</td>
<td></td>
<td></td>
<td>4.0 ± 4.1</td>
<td>1,546.2 ± 149.4</td>
<td>26</td>
<td>35.4 ± 1.4</td>
</tr>
<tr>
<td>MM.1S</td>
<td>Vehicle</td>
<td>7</td>
<td>Daily</td>
<td>s.c.</td>
<td></td>
<td>10.4 ± 1.4</td>
<td>2,667.2 ± 353.2</td>
<td>26</td>
<td>27.4 ± 1.7</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>7</td>
<td>Daily</td>
<td>d1,4/weekly × 9</td>
<td>i.v.</td>
<td></td>
<td>13.1 ± 7.9</td>
<td>717.6 ± 193.5</td>
<td>26</td>
<td>40.7 ± 4.2</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>5</td>
<td>Daily</td>
<td>d1,4/weekly × 9</td>
<td>0.5</td>
<td>i.v.</td>
<td>15.7 ± 3.9</td>
<td>1,589.1 ± 505.5</td>
<td>26</td>
<td>37.2 ± 4.5</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>7</td>
<td>Daily</td>
<td>d1,4/weekly × 9</td>
<td>0.1</td>
<td>i.v.</td>
<td>13.1 ± 3.2</td>
<td>2,574.7 ± 228.0</td>
<td>26</td>
<td>27.7 ± 1.21</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>7</td>
<td>Daily</td>
<td>3, s.c.</td>
<td></td>
<td></td>
<td>8.9 ± 4.6</td>
<td>1,803.8 ± 212.0</td>
<td>26</td>
<td>33.3 ± 1.6</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>7</td>
<td>Daily</td>
<td>3, s.c.</td>
<td></td>
<td></td>
<td>13.2 ± 3.1</td>
<td>956.1 ± 189.1</td>
<td>26</td>
<td>41.0 ± 2.6</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>7</td>
<td>Daily</td>
<td>d1,4/weekly × 9</td>
<td>0.5</td>
<td>i.v.</td>
<td>13.1 ± 7.9</td>
<td>717.6 ± 193.5</td>
<td>26</td>
<td>40.7 ± 4.2</td>
</tr>
<tr>
<td>CGS-21680</td>
<td>8</td>
<td>Daily</td>
<td>3, s.c.</td>
<td></td>
<td></td>
<td>13.0 ± 4.6</td>
<td>1,546.2 ± 149.4</td>
<td>26</td>
<td>35.4 ± 1.4</td>
</tr>
<tr>
<td>RPMI-8226</td>
<td>Bortezomib</td>
<td>10</td>
<td>Daily × 21</td>
<td>s.c.</td>
<td></td>
<td>7.7 ± 1.3</td>
<td>1,156.6 ± 67.7</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>10</td>
<td>Daily × 25</td>
<td>10</td>
<td>s.c.</td>
<td></td>
<td>7.9 ± 1.8</td>
<td>774.8 ± 93.4</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>10</td>
<td>Daily × 36</td>
<td>1</td>
<td>s.c.</td>
<td></td>
<td>−2.9 ± 1.0</td>
<td>56.7 ± 8.1</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>10</td>
<td>For 3 days × 6</td>
<td>1</td>
<td>i.v.</td>
<td></td>
<td>−7.0 ± 1.8</td>
<td>37.3 ± 7.4</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>10</td>
<td>For 3 days × 6</td>
<td>0.5</td>
<td>i.v.</td>
<td></td>
<td>3.7 ± 1.2</td>
<td>766.0 ± 79.7</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>10</td>
<td>Daily × 36</td>
<td>10</td>
<td>s.c.</td>
<td></td>
<td>−6.5 ± 0.9</td>
<td>29.1 ± 2.7</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>Daily × 36</td>
<td>1</td>
<td>s.c.</td>
<td></td>
<td></td>
<td>4.0 ± 4.1</td>
<td>1,546.2 ± 149.4</td>
<td>26</td>
<td>35.4 ± 1.4</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>Daily × 18</td>
<td>10</td>
<td>s.c.</td>
<td></td>
<td></td>
<td>4.0 ± 4.1</td>
<td>1,546.2 ± 149.4</td>
<td>26</td>
<td>35.4 ± 1.4</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>10</td>
<td>For 3 days × 6</td>
<td>1</td>
<td>i.v.</td>
<td></td>
<td>18.5 ± 3.1</td>
<td>341.0 ± 40.4</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>Salmeterol</td>
<td>10</td>
<td>Daily × 18</td>
<td>10</td>
<td>s.c.</td>
<td></td>
<td>4.0 ± 4.1</td>
<td>1,546.2 ± 149.4</td>
<td>26</td>
<td>35.4 ± 1.4</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>10</td>
<td>For 3 days × 6</td>
<td>0.5</td>
<td>i.v.</td>
<td></td>
<td>5.2 ± 1.8</td>
<td>341.0 ± 40.4</td>
<td>19</td>
<td>NA</td>
</tr>
</tbody>
</table>

NOTE: The activity of A2A AdR agonist CGS-21680 and β2AR agonist salmeterol in combination with multiple myeloma standard-of-care agents, dexamethasone and bortezomib. Activity was examined in 3 xenograft models.

Abbreviation: NA, not available.
confer a level of activity similar to that of a higher, more effective dose (0.8 mg/mL).

As our 18 multiple myeloma cell line panel is unlikely to fully capture the genetic complexity found in patients, it was important to examine patient tumor cells A2AR and β2AR drug sensitivity and combination activities \textit{ex vivo}. Our analysis of patient tumor samples with CGS-21680 or salmeterol plus dexamethasone (3 patients) and CGS-21680 or salmeterol plus bortezomib (1 patient) confirms that combination activity can also be observed \textit{ex vivo} (Fig. 3). In general, our results suggest that sensitivity of tumor cells to single agents increases the likelihood of combination activity. The combination effects observed, including absence of antagonism with standard-of-care drugs, suggest that A2A and β2AR agonists may provide benefit if added to current approved drug regimens.

As a first step toward understanding the molecular basis of combination drug synergy, microarray analysis was conducted to study drug-induced gene expression changes. The analysis provides insight into how the combination drugs potently kill multiple myeloma cells. We find that \textit{IRF4} is downregulated, an essential gene that when silenced by RNA interference results in the rapid cell death of multiple myeloma cell lines (22). MYC, a direct target of IRF4, is also downregulated. Like IRF4, silencing of MYC in multiple myeloma cells is toxic (22). RNA levels for some downstream targets of these transcription factors are also reduced. These genes play key roles in cell metabolism and growth and include HK2, PDK1, and CYCS (glycolysis); SCD (lipid synthesis); and CDK6 (cell-cycle regulation). Gene set enrichment analysis (GSEA) using a 36-member gene set of \textit{IRF4} target genes (Supplementary Fig. S10)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{heatmap.png}
\caption{Transcriptional profile for select genes upregulated or downregulated by combination drug treatment. The MM.1S cell line either untreated (lanes 1 and 2) or treated for 6 hours with 1 nmol/L salmeterol (lanes 3 and 4), 12.5 nmol/L CGS-21680 (lanes 5 and 6), low-dose dexamethasone (25 nmol/L; lanes 7 and 8), high-dose dexamethasone (2 μmol/L; lanes 9 and 10), or combinations (CGS-21680 or salmeterol in combination with low-dose dexamethasone; lanes 11-14). The data were normalized with Gene Pattern module ExpressionFileCreator using the GCRMA method. The color scheme in the heatmap represents the relative expression level of the gene detected by the probe set in each row.}
\end{figure}
further supports IRF4 pathway effects upon combination drug treatment.

In addition to IRF4 and MYC, the transcription factor MAF is downregulated. Exogenous expression of dominant-negative MAF inhibits multiple myeloma proliferation (23). Combination drug treatment upregulates the leucine zipper protein GILZ (TSC22D3) to a great extent than with dexamethasone alone. Previously, it has been shown that GILZ is upregulated when MM.1S cells are treated with glucocorticoids and that reducing expression with siRNA decreases glucocorticoid-induced cell death (24). These observations make GILZ an attractive candidate contributor to combination activity. Other genes where combination drug-specific changes in gene expression may adversely affect cell survival include PIM-2, TGFB1, PRKD2, CSNK1E, CDKN1C, GADD45A, ID2, BCL2L11, and BCL2L1 (25–32). Follow-up analysis of the temporal changes in gene expression after combination drug treatment will help distinguish primary from secondary events.

To our knowledge, our laboratory is the first to report the surprising discovery that A2A agonists synergize with oncology drugs to induce apoptosis in preclinical models of multiple myeloma and other B-cell malignancies. Other promising therapeutic areas for A2AR agonist development include inflammation, reperfusion injury, sepsis, and diabetic peripheral neuropathy (33, 34). At present, the only FDA-approved A2AR agonists are regadenoson and adenosine, for use in combination with a cardiac imaging agent for patients who are unable to exercise during cardiac stress tests (35). Regadenoson has a short half-life, which makes it ideal for cardiac imaging as effects can be rapidly terminated once the procedure has been completed. For use in treatment of multiple myeloma and other B-cell malignancies, molecules with longer transit circulation times coupled with dosing regimens that provide adequate exposure will be required. Our results suggest that A2AR agonists can synergize with multiple myeloma drugs at concentrations below those where cardiovascular effects arise, making them an attractive new class of drugs for the treatment of multiple myeloma.

Numerous β2AR agonist drugs have been developed for treatment of asthma and chronic obstructive pulmonary disease as they cause smooth muscle relaxation, resulting in the dilatation of bronchial passages (36). Most of the newer β2AR agonists have been formulated for delivery by inhalation and are not optimized for systemic exposure. Interestingly, β2AR agonists are often paired with corticosteroids (Advair Diskus, the combination of salmeterol and fluticasone, and Symbicort, paired with corticosteroids (Advair Diskus, the combination delivery by inhalation and are not optimized for systemic use). Each drug has distinct effects (β2AR agonist as a bronchodilator and corticosteroids anti-inflammatory), there may be beneficial synergy between these 2 classes of medication (37). Corticosteroids can enhance the expression of β2AR, which may provide some protection against desensitization and development of tolerance to agonists. β2 adrenergic agonists may amplify the anti-inflammatory effects of corticosteroids by facilitating nuclear translocation of the glucocorticoid receptor complex and enhance transcription and expression of steroid-inducible genes in proinflammatory cells (38). The plethora of β2AR agonist drugs suggest a quick path to potential proof-of-concept multiple myeloma clinical studies.

In recent years, drug discovery efforts have focused on the identification of agents that modulate validated therapeutic cancer targets (39–41). Given the complexity of roadblocks to cancer drug effectiveness, it is easy to see why the identification and deployment of combination therapies is such an important endeavor. The plasticity of tumor cell responses to drugs that affect growth and survival is best corralled using multitargeted therapies. Cancer cells have less opportunity to adapt and drug synergies are most likely to reduce tumor load. An additional benefit is that synergistic combinations of 2 or more drugs may improve therapeutically relevant selectivity and reduce the toxicity and other side effects associated with high doses of single drugs (14). The exquisite selectivity of A2AR and β2AR synergies for tumor cells of the B-cell lineage support the idea that context-dependent synergy can confer selectivity. Our use of a robust cHTS screening platform allows the analysis of many drug pairings to identify interesting combination effects and the determination of breadth of activity in cancers with different genetic backgrounds. The characterization of lead combinations in disease-relevant secondary assays such as ex vivo, microenvironment, and immune models will facilitate the identification of novel cancer combination therapies with increased chance of selectivity and translation.

Disclosure of Potential Conflicts of Interest

R.J. Rickles and M.S. Lee have ownership interest in Zalicus. D.W. McMillin has ownership interest in and is employed by Axios Biosciences as the founder. C.S. Mitsiades has received commercial research grants from Amgen, AVEO Pharma, OSI, EMD Serono, Sunesis, Gloucester Pharmaceuticals, Genzyme, Johnson & Johnson, licensing royalties from PharmaMar, and is a consultant for Millennium, Celgene, Novartis, Bristol-Myers Squibb, Merck & Co., Kosean Pharmaceuticals, Pharmion, Centocor, and Amnis Therapeutics. A.A. Borisy is employed by Zalicus and CombinatoRx (as the CEO); has an ownership interest in Zalicus, CombinatoRx, Forma Therapeutics, and Blueprint Medicines; and is a consultant/advisory board member for Gene Network Sciences. K.C. Anderson is a consultant/advisory board member for Celgene, Novartis, Millennium, BMS, Onyx, and Merck and is a founder of Acetylom. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

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References


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