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Bisbenzimidazoles: Anticancer Vacuolar (H\(^+\))-ATPase Inhibitors

Renukadevi Patil, Olivia Powrozek, Binod Kumar, William Seibel, Kenneth Beaman, Gulam Waris, Neelam Sharma-Walia and Shivaputra Patil

Abstract

Small molecule chemotherapeutic agents such as Imatinib, Gefitinib, and Erlotinib have played a significant role in the treatment of cancer. Although the unprecedented progress has been achieved in cancer treatment with these targeted agents, there is a strong demand for the development of selective and highly efficacious cancer drugs. V-ATPases are emerging as important target for the identification of novel therapeutic agents for cancer. Our screening and drug discovery processes have identified the bisbenzimidazole derivative (RP-15) as a potent anticancer V-ATPase inhibitor. In the present study, bisbenzimidazoles (compound-25, RP-11 and RP-15) have been tested for proton-pump inhibition activity in human hepatoma cell line (Huh7.5). RP-15 displayed comparable proton-pump inhibition activity to the standard Bafilomycin A1. We examined the antiproliferative activity of these analogs in two highly invasive and metastatic inflammatory breast cancer (IBC) cell lines (SUM 149PT and SUM190PT) along with Huh7.5. The compound-25 (SUM190PT: IC\(_{50}\) = 0.43±0.11 μM) and its structural analog RP-11 (SUM190PT: IC\(_{50}\) = 0.49±0.09 μM) have shown significant inhibition toward IBC cell lines. Additionally, RP-11 and RP-15 have demonstrated very good cytotoxicity toward the majority of cancer cell lines in the NCI 60 cell line panel.

Keywords: bisbenzimidazoles, anticancer, V-ATPase, proton-pump, inhibitors

1. Introduction

Since Paul Ehrlich’s introduction of the concept of chemotherapy, development of chemotherapeutic agents for cancer over the past several decades has seen marvelous records of accomplishments [1, 2]. Cancer is one of the major health problems globally and is second leading cause of death in the USA [3, 4]. Cancer is a very complex disease and our understanding towards it has been advanced tremendously over the last six decades since the first human cancer cell line HeLa identified in 1952 [5]. Over the past few years, the search for new anticancer drugs has changed dramatically. Advances in the molecular nature of drug action, new technology and more recently market considerations have produced new approaches to cancer drug discovery [6]. Recent advances in molecular biology, high throughput screening
(HTS), computer-aided drug design (CADD), and combinatorial chemistry technologies have allowed a combination of both knowledge around the drug receptor and large library screening to be used for anticancer drug discovery today [7–10].

As the understanding of human biology and new technologies progressed, the discovery and development process moved from a random pattern to a more predictable one. The development of a molecularly targeted anticancer drug has gained importance in recent years [11]. One of the important small molecule targeted therapy, Imatinib (Gleevec®), a tyrosine kinase inhibitor, achieved incredible advancement in cancer treatment [12–14]. Imatinib’s success stimulated the scientists to develop variety of targeted anticancer agents including Gefitinib (Iressa™) and Erlotinib (Tec евa®) for the treatment of different types of cancer patients (Figure 1). Targeted agents represented significant developments in cancer treatment and have increased the life expectancy of patients [15–18]. Despite the unprecedented progress achieved, the anticancer drug discovery research remains highly challenging and there is strong demand for the development of highly efficacious and safe anticancer drugs which can overcome cancer metastasis, and drug resistance.

Recent studies suggest that an acidic microenvironment in the tumor is responsible for cancer development, progression, and metastasis. Novel drugs that specifically target the mechanism by which V-ATPase lowers the pH of the tumor microenvironment are essential for cancer chemotherapy. Among the key regulators of the tumor, acidic microenvironment V-ATPases plays an important role in the regulation of the pH gradient. V-ATPases play a vital role in the maintenance of the tumor acidic microenvironment and are overexpressed in many types of metastatic cancers including breast cancer. V-ATPases are functionally expressed in plasma membranes of tumor cells and they have specialized functions in metastasis [19]. Recent research has demonstrated that the preferential expression of V-ATPase at the cell surface is important for the acquisition of invasiveness and the metastasis of breast cancer cells [19]. Therefore, V-ATPase is a potential target to investigate for metastatic breast cancer therapy. Discovery and development of easily synthesized,
cost-effective, and potent small molecule drugs targeting V-ATPase are needed to evaluate the therapeutic potential of V-ATPase inhibitors in metastatic breast cancer.

The V-ATPases are a family of ATP-driven proton pumps that couple ATP hydrolysis with translocation of protons across membranes. The V-ATPase proton pump is a macromolecular complex composed of at least 14 subunits organized into two functional domains, V1 is responsible for ATP hydrolysis and V0 provides the transmembrane proton channel [20–23]. The V-ATPases have been associated with cancer invasion, metastasis and drug resistance [19, 24–27]. Several pre-clinical studies have reported the anticancer effects of V-ATPase inhibitors [28–32]. V-ATPase inhibitors will be beneficial for cancer patients given either in combination with cytotoxic agents or dual-acting (anticancer and V-ATPase inhibitor) agents. Thus, V-ATPases are emerging as an important target for the identification of potential novel chemotherapeutic agents. Despite the clear involvement of V-ATPases in cancer, to date, therapeutic use of V-ATPase targeting small molecules have not reached the clinic. Natural products macrolide antibiotics, such as bafilomycin and concanamycin, potently inhibit V-ATPases [33–37] (Figure 2), but their use is complicated by non-specific effects on other targets. Moreover, these molecules have been difficult to synthesize in large quantities. Despite huge efforts by both academic and pharmaceutical industry medicinal chemists, development of useful V-ATPase inhibitors has been limited because of the complicated chemical structures of existing natural inhibitors.

We have been actively involved in the design and development of novel small molecular agents for different types of cancers. Past few years, we have reported the chromene-, chromenopyridine-, and imidazoquinoline-based pharmacophores as initial lead anticancer drug candidates through screening and drug development process [38–40]. Notably, we have identified the highly potent microtubule targeting anticancer agent (SP-6-27) for ovarian cancer [41]. Since then our laboratory has been active in identifying anticancer agents with different mechanisms of action. In continuation of our drug discovery research, we recently initiated a collaborative effort on the V-ATPases as anti-cancer targets. Successful identification of new lead small molecule drugs for ovarian cancer by screening and drug development processes [41] inspired us to screen the library of compounds based on the literature of known V-ATPase inhibitors. We identified the bisbenzimidazole scaffold from screening process. Bisbenzimidazoles are nitrogen heterocycles with wide spectrum of biological activities. We

Figure 2.

Natural potent V-ATPase inhibitors.
reported the focused set of bisbenzimidazoles as anticancer V-ATPase agents (Figure 3) [42]. Bisbenzimidazole derivatives (RP-3–RP-15) have been screened in selected human breast cancer (MDA-MB-231, MDA-MB-468, MCF-7) and ovarian cancer (cisplatin-sensitive A2780, cisplatin-resistant Cis-A2780 and PA-1) cell lines. Among this small set of bisbenzimidazoles, RP-15 demonstrated high potency towards the epidermal growth factor receptor (EGFR) over expressed triple negative breast cancer (TNBC) cell line, MDA-MB-468 (IC₅₀ = 0.04 ± 0.02 μM). Very interestingly, RP-15 is not toxic to normal breast epithelial cells. It is nearly 40 times less toxic in the normal breast epithelial cell line, MCF10A (IC₅₀ = 1.62 ± 0.14 μM). Furthermore, the bisbenzimidazole derivatives (Compound-25, RP-11 and RP-15) have demonstrated encouraging proton pump inhibition activity in MDA-MB-231. In particular our most efficacious anticancer analog RP-15 has shown comparable proton pump inhibition activity to standard agent Bafilomycin A1.

In the present study, we selected and screened top two bisbenzimidazole derivatives (RP-11 and RP-15) along with initial hit (compound 25) for proton pump inhibition activity in human hepatoma cells, Huh7.5 using pH indicator Lysosensor Yellow/Blue DND-160. These compounds have also been screened for their antiproliferative activity using BrDU incorporation assay in selected inflammatory breast cancer (IBC) cell lines (SUM149PT and SUM190PT) along with Huh7.5 human hepatoma cancer cell line. Additionally, RP-11 and
RP-15 have been tested in NCI Developmental Therapeutics Program (DTP) nine major (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer) 60 human cancer cell lines.

2. Methods

2.1 Chemical synthesis

We recently reported the synthesis and detailed characterization of all these new bisbenzimidazoles [42]. In brief, we developed a fast and efficient synthetic one pot procedure to prepare all these analogs (RP-3–RP-15). Condensation of 4-(6-(4-methylpiperazin-1-yl)-1H, 30H-[2, 50-bibenzo [d]imidazol]-20-yl) phenol with substituted alkyl halides in the presence of cesium carbonate in dimethyl formamide (DMF). For the more detailed synthesis and spectral and analytical characterization of all these compounds please see Ref. [42].

2.2 Proton pump inhibition activity in human hepatoma (Huh7.5) cell line

We used Huh7.5 cell line for proton pump activity. Briefly, the Huh7.5 cells were cultured in DMEM media supplemented with 10% serum to a confluency of 80%. The Huh7.5 cells were treated with the compounds (Compound-25, RP-11 and RP-15) at a concentration of 12 μM for 20 minutes followed by incubation with Lysosensor Yellow/Blue DND-160 (10 μM) for 10 minutes at 37°C. The cells were visualized under the microscope.

2.3 Antiproliferative activity

Cell proliferation ELISA BrdU colorimetric (assay no. 11647229001; Roche, Basel, Switzerland) was used to quantify cell proliferation by the measurement of BrdU incorporated during DNA synthesis. Cells from a 90% confluent T-25 flask were seeded 100 μL/well of 96-well plates and incubated overnight. Dimethyl Sulfoxide (DMSO) stock solutions of the compounds (Compound-25, RP-11 and RP-15) were diluted in pure F-12 media and exposed to different concentrations for 24 and 48 hours. Each concentration and controls were done in triplicates. The mean ± standard deviation (S.D.) was calculated and shown on the graph with untreated cells serving as a negative control, 20 minutes after adding the substrate, the absorbance was read at 370 nm. The compound concentration that inhibited cell growth by 50% of the untreated control (IC_{50}) was calculated from the dose response curves constructed by normalizing the data to percentages based of the negative control and a nonlinear regression analysis in GraphPad Prism Software 7 (GraphPad Software, San Diego, CA, USA). For the Huh7.5 cell line we used CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA).

2.4 The NCI 60 cell lines in vitro screening

The bisbenzimidazoles (RP-11 and RP-15) have been tested for growth inhibition against 60 human cancer cell lines from the NCI’s anticancer screening program. The NCI’s screening procedure has been given in detail elsewhere [43–47] and presently DTP uses the sulforhodamine B (SRB) assay.
3. Results and discussion

Inhibition of V-ATPase has shown the link between cell biophysical properties and proliferative signaling selectively in malignant hepatocellular carcinoma (HCC) cells, which provides a new strategy to combat HCC [48]. HCC is the third most common cause of cancer-related deaths worldwide. HCC is accounting for almost 90% of primary malignant hepatic tumors in adults. In continuation of our work on V-ATPase inhibition, we used Huh7 cells for the proton pump inhibition activity. We have performed proton pump inhibitory activity of selected bisbenzimidazole derivatives (Compound-25, RP-11 and RP-15) in Huh7 cells using Lysosensor Yellow/Blue DND-160 protocol [49]. The DND-160 is a pH indicator and cellular compartments with acidic pH elicit yellow fluorescence when stained, while the destabilized compartments with higher pH elicit blue fluorescence.

The compound RP-15 displayed maximum inhibition of the proton-pump activity of V-ATPase followed by compound-25 and RP-11. The untreated cells showed the strong intensity of yellow fluorescence (converted to pseudo-green in the Figure 4A) while the cells treated with bisbenzimidazoles (Compound-25, RP-11 and RP-15) showed the strong intensity of blue fluorescence representing varying degree of destabilization of pH due to impaired vacuolar ATPase activity (Figure 4A and B). Additionally, these compounds have been tested for their cytotoxicity towards Huh7.5 cells using the CellTiter-Glo Luminescent Cell Viability Assay. The IC_{50} were calculated based on the results obtained for these compounds treated for 24 hours only for Huh7.5 cells compared to breast and ovarian cancer cell lines where we treated all test compounds for 48 hours. Bisbenzimidazoles, RP-11 and RP-15 have demonstrated very moderate antiproliferative activity towards Huh7.5 cells for 24 hours (Table 1).

High potency of bisbenzimidazole analog (RP-15) against the EGFR over expressed TNBC cell line (MDA-MB-468) inspired us to explore the selected bisbenzimidazoles in other breast cancer cell lines for anticancer activity. We selected two IBC cell lines (triple negative SUM149PT and Het2 positive SUM190PT) for the in vitro screening process [50]. Both SUM149 and SUM190 cell lines have been established from primary IBC tumors. IBC is one of the highly invasive, metastatic and lethal variant of human breast cancer. Development of therapeutic targets and agents for IBC is still in very early stage and it represents an opportunity for medicinal chemists to develop novel (pre) clinical drug candidates.

In vitro screening of the bisbenzimidazoles (Compound-25, RP-11 and RP-15) towards these inflammatory cell lines has shown encouraging results (Figure 5 and Table 1). Very interestingly our initial hit, compound-25 (SUM149PT: IC_{50} = 0.80 ± 0.08 μM; SUM190PT: IC_{50} = 0.43 ± 0.11 μM) and its structural analog RP-11 (SUM149PT: IC_{50} = 0.91 ± 0.15 μM; SUM190PT: IC_{50} = 0.49 ± 0.09 μM) have shown very good inhibition, whereas our TNBC lead RP-15 (SUM149PT: IC_{50} = 1.77 ± 0.08 μM; SUM190PT: IC_{50} = 2.08 ± 0.56 μM) has demonstrated moderate inhibition towards these IBC cell lines. The high potency shown by compound-25 and RP-11 towards IBC has given us more insights to develop new anticancer agents for it and we plan to explore the structure-activity relationship (SAR) studies based on the bisbenzimidazole scaffold in very near future.

The Development Therapeutic Program (DTP) of the National Cancer Institute's 60 human tumor cell lines screen was developed as an in vitro drug discovery tool. We submitted both compounds (RP-11 and RP-15) to the NCI Developmental Therapeutics Program (DTP) anticancer drug screen. Both of them have been first tested for three cell lines (MCF-7 breast cancer; NCI-H460 large-cell lung cancer; SF-268 glioma) to advance to the 60 cell line screen. This pre-screen process eliminates the inactive compounds but preserves active agents for 60 cell line screening.
Both compounds have been advanced to 60 cell lines representing nine major cancers (leukemia, non-small cell lung, central nervous system, colon, melanoma, ovarian, renal, prostate, and breast). Compounds have been tested over a broad range of concentrations against every cell line in the panel (five 10-fold dilutions starting...
Figure 5.
The cell viability (%) of breast cancer cell lines (SUM190PT and SUM149PT) following the exposure of various concentrations of bisbenzimidazoles (Compound-25, RP-11 and RP-45) for 48 hours.

Table 1.
Half maximal inhibitory concentration of novel bisbenzimidazole analogs in different cancer cell lines.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>IC_{50} ± SD (μM)</th>
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<tr>
<td></td>
<td>SUM149PT</td>
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<tr>
<td>C-25</td>
<td>0.80 ± 0.08</td>
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<tr>
<td>RP-11</td>
<td>0.91 ± 0.15</td>
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<tr>
<td>RP-15</td>
<td>1.77 ± 0.08</td>
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<td>Baf A1</td>
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ND: not determined.

‡ Data from Ref. [42].

† The IC50 is calculated based on the results obtained from 24 hours drug treatment only.
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Figure 6. Dose response curves derived from screening of compound RP-11 (NSC: D-800436) in 60 cell line screen using nine major human cancer cell lines (leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer).

Figure 7. The mean graph representation of antitumor effects of compound RP-11 (NSC: D-800436). The GI_{50} (50% of growth inhibition), TGI (total growth inhibition) and LC_{50} (50% of lethal concentration) mean graphs are derived from the dose response curves using Figure 6 from the initial screening.
from $10^{-4}$ M concentration). Figures 6 and 8 describe the dose response curves for compounds RP-11 (NSC: D-800436) and RP-15 (NSC: D-800437) respectively. From these dose response curves three end points were calculated (GI50: 50% of growth inhibition; TGI: total growth inhibition; LC50: 50% of lethal concentration). Figures 7 and 9 demonstrate mean graph patterns for compound RP-11 and RP-15 respectively. Mean graphs are created for GI50, TGI, and LC50 by plotting positive and negative values termed as deltas generated from dose response curves. More sensitive cell lines are displayed as bars that project to the right of the mean, whereas the less sensitive cell lines are displayed with bars projected to the left. The length of each bar is proportional to the relative sensitivity of the agent with the mean determination.

Both bisbenzimidazole analogs, RP-11 and RP-15 demonstrated very good cytotoxicity towards the majority of cancer cell lines in the 60 cell line panel. Compound RP-11 displayed growth inhibition and total growth inhibition to low micromolar range and is moderate towards LC50 for MCF7 (GI50: 0.32 μM, TGI: 11.8 μM and LC50: 88.7 μM), MDA-MB-468 (GI50: 1.42 μM, TGI: 4.16 μM and LC50: 28.2 μM) and MDA-MB-231 (GI50: 2.25 μM, TGI: 6.49 μM and LC50: 60.4 μM).

Interestingly, it showed low micromolar range effects against other cell lines such as SR (GI50: 0.50 μM); NCI-H522 (GI50: 0.34 μM); COLO 205 (GI50: 0.37 μM); SF-268 (GI50: 0.58 μM); OVCAR-3 (GI50: 0.62 μM) and MDA-MB-435 (GI50: 0.62 μM) (Table 2). Compound RP-15 shows similar behavior as RP-11. Compound RP-15 exhibited GI50: 1.91 μM, TGI: 4.13 μM and LC50: 8.91 μM for the MDA-MB-468 cell line, whereas a similar trend is observed for the MDA-MB-231 cell line (GI50: 2.85 μM, TGI: 5.83 μM and LC50: 21.3 μM). Similarly, low micromolar growth inhibition was observed for other cell lines such as MDA-MB-435 (GI50: 1.97 μM), RXF

Figure 8.
Dose response curves derived from screening of compound RP-15 (NSC: D-800437) in 60 cell line screen using nine major human cancer cell lines (leukemia, non small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer and breast cancer).
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Figure 9. The mean graph representation of antitumor effects of compound RP-15 (NSC: D-800437). The GI\(_{50}\) (50% of growth inhibition), TGI (total growth inhibition) and LC\(_{50}\) (50% of lethal concentration) mean graphs are derived from the dose response curves using Figure 8 from the initial screening.

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<td>LC(_{50})</td>
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Chemistry and Applications of Benzimidazole and its Derivatives

393 (GI<sub>50</sub>: 1.91 μM), HT29 (GI<sub>50</sub>: 1.78 μM), LOXIMVI (GI<sub>50</sub>: 1.84 μM), DU-145 (GI<sub>50</sub>: 1.83 μM) and KM12 (GI<sub>50</sub>: 1.91 μM) (Table 2). Overall, the NCI 60 cell line results are encouraging for both new bisbenzimidazole derivatives.

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<td>TGI</td>
<td>LC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>GI&lt;sub&gt;50&lt;/sub&gt;</td>
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| NCI-H322M          | 3.07       | 9.93       | > 100              | 11.6       | 23.8       | 48.8
| NCI-H460           | 3.63       | 14.7       | > 100              | 2.12       | 3.98       | 7.44
| NCI-H522           | 0.34       | 2.36       | > 100              | 13.6       | 38.1       | > 100
| Colon cancer       |            |            | A498               | 1.21       | 3.39       | 9.54
|                  |            | 10.7       | > 100              | 9.20       | 22.8       | 54.2
| COLO 205           | 0.37       | 3.05       | > 100              | 12.9       | 30.0       | 69.8
|                  | 2.25       | > 100      | > 100              | 4.15       | 15.7       | 47.2
| HCC-2998           | 1.78       | 3.78       | 8.03               | 5.06       | 18.5       | 56.7
|                  | 1.80       | 3.63       | 7.32               | 1.91       | 3.84
| HCT-116            | 0.36       | 2.46       | > 100              | 1.82       | 3.44
|                  | 1.42       | 11.8       | > 100              | 4.00       | 15.4       | 66.2
| HCT-15             | 38.7       | > 100      | > 100              | 12.1       | 28.1       | 65.4
|                  | 4.74       | 40.2       | > 100              | 18.2       | 38.8       | 83.0
| HT29               | 0.58       | > 100      | > 100              | 1.78       | 4.07       | 9.30
|                  | 29.4       | > 100      | > 100              | 21.3       | 41.8       | 82.3
| KM12               | 2.58       | 14.3       | 65.4               | 1.91       | 4.20       | 9.26
|                  | 2.19       | 24.5       | > 100              | 2.63       | 5.81       | 21.8
| SW-620             | 0.90       | 10.2       | 36.5               | 2.12       | 3.96       | 7.37
|                  | 2.13       | > 100      | > 100              | 1.83       | 3.40       | 6.29
| CNS cancer         |            |            | DU-145             | 1.13       | 9.59       | > 100
|                  |            |            | MDA-MB-231/ATCC    | 2.25       | 6.49       | 60.4
|                  |            |            |                  | 2.85       | 5.83       | 21.3
| SF-268             | 0.58       | 5.66       | 62.9               | 6.58       | 25.4       | 81.0
|                  |            |            | Breast cancer      |            |            |            |
| SF-295             | 1.38       | 15.7       | > 100              | 2.34       | 6.22       | 48.4
|                  | 0.32       | 11.8       | 88.7               | 1.85       | 3.96
| SF-539             | 1.22       | 2.83       | 6.59               | 11.5       | 26.1       | 59.5
|                  | 2.25       | 6.49       | 60.4               | 2.85       | 5.83       | 21.3
| SNB-19             | 1.87       | 10.5       | 52.9               | 2.15       | 4.09       | 7.79
|                  | 2.94       | 15.9       | > 100              | 10.5       | 37.1       | > 100
| SNB-75             | 0.49       | 3.14       | 15.2               | 3.46       | 18.0       | 47.0
|                  | 1.76       | 3.96       | 89.1               | 17.5       | 40.1       | 91.7
| U251               | 0.66       | 10.2       | 36.5               | 2.16       | 3.81       | 6.74
|                  | 1.00       | 9.09       | > 100              | 14.2       | 41.1       | > 100
| Melanoma           |            |            |                  |            |            |            |
| LOX IMVI           | 0.85       | 2.83       | 8.66               | 1.84       | 3.67
|                  |            |            |                  |            |            |            |
| MALME-3 M          | 0.16       | 1.55       | 4.61               | 12.2       | 27.2       | 60.4
|                  |            |            |                  |            |            |            |
| M14                | 0.45       | 2.50       | 9.80               | 2.39       | 6.53       | 40.0
|                  |            |            |                  |            |            |            |

Table 2. The NCI 60 cancer cell line screening results.

4. Conclusions and future directions

In summary, our screening and drug discovery processes have identified the bisbenzimidazole (RP-15) as a potent anticancer V-ATPase inhibitor for TNBC and RP-11 as initial lead for the IBC. The compound RP-15 showed maximum inhibition of the proton-pump activity which is comparable to our standard agent Bafilomycin A1. The in vitro antiproliferative activity of these bisbenzimidazole analogs (Compound-25, RP-11 and RP-15) towards IBC cell lines revealed that compound-25 and its structural analog RP-11 could be possibly considered for further exploration in other IBC cell lines. Bisbenzimidazoles RP-11 (NSC: D-800436) and RP-15 (NSC: D-800437) have demonstrated very good cytotoxicity towards the majority of cancer cell lines in the NCI 60 cell line panel. Overall, our research identified efficacious and selective anticancer V-ATPase inhibitors for TNBC and
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IBC. We will continue to explore the SAR with this exciting pharmacophore to identify the highly selective and potent V-ATPase inhibitors which will ultimately lead to the generation of investigational new drug (IND) candidates for the clinical testing in TNBC and IBC patients.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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