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REVIEW ARTICLE

PAWI-2: A novel inhibitor for eradication of cancer

Jiongjia Cheng[1,2] · John R. Cashman[1,2]

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Abstract

Cancer is a major worldwide public health problem and is still the leading cause of death in the United States. There are many types of cancer treatment but completely successful results are oftentimes not attained. It remains a challenge to develop efficacious clinically useful cancer therapies. Therapies targeting dysregulated signal transduction pathways in cancer can be efficacious anti-cancer therapies with minimal adverse effects. In this study, we focus on novel small molecule **p**53 **A**ctivator **W**nt Inhibitor-**2** (PAWI-2) that was developed by optimizing potency and pharmaceutical properties. PAWI-2 is a nontoxic DNA-damage pathway inhibitor that shows a broad spectrum of potency and significant efficacy in vitro and in vivo. This study focuses on the application of PAWI-2 to four major types of cancers including colorectal cancer (CRC), breast cancer (BC), prostate cancer (PCa), and pancreatic cancer (PC). PAWI-2 shows a novel mechanism of action (MOA) by modulating two mechanisms of cancer invasion. In cancer with unimpaired p53, PAWI-2 activates DNA-damage checkpoint and mitochondrial p53-dependent apoptotic signaling. Consistently observed in most cancer types, PAWI-2 induces phosphorylation of optineurin (OPTN) to cause G2/M cell cycle arrest. These two mechanisms operate regardless of p53 variants and/or KRAS mutation status and also manipulate the effect of PAWI-2 to overcome tumor stemness and drug resistance in PC stem cells (PCSCs). This study summarizes the development of PAWI-2 as an attractive targeted therapeutic for mechanism-driven anti-cancer drug discovery.

Keywords Colorectal cancer · Breast cancer · Prostate cancer · Pancreatic cancer · Targeted therapy · PAWI-2

Introduction

Cancer is a major worldwide public health problem and is the second leading cause of death in the United States (US). In 2019, there were ~1,762,450 new cancer cases diagnosed and about 606,880 cancer deaths that occurred in the US (Siegel et al. 2020). There are many types of cancer treatment, including surgery, radiation therapy, chemotherapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplantation, and precision medicine (National Cancer Institute Types of Cancer Treatment 2020). These therapies can be applied either alone or in combination with other drugs in the treatment of cancer. However, many important challenges are still present in the field of cancer therapies that need to be met to improve treatment outcomes for patients.

To date, nonsurgical treatment of cancer (mainly conventional chemotherapy, targeted biological therapies, and radiotherapy) has not generated completely satisfactory results. Conventional chemotherapies have many problems including low target selectivity, drug resistance, inability to effectively address metastatic disease and oftentimes, severe side effects. Targeted biological therapies (i.e., monoclonal antibodies) are promising but are relatively expensive and are not broadly applicable. The pathogenesis of cancer is characterized by clinically relevant genetic alterations

Dedication: This study is dedicated to Professor Robert Hanzlik on the occasion of his retirement from the University of Kansas. As a graduate student working under Professor Hanzlik and later as a practicing scientist, I learned a great deal from Bob. He provided very thoughtful perspectives on science, teamwork, and professional networking. Most importantly, he taught me about the joy of science. He was kind, good-hearted, and firm—all virtues of a compassionate teacher and wonderful mentor. Because I worked on a sulfur-containing compound under his direction, it is only fitting that the story told herein is about a new approach to addressing cancer with sulfur-containing compound PAWI-2.

✉ Jiongjia Cheng
jcheng@hbri.org

[1] Human BioMolecular Research Institute, San Diego, CA 92121, USA

[2] ChemRegen, Inc., San Diego, CA 92121, USA

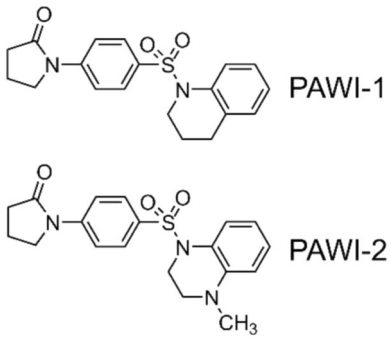

Fig. 1 Chemical structure of PAWI-1 and PAWI-2

leading to either activation of oncogenes or inactivation of tumor suppressor genes (e.g., inactivation of p53 function) (Anastas and Moon 2013; Kahn 2014; Lane et al. 2010; Levine and Oren 2009). Therapies targeting protein components of dysregulated signal transduction pathways can be efficacious anti-cancer therapies with minimal adverse effects (Anastas and Moon 2013; Kahn 2014; Lane et al. 2010; Levine and Oren 2009). For example, tumor suppressor protein p53 plays a critical role in cellular response to DNA damage and other genomic aberrations, and is an attractive target for mechanism-driven anti-cancer drug discovery (Lane et al. 2010). In some cases, targeting p53 has achieved good clinical responses that have affected survival in some cancers.

The present study focuses on a novel small molecule called **p**53 **A**ctivator **W**nt **I**nhibitor-**2** (i.e., PAWI-2, Fig. 1) that was developed after numerous rounds of medicinal chemistry refinement to optimize potency and pharmaceutical properties. As previously reported, PAWI-2 is a non-toxic DNA-damage pathway inhibitor that suppresses cancer cell growth and survival (Cashman et al. 2013; Cheng et al. 2018, 2019a, 2019b; Okolotowicz et al. 2018; Cheng and Cashman 2020) in several cancer types including colorectal cancer (CRC) (Cheng et al. 2018), breast cancer (BC) (Okolotowicz et al. 2018), prostate cancer (PCa) (Cheng et al. 2019a), and pancreatic cancer (PC) (Cheng et al. 2019b; Cheng and Cashman 2020). PAWI-2 shows a broad spectrum of potency and significant efficacy in vitro and in vivo. This study also provides overviews regarding the mechanisms of action of PAWI-2 in various types of cancer (Fig. 2).

SAR of PAWI compounds and ADMET of PAWI-2

"Hit" compound PAWI-1 (Fig. 1) was identified as an inhibitor of canonical Wnt/β-catenin-dependent transcription in a high-throughput screen. After numerous rounds of medicinal chemistry refinement (i.e., chemical synthesis of analogs of PAWI-1), PAWI-2 was identified and developed as a lead compound with greater potency and more promising pharmaceutical properties than hit PAWI-1 (Cashman et al. 2013; Okolotowicz et al. 2018). Compared with PAWI-1, PAWI-2 incorporates a tertiary amine HCl salt (Fig. 1). This increased aqueous solubility of PAWI-2. PAWI-2 also has improved potency for Wnt inhibition, inhibition of cancer cell proliferation (two-fold greater potency), improved modulation of physicochemical properties, and also improved pharmacological properties including ADMET (Cashman et al. 2013; Okolotowicz et al. 2018).

PAWI-2 possesses drug-like ADMET properties. PAWI-2 is chemically stable (i.e., >30 days, no change, pH 7.4). PAWI-2 is not extensively metabolized (i.e., stable for >200 min in mouse, rat, and human liver microsomes + NADPH) although minor (i.e., 5%) N-demethylation was observed in small animal and human liver microsomes supplemented with NADPH. In human liver microsomes, PAWI-2 does not inhibit the metabolism of testosterone suggesting it does not inhibit prominent human P-450s. PAWI-2 has acceptable pharmacokinetic properties including a favorable half-life (i.e., 16 h), T_{max}, and bioavailability (i.e., ~20%). PAWI-2 dose-dependently accumulated in tumors based on LCMS analysis. Compared with PAWI-1, the lack of metabolism and greater aqueous solubility of PAWI-2 affords more favorable pharmacokinetic properties. As described below, PAWI-2 is not detectably toxic to small animals. In our view, the efficacy of PAWI-2 to ameliorate multiple forms of cancer (i.e., prostate, breast, colon and pancreatic, and other cancers) points to the importance of inhibition of key cancer-causing signaling pathways and not to some arbitrary toxicity.

By applying the same anti-cellular viability study used in cancer cell studies, in normal cells, PAWI-2 did not affect cell growth (i.e., immortalized normal mouse epithelial cells) (Cheng et al. 2018). At a supermaximal concentration (i.e., up to 5 μM), PAWI-2 did not show any detectable inhibition of cell viability of normal intestine cells (IEC-6) (Cheng et al. 2018) or normal prostate epithelial cells (11220-hTERT) (Cheng et al. 2019a). The conclusion is that PAWI-2 only operates to selectively kill rapidly dividing cancer cells and not quiescent normal cells. In Ames-like tests, no direct or indirect genotoxicity or cytotoxicity was observed for PAWI-2 (i.e., up to 10 μM) with or without metabolic bioactivation (Cheng et al. 2018).

In small animal studies, it was shown that PAWI-2 was safe in vivo (no toxicity after 7 days, 30 mg/kg/day, i.p. or oral, mice) and nontoxic (at 1000 mg/kg, i.p., rats; 20 mg/kg/day, 30 days, mice). Histopathological and morphometric evaluation of tissues from animals dosed with large amounts of PAWI-2 (i.e., 1000 mg/kg for 24 h (rats) or

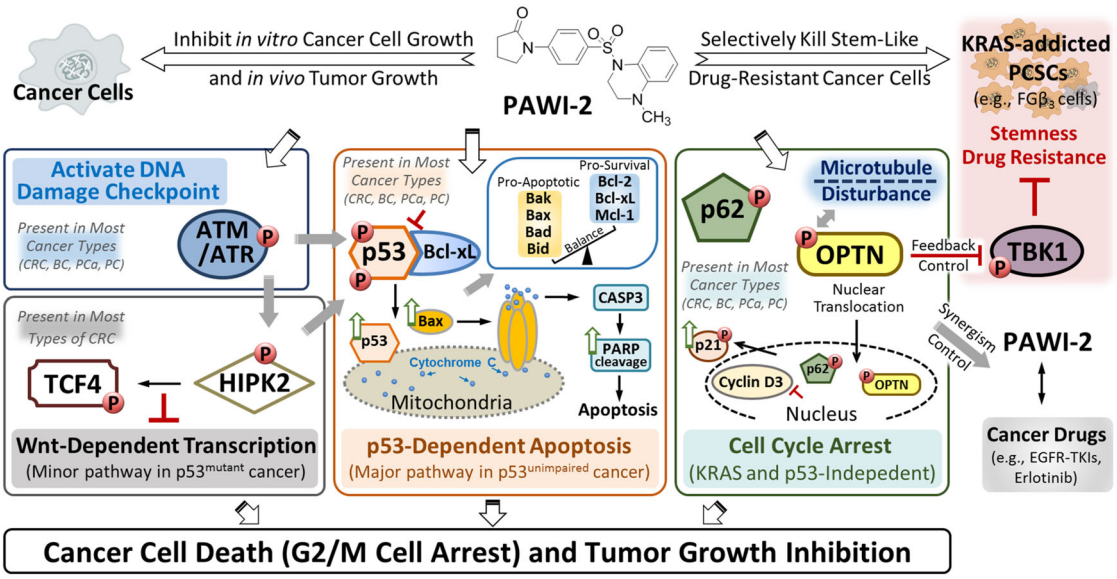

Fig. 2 Proposed model depicts a mechanism of PAWI-2 in various types of cancer. Green arrows, stimulations; red lines, inhibition. CRC colorectal cancer, BC breast cancer, PCa prostate cancer, PC pancreatic cancer, PCSCs pancreatic cancer stem cells, KRAS Kirsten rat sarcoma viral oncogene homolog, TBK1 TANK-binding kinase 1, ATM ataxia-telangiectasia mutated serine/threonine kinase, ATR ATM- and Rad3-related serine/threonine kinase, HIPK2 homeodomain interacting protein kinase 2, TCF4 transcription factor 4/immunoglobulin transcription factor 2 (ITF-2), Wnt wingless integration site, p53 tumor protein p53, Bcl-2 B-cell lymphoma 2 protein, Bcl-xL B-cell lymphoma-extra-large protein, Mcl-1 induced myeloid leukemia cell differentiation protein, Bak Bcl-2 homologous antagonist/killer protein, Bax Bcl-2-associated X protein, Bad Bcl-2 associated agonist of cell death protein, Bid BH3 interacting-domain death agonist protein, CASP3 caspase-3, PARP poly (ADP-ribose) polymerase, p62 (p62/SQSTM1) ubiquitin-binding protein/sequestosome-1, OPTN optineurin, p21 (p21/Cip1) cyclin-dependent kinase inhibitor 1/CDK-interacting protein 1, EGFR-TKIs epidermal growth factor receptor-tyrosine kinase inhibitors, G2/M Gap 2 phase/mitotic phase (subphase of interphase in the cell cycle)

20 mg/kg/day for 30 days (mice)) did not show any abnormalities. So far as we can see, PAWI-2 does not appear to show any toxicity at the doses examined in small animals.

Colorectal cancer

CRC is the third leading cause of cancer-related death in the US. It is anticipated to result in an estimated 53,200 deaths during 2020 (Siegel et al. 2020). Therapeutic options for CRC are limited to surgery, radiation, or chemotherapy and are often effective for early-stage disease but not for metastatic CRC. CRC drugs frequently cause untoward gastrointestinal and hematologic side effects with limited clinical benefit (Curtin 2013; Sridharan et al. 2014). Initiation and progression of CRC has been linked to dysregulation of several signaling pathways including Wnt/β-catenin (Morin et al. 1997; Su et al. 1993) and p53 pathways (Fearon 2010; Iacopetta 2003) and mutations of key molecular regulators that promotes a greater metastatic phenotype. Inhibition of Wnt/β-catenin signaling and regulators of p53 (i.e., MDM2, leading to p53 stabilization and activation) are considered attractive anti-CRC approaches (Lane et al. 2010; Novellasdemunt et al. 2015). However, various adverse effects (i.e., side effects, toxicities) limit long-term treatment with first generation anti-CRC drugs and have prompted ongoing development of less toxic drug-like molecules (Anastas and Moon 2013; Kahn 2014; Tisato et al. 2017).

PAWI-2 works against CRC cells targeting both Wnt signaling and ATM/p53

Shown by in vitro characterization, PAWI-2 potently inhibited cell growth of multiple types of CRCs (IC_{50}s, 10–20 nM) through inhibition of Wnt transcription, activation of p53, and binding of tubulin to afford its anti-mitotic and anti-proliferative activity. Although other anti-mitotic agents have been reported (Janssen and Medema 2011; Rao et al. 2012), the effect of PAWI-2 on Wnt and p53 pathways constitutes a novel and unprecedented mechanism. In contrast to other compounds that modulate the β-catenin complex, the action of PAWI-2 is due to its target downstream of Wnt inhibition at the β-catenin complex.

PAWI-2 activates mitotic stress signaling via ATM/ATR-kinase (Fig. 2) in CRC. Phosphorylated ATM (Ser1981) and p53 (Ser15 and Ser46) were induced by the treatment of PAWI-2, and this showed activation of DNA-damage-sensitive cell cycle checkpoints (Cheng et al. 2018). PAWI-2 induced p53-dependent cell apoptosis regardless of p53 mutation types (i.e., HCT-116, WT; DLD-1, Ser241Phe;

SW480, Arg273His/Pro309Ser) (Cheng et al. 2018), showing PAWI-2 could restore the tumor suppressor role of p53 in CRC cells with unimpaired p53 status (Fig. 2). Here, we define "unimpaired p53 status" as that standing for both wild-type (WT) p53 and missense mutations of p53 that retain some or all of p53 functional activity. The opposite of this definition is null p53 status. This distinguishes PAWI-2 from other p53-targeted cancer inhibitors (e.g., AMG-232), that work as MDM2 inhibitors but only promote restoration of critical p53 function in WT p53 tumors (Canon et al. 2015).

As has been found in >80% of sporadic CRCs (Novellasdemunt et al. 2015; Masuda et al. 2015; Yang et al. 2006), inactive adenomatous polyposis coli (APC) mutations (i.e., in DLD-1, SW480 cells) prevent β-catenin degradation. Generally, this limits use of Wnt inhibitors that target upstream of the core pathway (β-catenin complex) in clinical treatment of CRC. PAWI-2 modulates mitotic stress signaling and that leads to inhibition of Wnt responsiveness but works downstream of β-catenin by regulating disengagement of TCF proteins from chromatin (Fig. 2) (Cheng et al. 2018). CRC cell proliferation inhibition by PAWI-2 was independent of APC mutations. This suggests PAWI-2 may be useful as a tumor-specific Wnt inhibitor (i.e., Wnt-activated tumors with APC mutants) that works far downstream from the β-catenin complex.

For the mechanism of action (MOA) of PAWI-2, the linkage between inhibition of Wnt signaling and ATM/p53 activation was associated with activation of homeodomain interacting protein kinase 2 (HIPK2) by PAWI-2 (Fig. 2) (Cheng et al. 2018). HIPK2 activation caused inhibition of Wnt transcription via phosphorylation of TCF proteins (D'Orazi et al. 2002; Hikasa and Sokol 2011) and p53 activation via phosphorylation of p53 at Ser46 (D'Orazi et al. 2002). However, this linkage was observed in HCT-116 cells but not p53 null cells (Cheng et al. 2018). The lack of stimulation of cell apoptosis by PAWI-2 in p53-deficient cells (i.e., 10.1 cell line) (Cheng et al. 2018) showed that p53 played a significant role in the MOA of PAWI-2 to induce cell apoptosis (Fig. 2).

In CRC cells, PAWI-2 also induced immediate morphological cell rounding similar to paclitaxel and colchicine and arrested the cell cycle in the G2/M phase (Fig. 2) (Cheng et al. 2018). CRC cells treated with PAWI-2 showed a dose-dependent decrease in acetylated tubulin with a concomitant increase in tyrosinated tubulin (Cheng et al. 2018). These effects show one of the targets of PAWI-2 could be tubulin (De Brabander et al. 1986) via tubulin destabilization (Janke 2014; Janke and Bulinski 2011). We also showed that PAWI-2 bound to tubulin at a colchicine-binding site that is mechanistically distinct from paclitaxel or vinblastine (Cheng et al. 2018). These properties distinguish PAWI-2 from other reported tubulin inhibitors (Yoshimatsu et al. 1997). In addition, the inhibitory effect of PAWI-2 on CRC cell migration, invasion, and EMT processes further showed the utility of PAWI-2 as a candidate for the treatment of metastatic CRC (Kinzler and Vogelstein 1996).

PAWI-2 inhibited CRC tumor growth in xenograft models

PAWI-2 is chemically and metabolically stable with acceptable PK for efficient drug delivery/clearance and good bioavailability (Cheng et al. 2018). PAWI-2 did not cause any adverse behavioral or toxic effects. In a xenograft model, compared with vehicle control, PAWI-2 (20 mg/kg/day, 28 consecutive days, i.p.) decreased growth of implanted HCT-116 tumors greater than four-fold in nude mice (Fig. 3a, b) (Cheng et al. 2018). Serum clinical data suggested that treatment of animals with PAWI-2 was not toxic to liver, kidney, or blood. Post-xenograft tumor tissue analysis (i.e., immunoblotting and histology) showed a significantly greater apoptotic effect in the PAWI-2 treated group (Cheng et al. 2018). PAWI-2 was also more potent than vinblastine, another microtubule (MT) destabilizer, at inhibition of tumor growth (Koyanagi et al. 1994) but therapeutic use of vinblastine is limited because of its toxicity (Zhou and Rahmani 1992).

Concluding remarks for effects of PAWI-2 on CRC

PAWI-2 binds tubulin and potently activates mitotic stress signaling to stabilize p53 and inhibit Wnt/β-catenin transactivation of downstream genes in CRC cells. To our knowledge, PAWI-2 is the first reported potent small molecule that inhibits Wnt/β-catenin signaling and activates

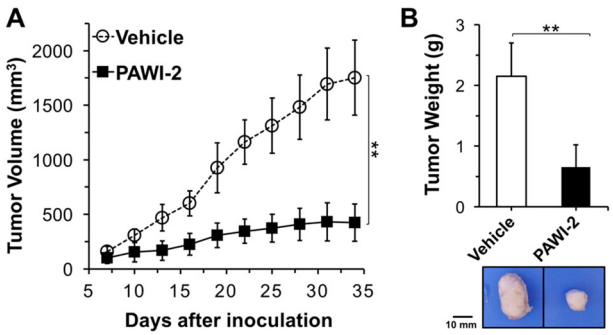

Fig. 3 Effect of PAWI-2 on HCT-116 CRC tumor growth in a subcutaneous xenograft model in nude mice. **a** The effect of 20 mg/kg/day PAWI-2 (solid squares, $n = 6$) or vehicle (open circles, $n = 5$) on tumor volume of HCT-116 CRC xenografts. Treatment was administered every day for 28 days starting on day 7 by intraperitoneal injection. **b** The weight of excised tumors at day 34 from the same animals of **a** and representative photographs of excised tumors. Data are mean ± SD. *P* values were estimated by Student's *t* test. This figure was revised based on Fig. 6 in Cheng et al. 2018

p53 signaling regardless of p53 mutation status without acute or chronic toxicity (Cheng et al. 2018). Therefore, the design of PAWI-2 for targeting multiple pathways to modulate cross-talk between molecular signaling pathways supports a novel CRC treatment strategy.

Breast cancer

BC is the most common cancer type and the second leading cause of cancer-related deaths in women in the US (Siegel et al. 2020). It is estimated that around 41,760 women died from this disease in 2019 (DeSantis et al. 2019). Although early-stage BC is curable, the 5-year survival rate of patients with metastatic BC is only 20% (DeSantis et al. 2019). Common anti-BC therapeutic modalities typically use a combination of surgery, radiation, and chemotherapeutics. Currently used chemotherapies include anthracyclines and taxanes (e.g., doxorubicin and paclitaxel) and are usually used in combination with other chemotherapeutics including fluorouracil and cyclophosphamide (Chemotherapy Medicines 2018).

Among all the BC subtypes, triple-negative BC (TNBC) is the most lethal subtype and is characteristically aggressive with significant recurrence, is metastatic, and has high mortality rates (Bauer et al. 2007; Foulkes et al. 2010). However, most of these drugs for TNBC treatment have problems with toxicity and may not be able to efficiently nullify activation of multiple growth-promoting pathways associated with TNBC (Carey et al. 2007). Due to the lack of druggable targets (i.e., estrogen receptor (ER), human epidermal growth factor receptor 2 (HER2)), surgery, radiation, and chemotherapy are still the "gold standards" in the treatment of TNBC. However, TNBC patients that do not respond to these therapies will eventually develop metastatic BC, which is virtually incurable (Carey et al. 2007; Dent et al. 2007; Liedtke et al. 2008). PAWI-2 is a nontoxic small molecule that uses a completely new approach to treat advanced BC (i.e., TNBC).

PAWI-2 works against BC cells regardless of BC types

PAWI-2 inhibits cell viability and stimulated apoptosis of non-TNBC (MCF-7) and TNBC (including MDA-MB-231, HS578T, BT-549, T47D, and MDA-MB-468) cells in vitro (IC_{50}s, 20–200 nM). In cisplatin (cP)-resistant MCF-7 cells, PAWI-2 also potently inhibited cell viability with an IC_{50} similar to that observed for parental MCF-7 cells (unpublished results). Specifically targeting drug-resistant BC cells is clinically important because drug resistance is a hallmark of metastatic BC. An increase in the expression level of phospho-ATM (an upstream kinase for p53 phosphorylation, Fig. 2) in both MCF-7 and MDA-MB-231 cells after treatment of cells with PAWI-2 (Okolotowicz et al. 2018) was also observed.

One of the most common chemotherapeutics for treatment of BC is doxorubicin and is usually given in combination with other chemotherapeutics, including paclitaxel or 5-fluorouracil (Chemotherapy Medicines 2018). PAWI-2 showed a synergistic effect on doxorubicin to inhibit proliferation of BC cells. Most patients with TNBC (78%) overexpress the transmembrane epidermal growth factor receptor (EGFR) (Arteaga and Truica 2004). This suggests EGFR is a potential therapeutic target. However, early phase clinical trials of anti-EGFR therapies failed to show significant efficacy in TNBC (Arteaga and Truica 2004). We tested synergism between PAWI-2 and 12 types of FDA-approved EGFR-tyrosine kinase inhibitors (TKIs) or vascular endothelial growth factor receptor (VEGFR)-TKI (i.e., erlotinib, afatinib, etc.) (National Cancer Institute Developmental Therapeutics Program 2020) in MDA-MB-231 cells and analyzed the results with Chou–Talalay synergism analysis (Chou 2010). PAWI-2 synergized 10 of the 12 drugs effectively to inhibit cell viability in MDA-MB-231 cells (lower value "combination index" < 1; unpublished result). This provides a powerful basis to develop PAWI-2 for the treatment of TNBC. To our knowledge, no EGFR therapies are efficient for treatment of TNBC (Nakai et al. 2016).

PAWI-2 inhibited BC tumor growth in xenograft models

PAWI-2 potently inhibited TNBC tumor growth in vivo when tested in a subcutaneous xenograft TNBC (MDA-MB-231 cells) tumor model (Fig. 4). Moreover, co-administration of PAWI-2 (20 mg/kg/day, 16 consecutive days, i.p.) plus doxorubicin (5 mg/kg/week, 16 days, i.p.) decreased tumor growth rate 8.8-fold compared with vehicle-treated animals (Fig. 4a) (Okolotowicz et al. 2018). At the end of the 16-day study treatment with PAWI-2 and doxorubicin dramatic, decrease in tumor volume and weight was observed compared with vehicle-treated mice (Fig. 4b, c) (Okolotowicz et al. 2018). Immunoblot analysis of tumor tissues showed considerably greater levels of p53 and phospho(Ser15)-p53 proteins and PARP cleavage ($P < 0.05$) compared with vehicle-treated samples (Okolotowicz et al. 2018).

Concluding remarks for effects of PAWI-2 on BC

PAWI-2 is a potent pathway modulator that suppressed BC cell viability and in combination with other chemotherapeutics or EGFR-TKIs also showed the ability to synergistically inhibit cell growth of MDA-MB-231 in vitro and

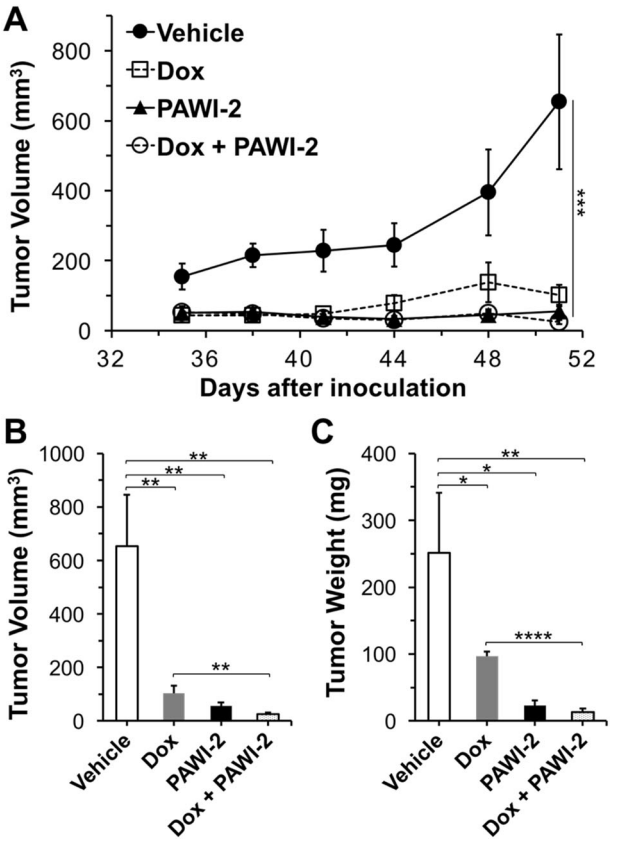

Fig. 4 Effect of PAWI-2 on MDA-MB-231 BC xenograft tumor growth in a subcutaneous model in nude mice. **a** Average tumor growth, **b** tumor volume, and **c** weight for excised tumors of animals treated with vehicle, doxorubicin, PAWI-2, or doxorubicin plus PAWI-2. Treatment was administered for 16 days starting on day 35 by intraperitoneal injection. Dose treatment: vehicle control (aqueous-PEG), $n = 7$; doxorubicin (5 mg/kg/week), $n = 8$; PAWI-2 (20 mg/kg/day), $n = 6$; or doxorubicin (5 mg/kg/week) plus PAWI-2 (20 mg/kg/day), $n = 5$; Dox doxorubicin. Data are mean ± SEM. P values were estimated by one-way ANOVA test in **a** (***$P < 0.001$) and by Student's t test in **b** and **c** (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$). This figure was revised based on Fig. 3 in Okolotowicz et al. 2018

in vivo. These studies warrant further preclinical investigation of PAWI-2 for the treatment of BC especially aggressive BC types (i.e., TNBC).

Prostate cancer

In 2019, PCa was the second leading cause of cancer-related death for men and resulted in an estimated 31,620 deaths (Siegel et al. 2020). PCa is often initially responsive to anti-androgen hormone therapies and thus characterized as castration-sensitive PCa. However, in 35% of patients, PCa recurs and is often transformed to castrate-resistant PCa (CRPCa), thus rendering hormone therapies ineffective (Gandhi et al. 2018; Howlader et al. 2019). However, effective drugs to treat CRPCa are lacking. Standard-of-care treatment options for CRPCa are limited to radiation or hormone therapy (e.g., enzalutamide) (Schalken and Fitzpatrick 2016; Tran et al. 2009) or in combination with chemotherapy (e.g., docetaxel) (Mukherji et al. 2014).

Androgen receptor (AR) signaling is a critical survival pathway for PCa cells. Expression of AR in PCa is heterogeneous. PCa cells are often classified as AR expressing (i.e., AR^+, LNCaP) and AR low- or nonexpressing (i.e., $AR^{-/lo}$, PC-3) (van Bokhoven et al. 2003). Blockade of AR was shown to be an effective PCa therapeutic strategy (Schalken and Fitzpatrick 2016; Tran et al. 2009). But $AR^{-/lo}$ PCa cells are resistant to most commonly used therapy for androgen ablation (Katzenwadel and Wolf 2015). Most AR^+ PCa reportedly respond to androgen ablation therapies but often leads to androgen-depletion independent status (Katzenwadel and Wolf 2015; Karantanos et al. 2013). This incurable stage of PCa (CRPCa) remains a challenge to treat (Jernberg et al. 2017; Ni et al. 2013; Ritch and Cookson 2016; Yang et al. 2009) and >90% of patients develop metastases that cause PCa-related deaths (Gandhi et al. 2018). For example, enzalutamide is an FDA-approved AR antagonist to treat PCa (Azvolinsky 2012). Combination of enzalutamide with abiraterone is the most widespread first-line treatment for CRPCa (de Bono et al. 2011). However, clinical studies showed this multi-component therapy only modestly extended overall survival with many untoward side effects (Gandhi et al. 2018) and high rate of relapse for patients (de Bono et al. 2011; Dhingra et al. 2013; Scher et al. 2012). Therefore, developing novel therapeutic approaches to overcome castrate-resistance are urgently needed in the treatment of CRPCa.

PAWI-2 works against both androgen-sensitive and androgen-independent PCa cells

We observed that PAWI-2 was effective against androgen-sensitive PCa (LNCaP) and androgen-insensitive CRPCa (PC-3). P53-dependence of PAWI-2 was also observed in PCa cells because apoptosis induced by PAWI-2 was significantly greater in LNCaP (WT p53) than that in PC-3 (similar to null p53 status) (Cheng et al. 2019a). However, there was no apparent relationship observed between p53 mutation status and potency of PAWI-2 for inhibition of in vitro PCa cell growth (similar IC_{50}s, ~15 nM) (Cheng et al. 2019a).

In the presence of PAWI-2, inhibition of PCa cell viability was markedly increased compared with enzalutamide alone or a combination of enzalutamide with abiraterone (Cheng et al. 2019a). This was consistently observed in both enzalutamide-sensitive LNCaP cells and enzalutamide-resistant PC-3 cells. PAWI-2 sensitized these cells to enzalutamide (but not abiraterone) (Cheng et al. 2019a). PAWI-2 inhibited PCa cell migration and invasion regardless of AR response status and also synergized/resensitized

the effect of enzalutamide. PAWI-2 is capable of interrupting highly invasive and metastatic properties of CRPCa. Considering cancer metastasis is a hallmark of malignancy in CRPCa, this is clinically relevant. However, the synergistic effect of PAWI-2 on enzalutamide in PCa cells was not completely dependent on p53 activation. Other effectors likely contribute to potency.

In PCa cells, PAWI-2 caused loss of mitochondrial membrane potential and affected mitochondrial membrane dynamics and import/translocation of proteins (degradation of several mitochondrial localized Bcl-2 family proteins) (Cheng et al. 2019a). This effect was independent of AR status and also highly associated with synergistic effects of PAWI-2 on enzalutamide. PAWI-2 selectively affected Bcl-2 family proteins (Fig. 2) that can be linked to non-genomic signaling of AR in PCa (i.e., control mitochondrial function and retrograde signaling (Massie et al. 2011; Zarif and Miranti 2016)). Accordingly, upregulation of pro-survival factors (i.e., Bcl-2, Bcl-xL, and Mcl-1) and acquired enzalutamide resistance is a hallmark of CRPCa (Li et al. 2018). The imbalance of pro-survival and anti-survival factors caused by PAWI-2 (Fig. 1) through affecting mitochondrial membrane potential/function may be a controlling mechanism in synergizing/resensitizing the effect of enzalutamide to overcome enzalutamide resistance.

PAWI-2 inhibited PCa tumor growth in xenograft models

Efficacy of PAWI-2 was examined either as a single agent or in combination with enzalutamide in a PC-3 xenograft animal model. PAWI-2 (20 mg/kg/day, 21 consecutive days, i.p.) or enzalutamide (5 mg/kg/day, 21 consecutive days, i.p.) with PAWI-2 decreased PC-3 tumor growth rate in mice but at the dose examined, enzalutamide alone did not inhibit PC-3 tumor growth (Fig. 5a–c) (Cheng et al. 2019a). Thus, PAWI-2 significantly decreased PC-3 tumor growth in vivo and also resensitized the effect of enzalutamide inhibition without any apparent abnormal effect on liver or kidney function (Cheng et al. 2019a). Amongst the few medications or treatments registered for CRPCa (including docetaxel, cabazitaxel, abiraterone, enzalutamide, and radium-223) (Karantanos et al. 2013), enzalutamide is well tolerated and has a favorable toxicity profile (Tran et al. 2009). However, enzalutamide treatment outcome remains modest. It is notable that PAWI-2 was more efficacious than the most commonly used clinical treatment in an animal model of PCa.

Concluding remarks for effects of PAWI-2 on PCa

PAWI-2 is a highly efficacious compound for PCa with decreased side effects. PAWI-2 also provides a molecule for both androgen-dependent and androgen-resistant PCa treatment. PAWI-2 showed considerable synergism with enzalutamide. PAWI-2 effectively inhibited tumor growth in a xenograft model of CRPCa cells (PC-3) as a single agent and also in combination with enzalutamide. Because of its novel MOA, PAWI-2 has broad utility to treat more aggressive CRPCa.

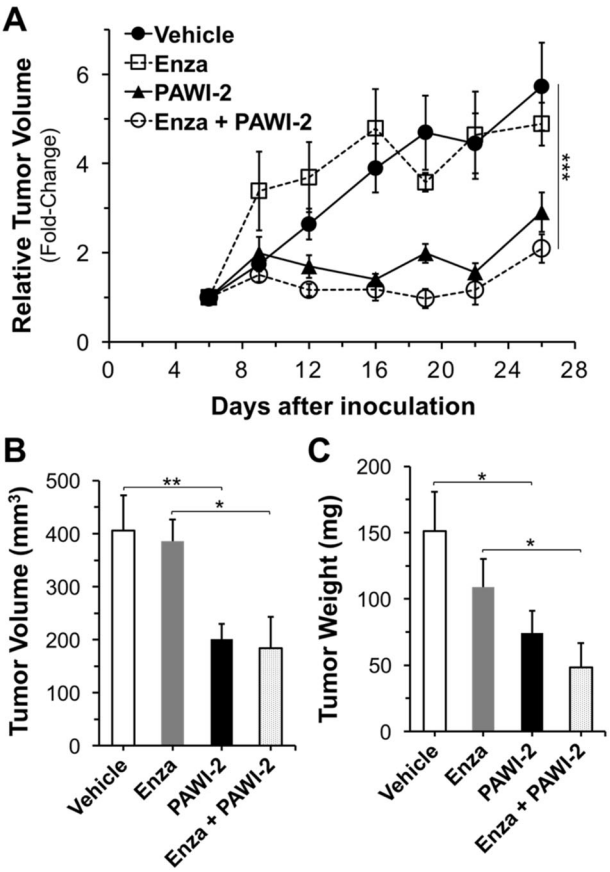

Fig. 5 Effect of PAWI-2 on PC-3 PCa tumor growth in a subcutaneous xenograft model in nude mice. **a** Average tumor growth, **b** tumor volume, and **c** weight for excised tumors of animals treated with vehicle, enzalutamide, PAWI-2, or enzalutamide plus PAWI-2. Treatment was administered every day for 21 days starting on day 6 by intraperitoneal injection. Dose treatment: vehicle control (aqueous-PEG), $n = 9$; enzalutamide (5 mg/kg/day), $n = 9$; PAWI-2 (20 mg/kg/day), $n = 9$; or enzalutamide (5 mg/kg/day) plus PAWI-2 (20 mg/kg/day), $n = 6$; Enza enzalutamide. Data are mean ± SEM. P values were estimated by one-way ANOVA test in **a** (***$P < 0.001$) and by Student's t test in **b** and **c** (*$P < 0.05$, **$P < 0.01$). This figure was revised based on Fig. 4 in Cheng et al. 2019a

Pancreatic cancer

PC has the poorest prognosis of any major malignancy with a 5-year survival rate about 5% (Siegel et al. 2020). PC is the third leading cause of cancer death in the US and soon will be the second most common cause of mortality due to

cancer (Siegel et al. 2020; Rahib et al. 2014). Pancreatic ductal adenocarcinoma (PDAC) is the most common form of PC (>95%) (Siegel et al. 2020). Patients with PDAC are often diagnosed at late stages with extensive local tumor invasion and early metastasis, presenting a major obstacle to all forms of therapy. First-line chemotherapy (i.e., gemcitabine, 5-fluorouracil or FOLFIRINOX, etc.) has made minimal impact on PDAC treatment (Burris and Storniolo 1997; Burris et al. 1997; Conroy et al. 2011; Moore et al. 2007) and a majority of PC patients are often resistant to clinical therapies (Burris et al. 1997). Thus, it remains a challenge to develop an efficacious clinically useful PC therapy.

Mutations of tumor suppressor p53 are among the most common genetic changes in cancer (Deer et al. 2010). Approximately 75% of PDAC patients harbor intragenic p53 mutations (Moore et al. 2007; Fiorini et al. 2015) that makes PDAC cells resistant to chemotherapeutic regimens (Fiorini et al. 2015) due to loss of functional effects activated by p53 (i.e., growth arrest, apoptosis, and senescence). Mutations of p53 in tumor cells can cause loss of WT p53 and gain of novel oncogenic functions (i.e., regulation of DNA-damage-induced apoptotic response), leading to metastasis of cancer cells (Freed-Pastor and Prives 2012) and poor clinical response to cancer chemotherapies (Xu et al. 2014). An overarching challenge is to develop a drug that potently inhibits PDAC growth by restoring tumor suppressor function. Extra-nuclear p53 apoptotic cell death mechanisms are dependent on transactivation-deficient p53 localization to cytosol or mitochondria-associated membrane and/or ER (Chipuk and Green 2004; Moll et al. 2005; Vaseva and Moll 2009). In mitochondrial-controlled cell apoptosis (intrinsic pathway), trafficking of mitochondria-bound Bcl-2 family members (i.e., Bax, Bad, Bak, etc.) cause the release of mitochondrial cytochrome c into cytosol to induce further cell apoptosis (Chipuk et al. 2006; Suen et al. 2008).

Recently, cancer stem cells (CSCs) have come into focus as potential therapeutic targets in multiple cancers (Nassar and Blanpain 2016). Accumulation of mutations in CSCs enhances chemo/radiation resistance that often ablates the effect of therapy, leading to cancer recurrence, characteristic of PDAC (Hermann et al. 2007). Conversely, because CSCs are a unique subset of a tumor cell population, targeting these cells may lead to the identification of effective drugs for poorly treatable cancers such as PDAC (Nassar and Blanpain 2016). Human PC stem cells (hPCSCs) reported previously (i.e., FGβ_3 cells) are a validated human CSC model (Desgrosellier et al. 2009; Seguin et al. 2014, 2017) that overexpress integrin $\alpha_v\beta_3$. In FGβ_3 cells, integrin $\alpha_v\beta_3$ recruits Kirsten rat sarcoma viral oncogene homolog GTPase (KRAS) and RAS like proto-oncogene B (RalB) to activate serine/threonine kinase TANK-binding kinase 1 (TBK1, IκB kinase (IKK)-related kinase) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to trigger dysregulated KRAS-RalB-NF-κB. This pathway was reported to be a pharmacological target to reverse CSC-like properties or resensitize drug resistance for established FGβ_3 tumors (Desgrosellier et al. 2009; Seguin et al. 2014, 2017).

PAWI-2 potently decreased PDAC cell proliferation by activating DNA-damage checkpoint and apoptotic pathways (e.g., the mitochondrial (intrinsic) control of apoptosis) (Cheng et al. 2019b). PAWI-2 inhibited tumor growth of a syngeneic, orthotopic model of PDAC (Cheng et al. 2019b). PC stem cells (PCSCs) may provide an important target of significant clinical utility to treat PC. In this context, it is notable that PAWI-2 was observed to afford a novel treatment strategy that targeted hPCSCs or their extrinsic and intrinsic regulators. PAWI-2 ameliorates drug-resistant hPCSCs (i.e., FGβ_3 cells) and synergizes erlotinib by targeting optineurin (OPTN)-dependent cell cycle arrest (Cheng and Cashman 2020). Development of PAWI-2 as an anti-PC drug candidate addresses an unmet clinical need. PAWI-2 could improve standard of care for patients because it synergizes eradication of hPCSCs.

PAWI-2 inhibits invasive PC by restoring cell apoptosis through activation of mitochondrial p53

PAWI-2 showed significant potency in inhibition of cell proliferation and activation of cell apoptosis in PDAC cell models (i.e., MIA PaCa-2, HPAC-1, BxPC-3, etc.) including two patient-derived PDAC cell lines (i.e., 779E and 1334E) (Cheng et al. 2019b). Resistance to other chemotherapy (i.e., gemcitabine, 5-fluorouracil, etc.) normally observed in cancer cells (Burris and Storniolo 1997; Burris et al. 1997; Conroy et al. 2011; Moore et al. 2007) with mutant p53 was not observed in PDAC cells (with different p53 mutant status) treated with PAWI-2. The dominant MOA through upstream DNA damage via ATR/ATM-kinase activation and p53-dependent apoptosis was still observed for PAWI-2 in PC (Fig. 2) (Cheng et al. 2019b) as we observed in CRC cells (Cheng et al. 2018). Shown by synergism analysis, p53 activation of apoptosis contributes to the MOA of PAWI-2 in the presence of chemotoxins. Specifically, phosphorylation of p53 at Ser15 likely is a factor that controls different synergistic effects of PAWI-2 observed in different PDAC cells (Cheng et al. 2019b).

Compared with WT p53 cells (i.e., HPAC), cell apoptosis of mutant p53 cancer cells (i.e., LM-P, MIA PaCa-2, etc.) was induced by PAWI-2 but without significant upregulation of p53 or phosphorylation of p53 (Cheng et al. 2019b). PAWI-2 activated p53 mediated transcription-independent mitochondrial-apoptotic pathways in both WT p53 and mutant p53 PDAC cells (Fig. 2) (Cheng et al. 2019b). PAWI-2 disrupts the interactions of pro-survival factors (i.e., Bcl-xL) and pro-apoptotic Bcl-2 family members (i.e., Bax) and p53. This action releases p53/Bax to

activate translocation of pro-apoptotic Bcl-2 family proteins from the cytosol to the mitochondria (Cheng et al. 2019b). Activation of apoptotic systems by treatment of PAWI-2 in PDAC cells also involves loss of mitochondrial membrane integrity and opening of permeability transition pores related to mitochondrial outer-membrane permeabilization. As a result, this further induces cytochrome c, Smac, and HSP60 release into cytosol that neutralizes inhibition of apoptosis (caspases) and causes incipient apoptotic signaling (i.e., PARP cleavage) (Vaseva and Moll 2009; Chandra et al. 2002; Galluzzi et al. 2008). Such a working model differentiates PAWI-2 from other p53-targeted inhibitors in tumor suppression. P53-dependent cell death checkpoint inhibitors (e.g., nutlin-3a and pfithrins) do not induce apoptosis in mutant p53-bearing PCs (Sriraman et al. 2016).

As observed in CRC cells, PAWI-2 exerts its effect on Wnt signaling inhibition via HIPK2 and TCF proteins linked to p53 activation via phospho(Ser46)-p53 (Cheng et al. 2018). However, in PDAC cells with mutant p53 status, no detectable effect of PAWI-2 on Wnt target gene expression was observed. Likewise, no activation of phospho(Ser46)-p53 or phosphorylated-HIPK2 and TCF3 by PAWI-2 was observed in PDAC cells examined (Cheng et al. 2019b). Inhibition of Wnt-dependent transcription by PAWI-2 is unlikely to be a major pathway in the MOA of PAWI-2 in PDAC cells (Fig. 2).

PAWI-2 overcomes tumor stemness and drug resistance via cell cycle arrest in PCSCs

PAWI-2 is selective for killing hPCSC tumor spheroids responsible for hPCSC drug resistance (Cheng and Cashman 2020). In a well-established hPCSC model (FGβ_3 cells) (Desgrosellier et al. 2009; Seguin et al. 2014, 2017), association of TBK1 with RalB of the major oncogene (RAS) in a dysregulated integrin $\alpha_v\beta_3$-KRAS-NF-κB signaling pathway promotes tumorigenesis and CSC-like properties (Desgrosellier et al. 2009; Seguin et al. 2014, 2017). We observed that PAWI-2 inhibited KRAS-NF-κB-RalB signaling regardless of KRAS or Ral status (Cheng and Cashman 2020). Given the fact that >90% of KRAS is activated by mutations in PC (Deer et al. 2010) and RAS or Ral inhibitors have not proven effective clinically (Seguin et al. 2014), this suggests that PAWI-2 may possess advantages in the clinic.

TBK1 is a serine/threonine kinase that phosphorylates p62 or OPTN (Pilli et al. 2012; Wild et al. 2011). TBK1 is involved in tumor suppression via a mitophagy pathway (Richter et al. 2016). However, we excluded the role of mitophagy initiated via TBK1/OPTN for the MOA of PAWI-2. Instead, phosphorylation of OPTN at Ser177 plays a pivotal role in mitotic progression and induces OPTN translocation into the nucleus (Ying et al. 2010). OPTN-dependent G2/M cell cycle arrest induced by PAWI-2 in FGβ_3 cells parallels this process (Cheng and Cashman 2020). PAWI-2-induced OPTN phosphorylation negatively regulates TBK1 functional activity (dephosphorylation) that further regulated KRAS-NF-κB signaling. Moreover, phosphorylation of OPTN also controlled synergism between PAWI-2 and other validated drugs (i.e., erlotinib) (Cheng and Cashman 2020). OPTN may work as an overarching branch point for PAWI-2 inhibition of cell viability to overcome self-renewal capacity in FGβ_3 cells and also to synergize other pathway inhibitors (Fig. 2).

Anti-mitotic agents may perturb the mitotic spindle through either disruption (e.g., vinblastine) or stabilization (e.g., paclitaxel) of MTs (Janssen and Medema 2011). PAWI-2 was previously found to disrupt MT structure in PDAC cells in a similar manner to that observed in other cancer types (Cheng et al. 2018, 2019b). However, we observed PAWI-2 was a dose-dependent MT stabilizer (<50 nM) and destabilizer (>100 nM) (Cheng and Cashman 2020). This further differentiates PAWI-2 from other MT disturbing agents that may also contribute to the considerable efficacy and lack of toxicity observed for PAWI-2 (Cheng et al. 2018). Phosphorylation of OPTN was closely associated with MT stabilization because this effect was also observed in cells treated with other MT stabilizers (e.g., paclitaxel or docetaxel). Accumulation of pS177-OPTN in the presence of MT stabilizers may be due to the essential role of MTs in coordinating and organizing many crucial cellular steps (Brouhard and Rice 2018). Thus, OPTN phosphorylation induced by PAWI-2 or other MT stabilizers could modulate synergism effects to overcome drug resistance and combat more aggressive CSCs.

PAWI-2 inhibited tumor growth in an orthotopic model of invasive PDAC

The efficacy of PAWI-2 was examined in an orthotopic PDAC (LM-P, invasive PDAC murine cell line) animal model (Tseng et al. 2010). PAWI-2 significantly decreased PDAC growth in vivo. Compared with vehicle-treated mice, excised tumor volumes and tumor weights from PAWI-2-treated mice (20 mg/kg/day; 28 days; i.p.) were significantly lower (i.e., $P < 0.05$; 65% and 41%, respectively; Fig. 6) (Cheng et al. 2019b). Compared with vehicle-treated animals, no difference was observed for tumor tissue morphology but a significant apoptotic effect (TUNEL staining) was observed in PAWI-2 treated animals (Cheng et al. 2019b). In clinical treatment of PDAC tumors, gemcitabine plus *nab*-paclitaxel is a standard systemic chemotherapy (Frese et al. 2012). PAWI-2 was equal or more efficacious compared with this most commonly used clinical combination treatment (Von Hoff et al. 2011, 2013) in an animal model of PDAC.

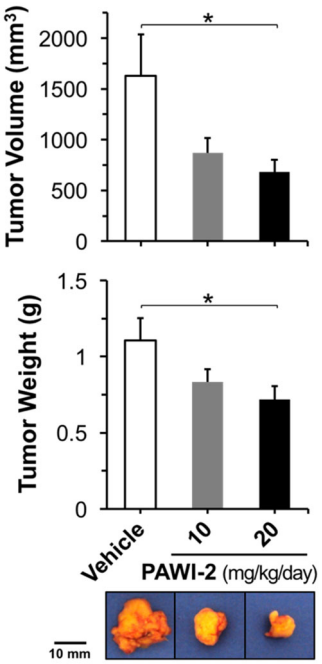

Fig. 6 Effect of PAWI-2 on LM-P PDAC tumor growth in a syngeneic orthotopic model in mice. Tumor volume and weight for tumor samples excised from orthotopic mice treated with vehicle or two different doses of PAWI-2 (10 or 20 mg/kg/day) and representative photographs of excised tumors. Treatment was administered every day for 28 days starting on day 6 by intraperitoneal injection. Dose treatment: vehicle control (aqueous-DMSO-captisol), $n = 7$; PAWI-2 (10 mg/kg/day), $n = 6$; or PAWI-2 (20 mg/kg/day), $n = 7$. Data are mean ± SEM. P values were estimated by Student t tests ($^*P < 0.05$). This figure was revised based on Fig. 4 in Cheng et al. 2019b

Concluding remarks for effects of PAWI-2 on PC

In summary, PAWI-2 is a nontoxic, highly efficacious treatment of PDAC that activates damage checkpoint and mitochondrial p53-dependent apoptosis. PAWI-2 showed considerable synergism with commonly used combination therapy in the treatment of PDAC and also showed synergism with specific pathway inhibitors (e.g., TBK1 inhibitors, EGFR inhibitors) against PCSCs. PAWI-2 inhibited tumor growth in an orthotopic model of invasive PDAC cells (LM-P). Moreover, selective pharmacological potency of PAWI-2 against PCSCs showed the utility of PAWI-2 to inhibit CSCs versus bulk cancer cells. This observation provides a basis for PAWI-2 as an efficient treatment of PC, especially in highly aggressive/metastatic cancer with stem-like properties and intrinsic or acquired drug resistance.

Overall conclusion

Cancer is still the leading cause of death in the US. Development of new ways to treat cancer is a significant challenge. Late-stage diagnosis of cancer renders current therapies ineffective often due to their drug-resistant nature. The effectiveness of relatively new "targeted treatments" remains to be shown. PAWI-2 affords a completely different focus on inhibition of key molecular pathways by a safe compound. PAWI-2 showed great efficacy in four relevant but different preclinical cancer models. PAWI-2 does not depend on any particular cancer cell mutation profile to work effectively. PAWI-2 may synergize existing standard of care thus increasing efficacy of standard of care anticancer agents with improved safety. Our work on PAWI-2 provides fundamental information about a novel therapeutic strategy to treat cancer and will further enhance standard of care with minimal side effects.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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