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ORIGINAL RESEARCH

Enhanced Accumulation of Cisplatin in Ovarian Cancer Cells from Combination with Wedelolactone and Resulting Inhibition of Multiple Epigenetic Drivers

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Purpose: Cisplatin resistance is a major concern in ovarian cancer treatment. The aim of this study was to investigate if wedelolactone could perform better in resistant ovarian cancer cells when used in combination with cisplatin.

Methods: Growth inhibitory potential of wedelolactone and cisplatin was investigated through MTT reduction assay in ovarian cancer cell lines including A2780 (sensitive), A2780^{cisR} (cisplatin resistant) and A2780$^{ZD0473R.}$ Resistance factor (RF) of drugs was determined in these three cell lines. Combination index (CI) was calculated as a measure of combined drug action. Effect of this combination on changes in the cellular accumulation of platinum levels and platinum-DNA binding was also determined *in vitro* using AutoDock Vina while the effect of wedelolactone on inhibition of possible key culprits of resistance including Chk1, CD73, AT tip60, Nrf2, Brd1, PCAF, IGF1, mTOR1 and HIF2α was investigated *in silico*.

Results: Cisplatin and wedelolactone showed a dose-dependent growth inhibitory effect. RF value of wedelolactone was 1.1 in the case of A2780^{cisR} showing its potential to bring more cell death in cisplatin-resistant cells. CI values were found to vary showing antagonistic to additive outcomes. Additive effect was observed for all sequences of administration (0/0, 0/4 and 4/0 h) in A2780^{cisR}. Enhanced cellular accumulation of cisplatin was observed in parent and resistant cells on combination. Docking results revealed that among the selected onco-targets, Chk1, CD73, Nrf2, PCAF and AT tip60 were more vulnerable to wedelolactone than their respective standard inhibitors.

Conclusion: These findings have shown that additive outcome of drug combination in A2780^{cisR} and raised levels of platinum accumulation followed a clear pattern. This observation indicates that the presence of wedelolactone might have contributed to sensitize A2780^{cisR}. However, *in silico* results point to the possible effects of this compound on epigenetic factors involving tumor microenvironment, epithelial mesenchymal transition, and immune-checkpoint kinases.

Keywords: coumestan, chemotherapy resistance, growth inhibition, A2780

Introduction

Ovarian cancer is the most lethal gynecological malignancy, with 5-year survival rate less than 50%.[1] It is more common in the aged population.[2] According to National Cancer Institute (NCI) report, 21,750 new cases of ovarian cancer and 13,940 deaths were estimated in 2020. Though there is improvement in 5-year survival, this still remains less than 50% in most prevalent cases, as evident in

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Figure 1.[3] Survival rate is very low unless detected at an early stage but it is often detected at an advanced stage, being asymptomatic at earlier stages. More than 75% patients are diagnosed at stage III or IV (localized or regional) when the disease spreads to the abdominal cavity, and often associated with low survival rate as shown in Figure 1.[4] Debulking surgery followed by platinum-based chemotherapy remains the main option, but unfortunately high recurrence (70%) and acquired drug resistance are frequent events.[5] Unclear etiology is also a hurdle in the treatment of ovarian cancer. Almost all the cells of the ovary can become cancerous, but epithelial ovarian cancer accounts for up to 90% of cases while 3–9% are associated with germ cell and sex cord stromal cells.[6] Epithelial ovarian cancer (EOC) is further divided into a number of subtypes namely serous, mucinous, clear cell, transitional, endometrioidal and undifferentiated. As far as the question of origin of EOC is concerned, the ovarian surface epithelium itself, its cysts and non-ovarian structures (including peritoneum, endometriotic lesions and fallopian tubes) have all been hypothesized as being its source, although knowledge of its etiology remains poor. Lifestyle, social and environmental factors are also considered to be important risk factors evident from its high incidence in the industrialized world. It is more common in Northern Europe, America and Canada than in Asia. Obesity is also a risk factor while results on consumption of meat and alcohol appear to be contradictory. Ovarian cysts arise due to penetration of surface epithelium into stromal region. Unless these invade the stroma and metastasize to lymph nodes or peritoneum, they are categorized as low malignant potential tumors (LMP). EOC remains asymptomatic until it reaches an advanced stage where symptoms including abdominal discomfort, flatulence and gastrointestinal disturbances become evident while vaginal bleeding is the most common symptom in the case of fallopian tube malignancies.[7] Despite recent advances in immunotherapy, platinum drugs are the most commonly used clinical agents in first-line chemotherapy against ovarian cancer, used as single agents or in combination with other drugs. Cisplatin is among the most effective clinical agents. It enters cells uniquely through a distinctive mode involving multiple pathways and effecting several genetic and epigenetic drivers. The two major problems associated with cisplatin are development of resistance to the drug and the serious adverse effects.[8] Understanding and exploring both resistance factors and how resistance develops, is critical to design better and effective therapies. Resistance results from complex genetic and epigenetic changes involving multiple alterations in genetic and protein expression.[9] To cover all possible molecular characters is not possible here but we have selected a few emerging targets which are increasingly being focused on in research as possible key players in development of resistance. CD73 (Ecto-5-nucleotidase) is a plasma membrane-bounded protein, found to be overexpressed in a variety of invasive and resistant tumor cells and biopsies of breast, ovarian and many other malignant tissues. It is also the focus of research as a novel checkpoint inhibitor target and an immune regulatory molecule.[10,11] Tip60 belongs to an important family of histone acetyltransferases (HATs) which reportedly functions in cell cycle regulation, DNA damage repair, apoptosis and signaling. It has also been suggested variously in literature to contribute to the

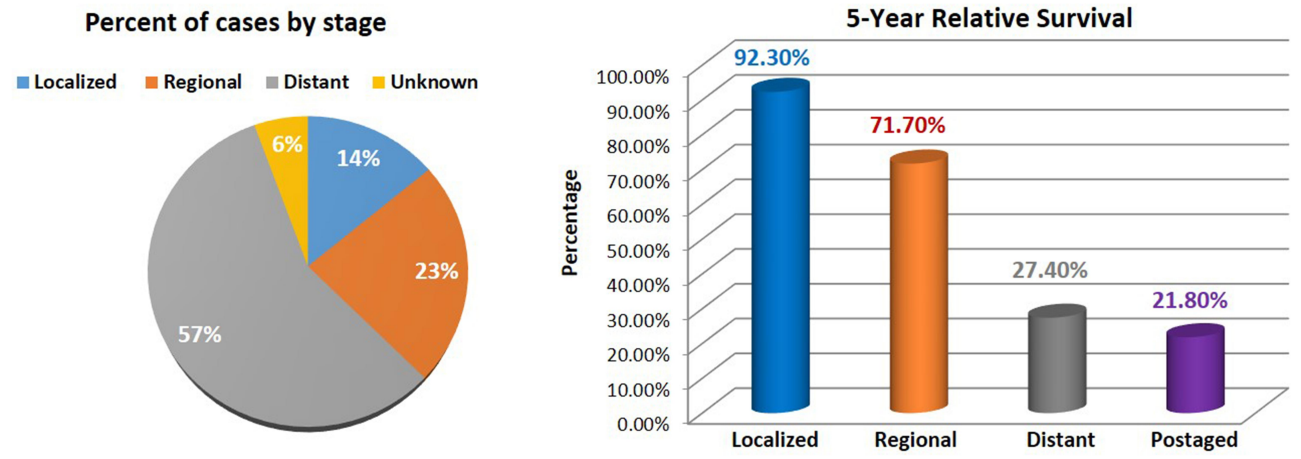

Figure 1 Percentage of ovarian cancer diagnosed at different stages and its correlation with survival.

development of chemo-resistant cancers. Only a small number of Tip60 inhibitors have been reported. The natural products anacardic acid and garcinol are the best known Tip60 inhibitors. Although inhibitors of Tip60 are emerging as potential therapeutics in cancer treatment, development of targeted small molecule Tip60 inhibitors is still in the very early stages.[12] Nrf2, considered classically as a cancer-preventing factor, is now consistently being reported as a tumor-promoting agent. Activation of the Nrf2 pathway is associated with chemo-resistance and Nrf2 inhibitors are emerging as novel drugs in treating resistant cancers.[13] Cisplatin is a DNA-damaging agent. Increased replication stress and resulting instability at genomic level may induce disruption of DNA-damaging response resulting in tumor survival and dependence on checkpoint controls. Drugs targeting such checkpoints, Chk1 being one of them, are the focus of multiple clinical trials.[14] Decreased accumulation of platinum drugs is one of the mechanisms of the development of resistance to the drugs by the cells. The drug wipes out the sensitive cells but leaves the resistant ones. Application of comparatively less toxic phytochemicals in combination with cytotoxic drugs is a pragmatic approach to overcome side effects and even drug resistance. It is very likely that drug-resistant cells may be present in a tumor and use of anticancer drugs in combination makes sense because it may increase the chances of finding and targeting a weakness in the cell. There are multiple reports supporting the use of small molecules, both natural and synthetic, in combination with antitumor drugs to revert resistance.[15–19] Wedelolactone is an emerging potential chemopreventive agent with multiple reported actions.[20–23] It has been reported in our previous work that the combination of wedelolactone with cisplatin acts synergistically in a cervical tumor model.[24] The presented work has further explored the interesting aspects of this combination in ovarian tumor models.

Materials and Methods

Chemicals and Cell Lines

Cisplatin was synthesized at Discipline of Biomedical Science, School of Medicine, The University of Sydney.[25] Wedelolactone was purchased from Sigma Aldrich, Sydney, Australia. All other solvents or chemicals used in this study were of analytical grade. Human ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473} resistant were purchased from ECACC (93112519, 93112517 for A2780 and A2780^{cisR} respectively). A2780^{ZD0473R} cell line has been developed by *in vitro* exposure of A2780 (ECACC no: 93112519) cells to increasing concentration of the drug ZD0473 from 0.5 to 12.5 uM.

MTT Reduction Assay

The whole experimental study was conducted at Discipline of Biomedical Science, School of Medicine at The University of Sydney, Australia.[26] MTT reduction assay is a quantitative colorimetric assay used to quantify living cells. It can be used to measure cytotoxicity, cell proliferation and activation. Tetrazolium salt is cleaved only by active mitochondria of living cells. MTT (3-(4, 5-dimethylthiazole-2yl)-2, 5-diphenyl tetrazolium bromide) assay was performed after 24 hof incubation. MTT solution was prepared by dissolving MTT powder in serum free –RPMI-1640 medium at 1 mg/mL and filtered (0.22 µM). Stock solutions of cisplatin and wedelolactone were made and stored at −20°C. 1mM solution of cisplatin was made (0.00150 g/5 mL) by dissolving powder in DMF followed by mQ water (1:4). 10mM solution of wedelolactone was prepared by dissolving 1 mg/0.32 mL of DMSO. All stock solutions were (weighed and) prepared *in vitro* and filtered using 0.22 µM membrane filters. All stocks were prepared in 5 mL tubes. From stock, each solution was divided into 10 parts and distributed in 10 (5 mL) tubes (500 µL) from filtered stock and was stored at −20°C.

The stock solutions were serially diluted with freshly prepared RPMI-1640. The cytotoxicity was determined through 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay.[21] RPMI-1640 medium was used to culture cells (density of 4000–6000 cells/well) in 96-well culture plates. Each drug was added at four doses in three wells and incubated (37°C, 5% CO_2 in air) for 72 h. Cisplatin and wedelolactone were prepared in desired concentrations (0.16–20 µM for cisplatin and 0.80–100 µM for test compound) followed by addition of 100 µL of each drug in triplicate wells. The cells were then left to incubate under normal growth conditions for 72 h at 37°C in a humidified atmosphere. In the case of combinations, cells were treated with increasing concentrations of compounds at constant ratios of their IC_{50} values using the sequences 0/0 h (cisplatin and wedelolactone were added immediately after seeding), 0/4 h (cisplatin immediately after seeding; wedelolactone 4 h after seeding) and 4/0 h (wedelolactone immediately after seeding; cisplatin 4 h after seeding). After 72 h of

incubation, the MTT reduction assay was performed. For single compounds, IC_{50} was calculated as mean ± SD of at least five experiments. Cell survival fraction was calculated as follows:

Cell survival fraction = absorbance sample/absorbance blank

The one-way analysis of variance (ANOVA) was used to decide whether there were any statistically significant differences between the means of different study groups with significance defined as P value of <0.05 (Figure 3). The analysis of combined drug action was based on median effect.[27–30] Combination index (CI) was calculated as average of five repeats. The CI was calculated following the equation given below:

$$CI=D1/D1x + D2/D2x$$

Cellular Accumulation of Platinum

Since the platinum drugs are assumed to act through DNA binding, platinum accumulation was measured to determine if there was any correlation between the combined drug action and the level of platinum. Both cisplatin and WDL were added to the culture (A2780, $A2780^{CisR}$ cell density= 1×106 cells per mL) in 5 mL 10% PBS/RPMI 1640 culture medium. The cell monolayers were trypsinized after 24 h of incubation. After 24 h of incubation, cell monolayers were scratched and transferred to centrifuge tube and spun at 35,000 rpm (RCF = 163268) for 2 min at 4°C. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at −20°C until assayed. Cell pellets were suspended in 0.5 mL 1% triton-X while sonicated (held on ice). Total intracellular platinum contents were determined by graphite furnace AAS using a Varian SpectrAA-240 plus with GTA 120 atomic absorption spectrophotometer.

Platinum–DNA Binding

DNA was isolated from cell pellets using H440050 JETQUICK Blood Spin Kit/50 (Austral Scientific Pty Ltd). DNA content was determined by UV spectrophotometry (260 nm) with Varian 50 BIO UV-Visible spectrophotometer coupled with Varian 50 MPR Microplate Reader and platinum content was determined by graphite furnace AAS. DNA concentration was calculated according to the equation: concentration = Absorbance at 260nm x 50 ng/µL.

Molecular Docking

Auto Dock Vina program was used for docking through PyRx[31,32]. Affinity of best conformational pose of ligand-protein complex was measured by E-value (Kcal/mol). 3D-structures of selected protein targets were retrieved from online protein data bank (http://www.rcsb.org/pdb/home/home.do). The selected protein targets in cancer pathways include checkpoint kinase 1 (PDB ID: 1ia8), ecto-5′-nucleotidase (PDB ID: 4h1s), mammalian target of rapamycin (PDB ID: 5h64), nuclear factor erythroid 2–related factor 2 (PDB ID: 2flu), hypoxia-inducible factor 2-alpha (PDB ID: 3flp), heat shock protein 60 (PDB ID: 4pj1), insulin-like growth factor (PDB ID: 1gzr), P300/CBP-associated factor (PDB ID: 5mkx), bromodomain protein 1(PDB ID: 5fg6), and acetyl transferase (PDB ID: 2ou2). Protein targets were then cleaned off the ligands and water molecules by using Biovia Discovery Studio Visualiser 2016. The 2D structures of wedelolactone and reference drugs were retrieved from online data base PubChem (https://pubchem.ncbi.nlm.nih.gov/search/) in xml format and Open Babel JUI was used to convert xml to PDB format. PDB files of wedelolactone, standard drugs and protein targets were transformed to PDBQT via AutoDock Tools (Version 1.5.6 Sep_17_14). PDBQT forms of wedelolactone, reference drugs and target proteins were loaded and docked in PyRx and binding affinity was shown in Kcal/mol. Post dock interactions were evaluated through Discovery Studio Visualizer to determine the number of hydrogen bonds (conventional and non-conventional), π-π bonds and other hydrophobic interactions by amino acid residues: alanine (ALA), asparagine (ASN), arginine (ARG), aspartic acid (ASP), cysteine (CYS), glutamine (GLN), glutamic acid (GLU), glycine (GLY), histidine (HIS), leucine (LEU), lysine (LYS), serine (SER), threonine (THR), tryptophan (TRP), tyrosine (TYR), valine (VAL), and phenylalanine (PHE) as illustrated in the 2D interaction images.

Statistical Analysis

Data were analyzed through two-way ANOVA followed by post-hoc Bonferroni multiple comparisons test using Graph Pad Prism-6 software for significant differences between control and treatments in cell survival fraction experiment. The significance is indicated as *P < 0.05, **P < 0.001 and ***P <0.001. The median effect analysis was done to determine combination index (CI).

Results

Growth Inhibitory Effects in Terms of IC_{50} and RF Values

Growth inhibition (in terms of IC_{50} values) and resistance factor (RF) for cisplatin and wedelolactone as calculated in

the ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473R} are given in Table 2 and Figure 2A (IC_{50} values) and 2B (RF values). RF is the ratio of drug concentration for 50% cells killed in resistant cell lines to that in parent cell lines. RF value less than 2 for a phytochemical is an indication of ability of that compound to bring about more cell death than standard drug in resistant cells (Figure 2). RF for wedelolactone was 1.1 in resistant cell lines A2780^{CisR} while 1.7 in A2780^{ZD0473R}. Cell survival fraction values at different administered doses of cisplatin and wedelolactone in the case of all three cell lines are given in Figure 3. Dose-dependent growth inhibition was evident in all cases except at lowest concentration in the case of both drugs. It can be observed that wedelolactone is more effective than cisplatin in bringing down the survival of resistant cells at almost all the tested concentrations (Figure 3).

Results of Combination Study

Combination studies were carried out to further explore combined drug action in terms of synergism, additive effect and antagonism resulting from the combination of wedelolactone with the platinum drug cisplatin as applied to the parent ovarian cancer cell line A2780 and cisplatin-resistant ovarian cancer cell line A2780^{cisR}. Combination Indices (CI) were used as measure of combined drug action. The CI of < 1, = 1 and > 1 indicates synergism, additive and antagonistic effect in combined drug action, respectively. Dose-effect was taken as a function of sequence of administration of drugs (0/0, 0/4 and 4/0 h) and their concentration. Median-effect dose (ED_{50}), shape (sigmoidicity) and conformity (linear correlation coefficient) were taken as dose-effect parameters, represented as Dm, m and r as shown in Table 1. The combination indices (CIs) at ED_{50} in the case of the ovarian cancer cell lines A2780 and A2780^{cisR} are also shown in Table 1. The results show that the combination was nearly additive (for more details interested readers may consult reference 24 where explanation is provided in supplementary Table 1) for all the sequences of administration in cisplatin-resistant cell line A2780^{cisR}. However, in the case of parent cell line A2780, the combination was additive for bolus (0/0 h) and moderately antagonistic for the other sequences (0/4 h and 4/0 h).

Determination of Cellular Platinum (Pt) Accumulation and Pt-DNA Binding

The effect of this combination of wedelolactone and cisplatin on cellular accumulation of platinum and level of Pt-DNA binding as applied to three sequences

Table 1 Combination Indices (CIs) and Dose-Effect Parameters Applying to the Combination of Wedelolactone with Cisplatin in A2780 and A2780cisR

Cell Lines	Drugs	CIs at ED50	Nature of Combination	Dm	M	R
A2780	Cisplatin	N/A		1.11	0.52	1.00
	Coumestan	N/A		51.8	1.44	0.99
	0/0	1.08	Nearly additive	0.63	0.57	0.99
	0/4	1.49	Moderately antagonistic	0.93	0.77	0.99
	4/0	1.40	Moderately antagonistic	0.88	0.87	0.99
A2780cisR	Cisplatin	N/A		7.94	0.77	1.00
	Coumestan	N/A		31.59	1.23	0.95
	0/0	1.07	Nearly additive	5.83	1.39	1.00
	0/4	1.05	Nearly additive	5.75	0.85	1.00
	4/0	0.95	Nearly additive	5.18	1.03	0.97

Note: Median-effect dose (Dm), shape or sigmoidicity (m or M) and conformity (linear correlation coefficient abbreviated as r or R) were taken as dose-effect parameters.

Table 2 Cytotoxic Activity in Terms of Inhibitory Concentration (IC_{50}) and Resistance Factor (RF) of Cisplatin and Wedelolactone

Drugs	IC50(μM) A2780	IC50(μM) A2780cisR	RF	IC50(μM) A2780ZD0473R	RF
Cisplatin	1.40 ± 0.11	7.39 ±1.27	5.3	5.44 ± 0.82	3.9
Wedelolactone	11.97	13.41 ± 0.99	1.1	20.24 ± 2.20	1.7

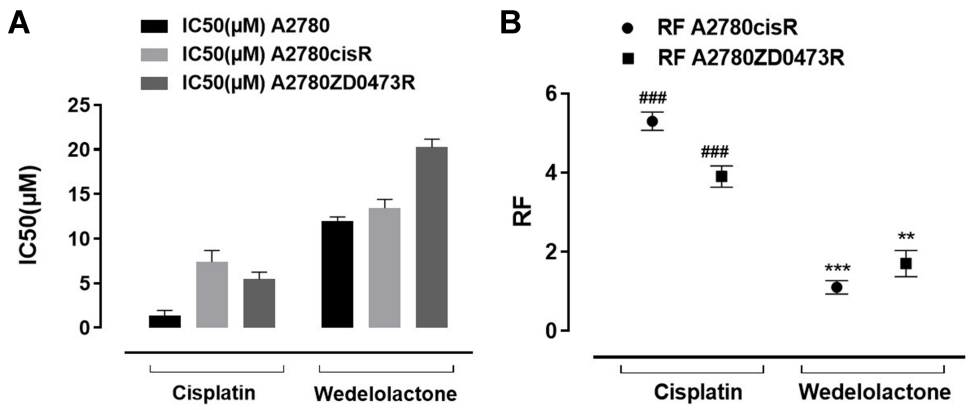

Figure 2 (**A**) Illustrates IC_{50} (n=7) values while (**B**) illustrates RF values. ### indicates cisplatin (as control) whereas ** indicates P <0.01 and *** indicates P <0.001.

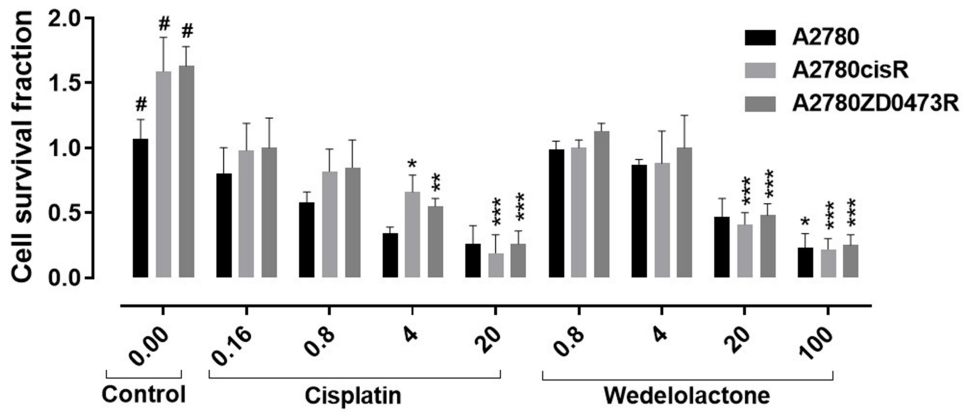

Figure 3 Cell survival fraction values as calculated in ovarian cancer cell lines including A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ in response to cisplatin and wedelolactone. Data are expressed as means ± SEM (n = 7); and interactions examined by ANOVA (two-way) pursued by multiple comparison test (post hoc Bonferroni) using the software Graph Pad Prism-6. * indicates P < 0.05, ** indicates P <0.01 and *** indicates P <0.001. # shows significant difference relative to the control.

of administration (0/0, 0/4 and 4/0 h) in A2780 and $A2780^{cisR}$ was determined. It was found that the cellular accumulation of platinum for each sequence of administration was greater in the resistant cell line $A2780^{cisR}$ than the parent cell line A2780 (Figure 4). Overall, an increased uptake of platinum was evident in both cell types at any sequence of combination. The enhanced accumulation of platinum has been reported previously in the case of many phytochemicals to be associated with enhanced binding of platinum with DNA, hence, resulting in DNA damage. We also investigated the effect of application of wedelolactone on the binding of platinum with DNA in the case of parent and platinum-resistant cells. In our investigation, however, this combination resulted in reduced levels of platinum-DNA binding in all sequences of administration in resistant

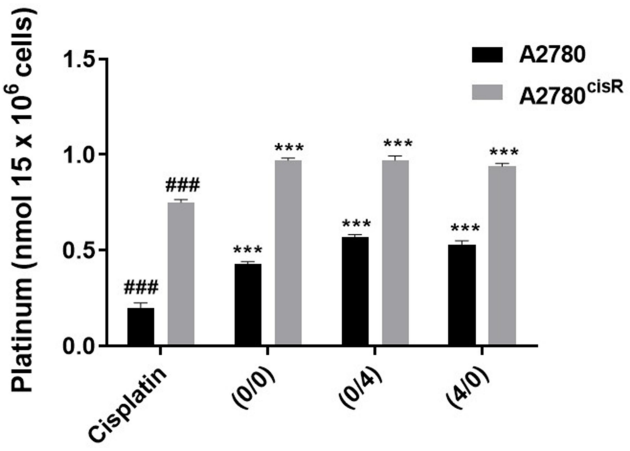

Figure 4 Intracellular platinum accumulation levels in A2780 and $A2780^{cisR}$ calculated in response to sequential (0/0, 0/4 and 4/0 h) drug administration; Data are expressed as means ± SEM (n = 3); and analyzed by ANOVA (two-way) pursued by multiple comparison test (post hoc Bonferroni) using the software Graph Pad Prism-6. *** indicates P <0.001. ### shows significant difference relative to the control through two-way ANOVA followed by post hoc Bonferroni multiple comparisons test. ***P <0.001 compared with the cisplatin uptake in untreated control.

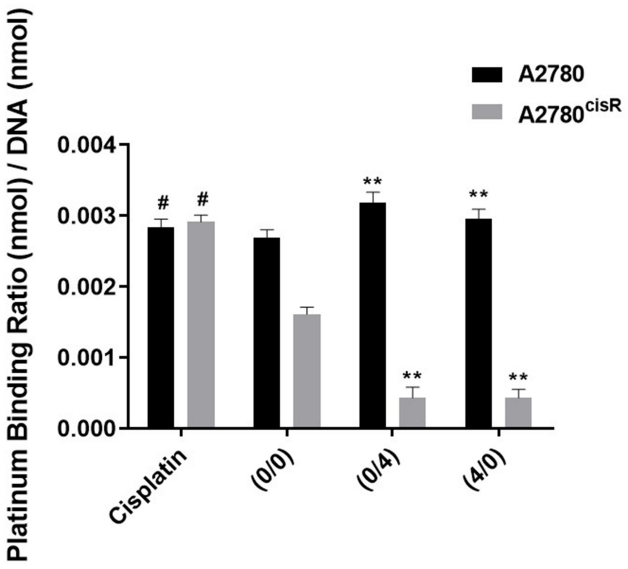

Figure 5 Intracellular platinum-DNA binding levels in A2780 and A2780^{cisR} calculated in response to sequential (0/0, 0/4 and 4/0 h) drug administration; data are expressed as means ± SEM (n = 3); and analyzed by ANOVA (two-way) followed by multiple comparison test (post hoc Bonferroni) using the software Graph Pad Prism-6. ** indicates P <0.01 and # shows significant difference relative to the control through two-way ANOVA followed by post hoc Bonferroni multiple comparisons test.

cell line. Contrarily, however, this combination resulted in increased levels of platinum-DNA binding in parent cell line A2780 (Figure 5).

In silico Investigation of Effects of Wedelolactone on Epigenetic Factors

The binding affinity of ligands in this study was evaluated on the basis of E-value (kcal/mol), number of hydrogen bonds, pi-pi bonds and other hydrophobic interactions against selected targets that are reported to be involved in development of resistance of cancer cells against cisplatin (Figure 6; Table 3). Moreover, 2D images of the most probable interaction of wedelolactone and reference drugs with selected targets are represented in Figure 7 to 10. Results of post hoc analysis revealed that the E-values (Kcal/mol) of the best pose of wedelolactone with selected targets are comparable with the respective reference drugs. Binding energy (Kcal/mol) for interaction of wedelolactone with checkpoint kinase 1 (Chk1) lower than standard drug prexasertib while more pi-pi interactions (5 compared with 2) are evident (Table 3). Similarly more favorable binding energy for interactions with ecto-5-nucleotidase CD73 was observed in the case of wedelolactone (−8.3 Kcal/mol) than respective standard (−6.1 Kcal/mol). This may be the outcome of a lower number of unfavorable interactions of test compound with CD73 than standard (Table 3). Acetyl transferase Tip60 was also selected as potential target. 2D interactions and binding energy values both point towards better interaction and binding of wedelolactone than standard inhibitor anacardic acid (Figure 8C and D). Tested compound came out as a stronger ligand for Nrf2 than standard drug trigonelline with low binding energy (−10 Kcal/mol) than standard (−6.3 Kcal/mol), however the possible reason can be the formation of an additional pi-pi bond or absence of Pi-anion bond which is present in the case of standard inhibitor (Figure 9C and D). In the case of another selected target, mTOR1, standard drug sirolimus showed higher binding energy than 7- wedelolactone despite an additional H-bond and pi-pi interactions (Figure 8A & B). The interaction of wedelolactone with another important culprit of resistance development, HIF-2α, was less favorable that its ligand PT2385. The reason could be two additional pi-pi bonds in the case of latter (Figure 8E and F Figure 9E and F). The better interaction of wedelolactone than standard is evident in the case of Brd1 which can be possibly attributed to stronger H-bonding with PHE: A 26 (Figure 8A and B)(Figure 7C and B).

Discussion

Ovarian cancer is one of the most lethal malignancies responsible for thousands of deaths annually. Populations at risk are continually on the rise due to acquired drug resistance, high relapse rate after surgery, incomplete knowledge regarding the etiology, interaction with other gynecological malignancies and diagnosis at an advanced stage when the disease is likely to have spread beyond the ovaries. This study was designed to investigate the effectiveness and potential of a natural compound wedelolactone in combination with the clinical agent cisplatin in ovarian cancer cell line A2780 and its cisplatin resistant subtype A2780^{cisR}. For this purpose, IC_{50} and RF values of cisplatin and wedelolactone were first determined before combination studies (Figure 2A and B). Whereas IC_{50} is a measure of the ability of a compound to induce cell death, RF value indicates its relative ability to induce cell death in parent and resistant cell lines. Knowing that cisplatin has an RF value of about 5, anything less than that means that the compound would be better able than cisplatin to

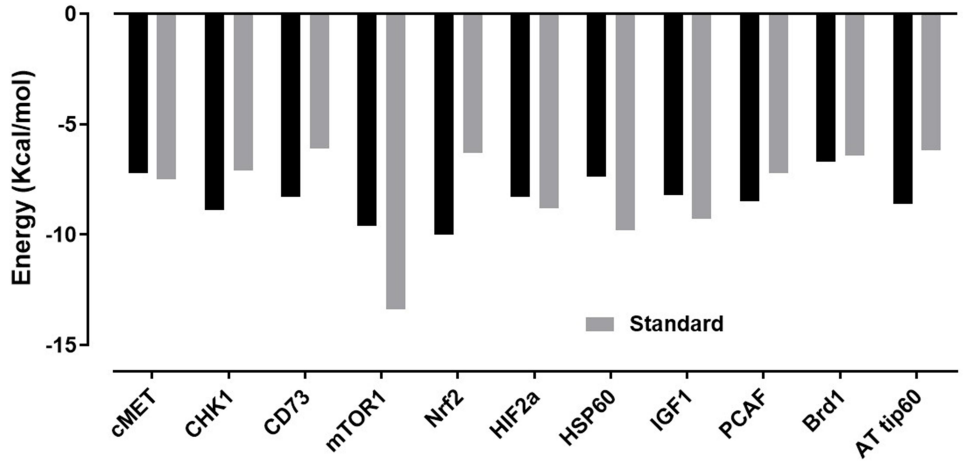

Figure 6 Illustrates E-values (Kcal/mol) of best docked poses of wedelolactone and standard drugs with hepatocyte growth factor receptor (cMET), checkpoint kinase 1 (Chk1), ecto-5′-nucleotidase (CD73), mammalian target of rapamycin (mTOR1), nuclear factor erythroid 2–related factor 2 (Nrf2), hypoxia-inducible factor 2-alpha (HIF2α), with heat shock protein 60 (HSP60), insulin-like growth factor (IGF1), P300/CBP-associated factor (PCAF), bromodomain protein 1 (Brd1) and acetyl transferase tip (AT tip60).

overcome drug resistance.[33] Thus, RF value of 1.1 for wedelolactone compared with 5.3 for cisplatin indicates its greater relative ability to cause more cell death than cisplatin in cisplatin-resistant cell line A2780^{cisR} (Table 2). In the next step, combination of wedelolactone with cisplatin was administered in a sequence- and time-dependent manner in both ovarian cancer cell lines (Table 1). The idea behind the sequential administration is the recognition that plant-derived compounds can sensitize tumor cells towards a toxic clinical agent, cisplatin in this case, resulting in a lesser dose of drug (which is reduced to half in combination), thus resulting in reduced toxicity and side effects. The rationale behind the time gap (4 h in our experiment) to add the phytochemical either before or after the cisplatin is to see if the phytochemical agent of interest can sensitize cells in a way that a lesser amount of platinum drug can bring the same cytotoxic effect. In our experiment, CI (1.07 and 1.08 in the case of resistant and parent cell lines, respectively) at 0/0 h shows that when cisplatin and wedelolactone were added to cells immediately after cell seeding, the outcome was additive in both types of cells irrespective of their sensitive or resistant nature. As evident in Table 1, the time gap was not effective (as it was moderately antagonistic) in either 0/4 or 4/0 h in the case of parent cell line (A2780); however in the case of cisplatin-resistant A2780^{cisR} cells, CI values (1.05 and 0.95 at 0.4 and 4/0 h) show an additive effect which implies that the addition of wedelolactone in either case (0/4 or 4/0 h) could somehow sensitize the cells. In our earlier work, however, we have seen a synergistic outcome of combination of wedelolactone with cisplatin in HeLa cell line.[24] In this study, the additive effect was observed in the resistant cell line irrespective of sequence of administration while the antagonistic effect observed in the parent cell line at 0/4 and 4/0 h suggests that the combination is more effective in the resistant cell line than in the parent one. This finding supports the results (Table 2) where significantly lower RF value of wedelolactone (1.1) than cisplatin (5.3) in the case of A2780^{cisR} was observed. This finding gets further support from the platinum accumulation and platinum-DNA binding study (Figures 4 and 5). As to the question why a particular combination is additive in the resistant cell line but antagonistic in the parent cell line, it is suggested that the difference may be associated with differential expression of some key proteins, some of which we selected in this study. The function of such proteins can be restored to normalcy in response to successful drug combinations. Nrf2 is reputed as a tumor suppressor in initial stages of cancer development but consistently increasing data have portrayed it as an oncogenic factor leading to resistance to chemotherapy while hyperactivated. The extent of resistance in human cisplatin resistant ovarian cancer cell line SKOV3 has been shown previously to be associated with increased

Table 3 Binding Energy (kcal/mol) and Post-Dock Analysis of the Best Pose of Wedelolactone with Hepatocyte Growth Factor Receptor (cMET), Checkpoint Kinase 1 (Chk1), Ecto-5′-Nucleotidase (CD73), Mammalian Target of Rapamycin (mTOR1), Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2), Hypoxia-Inducible Factor 2-Alpha (HIF2α), Heat Shock Protein 60 (HSP60), Insulin Like Growth Factor (IGF1), P300/CBP-Associated Factor (PCAF), Bromodomain Protein 1 (Brd1) and Acetyl Transferase Tip (at Tip60)

WDL	E-Value (kcal/mol)	Amino Acids Forming H Bonds	Amino Acids Forming Pi-Pi Bonds	Amino Acids Forming Other Hydrophobic Interactions	Standard Drugs	E-Value (kcal/mol)	Amino Acids Forming H Bonds	Amino Acids Forming Pi-Pi Bonds	Amino Acids Forming Other Hydrophobic Interactions
Brd1	−7.2	_	1(ILE1084), MET1160, ALA 1108, VAL1092, MET1108	(MET1211)	Crozotinib	−7.5	1(HIS1162)	1(MET1211), 1 (ILE1084)	_
AT Tip60	−8.9	_	2(LEU15, 137), 1 (ALA36), 2(LYS38)	1(VAL23)	Prexasertib	−7.1	1(GLU91), 1 (LEU15), 1 (CYS87), 1 (SER147)	1(VAL23), 1 (LEU137)	GLY90
CD73	−8.3	1(ALA72)	PRO51	-	AMP-CP	−6.1	GLU231, 1 (GLY562), 1 (LYS187), 1 (TYR253)	_	LYS232
mTOR1	−9.6	1(ASN381)	ALA47	1(CYS317)	Sirolimus	−13.4	_	TRP2239	-
Nrf2	−10	1(GLY511)	2(ALA366; VAL465)	-	Trigonelline	−6.3	1(ASN381), 1 (ASP38)5	1(TYR572), 1 (PHE577)	TYR572
HIF2α	−8.3	_	1(VAL46), 1 (ARG58)	1(GLU19)	PT2385	−8.8	_	1(PHE61),1 (LEU57), 1 (VAL46),1(ARG58)	
Chk1	−8.6	1(SER361)	1(THR 320), 1 (ARG326)	_	Anacardic acid	−6.2	1(SER361)	1(ILE318)	_
HSP60	−7.4	1(LYS272)	2(VAL271, 240), 1 (LEU237), 1 (ALA241)	_	Myrtucommulone	−9.8	1(LYS272)	3(VAL240, 271, 273), 1(LEU237), 1 (ALA241)	_
IGF1	−8.2	2(MET1082, PHE1154)	1(VAL1063), 1 (MET1082), 1 (LEU1005)	2(MET1142)	PPP	−9.3	1(GLY1155), 1 (TYR1165), 1 (ARG1158)	-	1(ASP1185)

(*Continued*)

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Table 3 (Continued).

WDL	E-Value (kcal/mol)	Amino Acids Forming H Bonds	Amino Acids Forming Pi-Pi Bonds	Amino Acids Forming Other Hydrophobic Interactions	Standard Drugs	E-Value (kcal/mol)	Amino Acids Forming H Bonds	Amino Acids Forming Pi-Pi Bonds	Amino Acids Forming Other Hydrophobic Interactions
PCAF	−8.5	1(ASP610)	1(ALA643), 1 (LEU526), 1 (ARG528)	1(MET529)	Anacardic acid	−7.2	1(THR607), 1 (CYS574), 1 (ALA609)	1(LEU526),1 (VAL576)	TYR608
CMET	−6.7	1(PHE26)	1(LEU25)		Olinone	−6.4	1(GLY47), 1(VAL 23),1(SER18), 1 (ILE24)	2(PHE26)	-

Abbreviations: ALA, alanine; ASN, asparagine; ASP, aspartic acid; ARG, arginine; CYS, cysteine; GLU, glutamic acid; GLY, glycine; GLN, glutamine; HIS, histidine; LYS, lysine; PHE, phenylalanine; SER, serine; THR, threonine; TRP, tryptophan; TYR, tyrosine; VAL, valine.

level of Nrf2. The inhibition of Nrf2 has been found to result in increased sensitivity while its activationresults in increased resistance in human cisplatin resistant cancer cells.[34] Overall, the enhanced level of Nrf2 seems to be preventive in initial stages while in advanced or malignant stages of cancer increased levels have been shown to protect the tumor microenvironment. Patricia Cho et al. (2019) working at Harvard Medical School recently conducted a study in a 3D spheroid model where Nrf2 hyper-activation in lung cancer enabled the cancer cells to bypass the death program through enhanced antioxidant role of cancer cells; knocking down Nrf2 through CRISPR resulted in cell death in inner cells of spheroids which previously escaped death.[35] Wedelolactone showed more inhibition of Nrf2 than standard as observed *in silico* (Table 3; Figure 9). The better response of wedelolactone in resistant cells may be the outcome of its inhibitor-like effect on check point kinase 1 (Chk1) which may contribute towards its additive outcome when applied in combination with cisplatin. Recently Parmar et al. (2019) in a preclinical study have reported that combination of a Chk1 inhibitor prexasertib with PARP inhibitor olaparib in an olaparib-resistant model of high grade serous ovarian cancer was synergistic.[36] However, since AutoDoc Vina only gives a rough idea of binding and simulation studies can give more reliable speculations, while only *in vitro* studies are authoritative to conclude the involvement of actual players, further studies are needed to explore the actual mechanisms and pathways involved.

Our study points towards possible action of wedelolactone on these selected protein targets associated with resistance (Figures 6 and 11). Additive combinations at 0/0 h, 0/4 h and 4/0 h sequences were found to be associated with higher levels of platinum accumulation/uptake in both cell lines (Figure 4). However, it is clear from the platinum-DNA binding experiment (Figure 5) that combination of wedelolactone resulted in reduced binding of platinum to DNA in cisplatin-resistant A2780^{cisR} cells irrespective of any time gap. It is worth noting that while platinum-DNA binding is considered a crucial step in cell death, cell apoptosis is a joint venture resulting from downstream expression of several cellular proteins. It can be interpreted from these results that higher platinum content has been compartmentalized somewhere from where it could not reach DNA in case of A2780^{cisR}. The apoptotic effect of

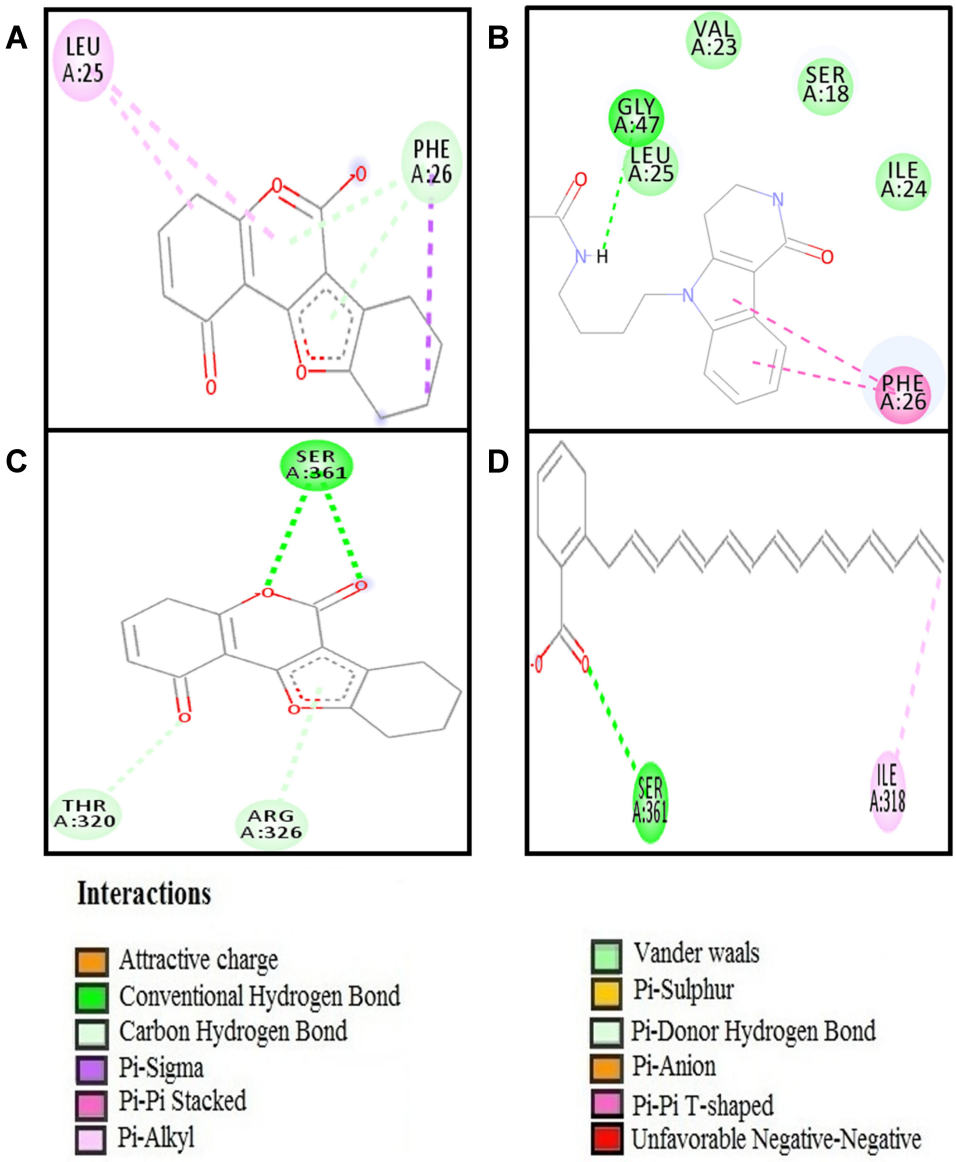

Figure 7 Shows 2D best pose of (**A**) hepatocyte growth factor receptor (cMET) with wedelolactone; (**B**) hepatocyte growth factor receptor (cMET) with crozotinib; (**C**) checkpoint kinase 1 (Chk1) with wedelolactone; (**D**) checkpoint kinase 1 (Chk1) with anacardic acid.

wedelolactone on A2780^{cisR} as well as its better action towards curbing cisplatin resistance may be an outcome of its effect on enhanced accumulation of cisplatin and down-regulation of Nrf2 on one hand, while inhibiting CD73 cascade and AT Tip60 in an interconnected sequence of events, as hypothesized in Figure 11. Wedelolactone might have inhibited cell cycle progression, gene transcription and DNA damage repair.

To be able to develop and test subtype-specific treatments in the correct model, it is imperative to know which histological subtype each cell line represents. All three cell lines used in this study are of endometrioid clear cell subtype which are non-serous. A2780 has been described as' 'R (round) while A2780cisR as 'S' (spindle shaped). The transformation from round to spindle shape cluster is indeed an outcome of adaptation of invasiveness and

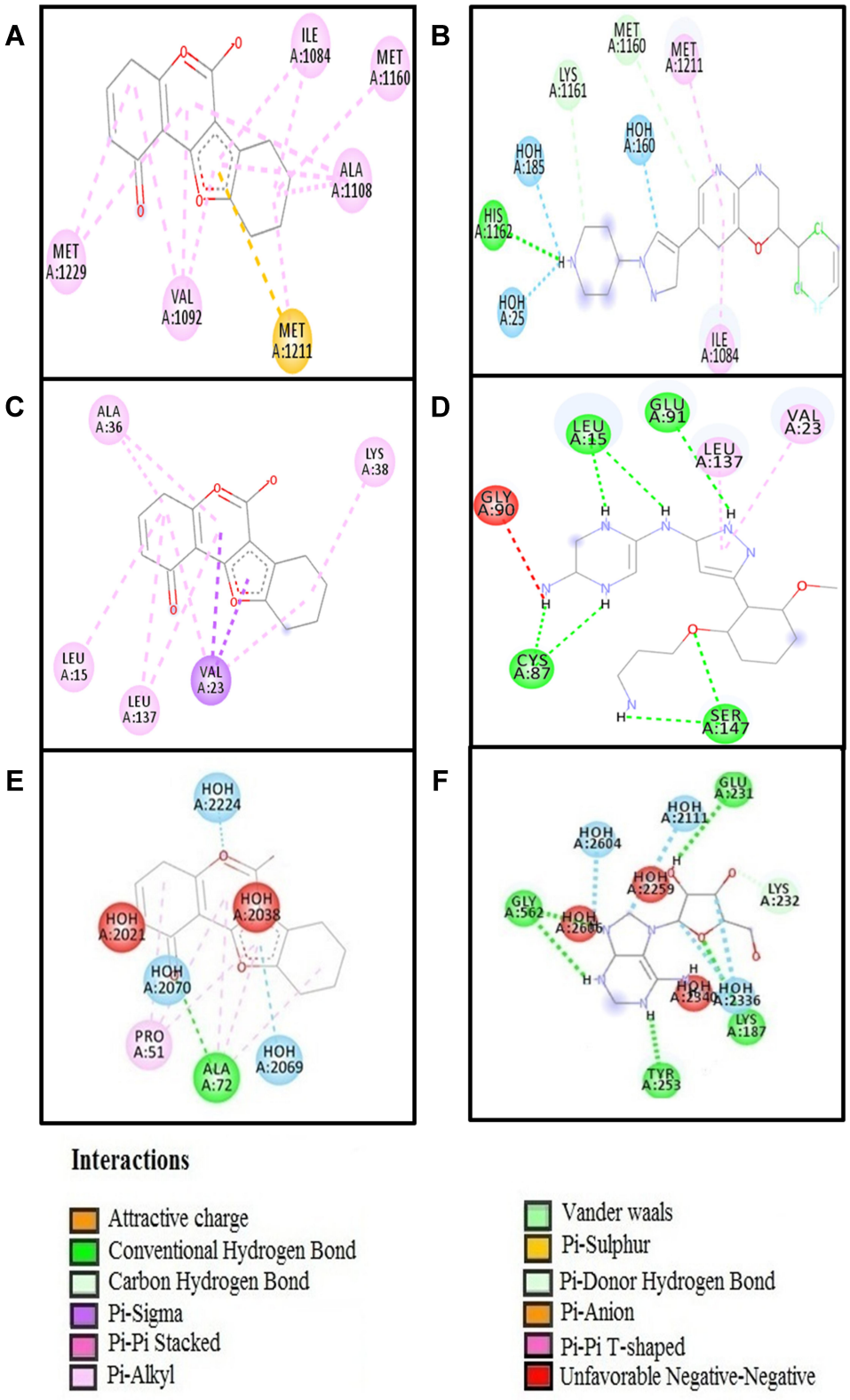

Figure 8 Shows 2D interactions of the best pose of (**A**) bromodomain protein 1 (Brd1) with wedelolactone; (**B**) bromodomain protein 1 (Brd1) with olinone; (**C**) acetyl transferase tip60 (AT tip60) with wedelolactone, (**D**) acetyl transferase tip60 (AT tip60) with anacardic acid. (**E**) CD73 with wedelolactone; (**F**) CD73 with Pt2385.

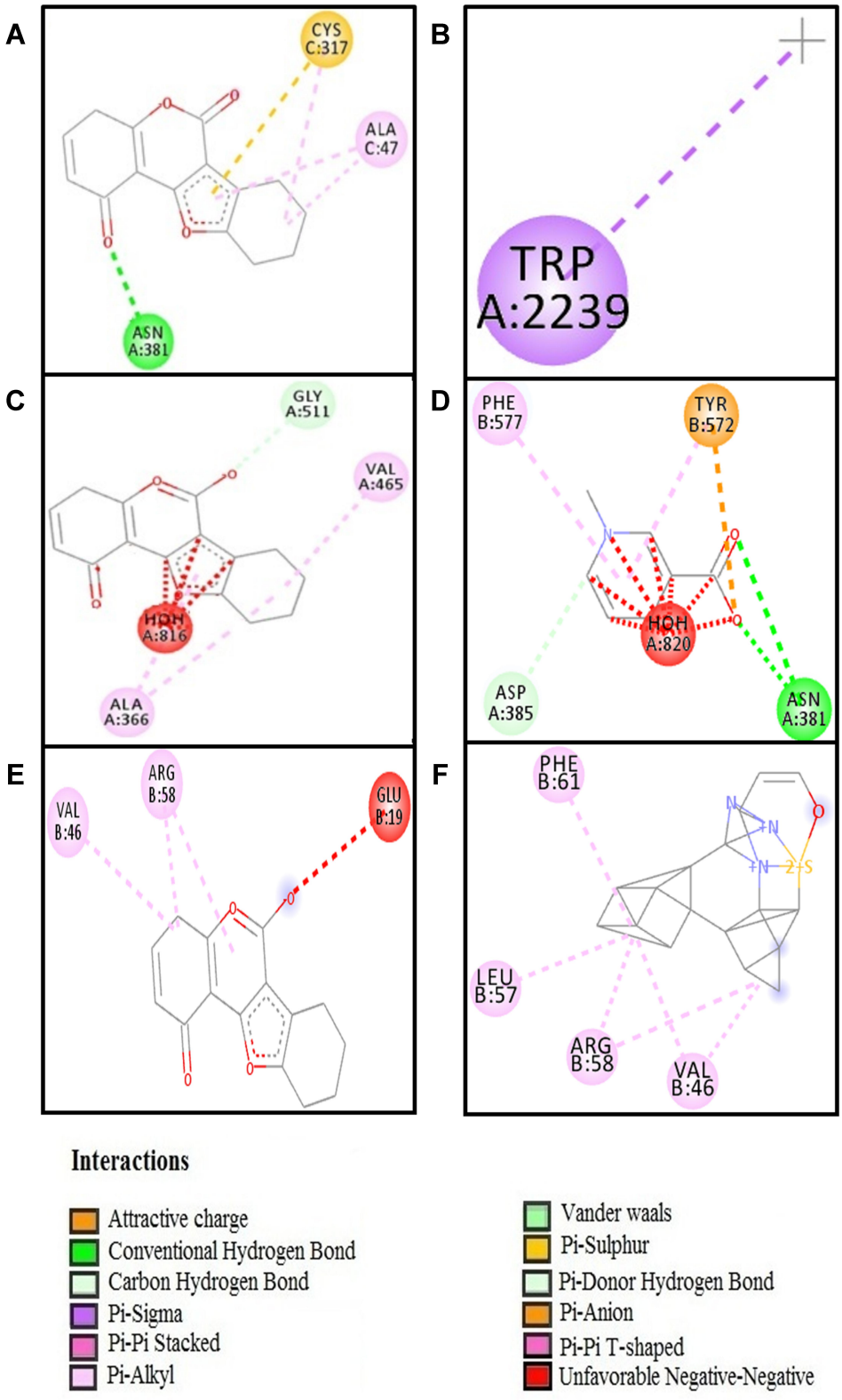

Figure 9 Shows 2D interactions of the best pose of (**A**) mammalian target of rapamycin (mTOR1) with wedelolactone; (**B**) mammalian target of rapamycin (mTOR1) with sirolimus; (**C**) nuclear factor erythroid 2–related factor 2 (Nrf2) with wedelolactone; (**D**) nuclear factor erythroid 2–related factor 2 (Nrf2) with trigonelline; (**E**) hypoxia-inducible factor 2-alpha (HIF2α) with wedelolactone; (**F**) hypoxia-inducible factor 2-alpha (HIF2α) with PT2385.

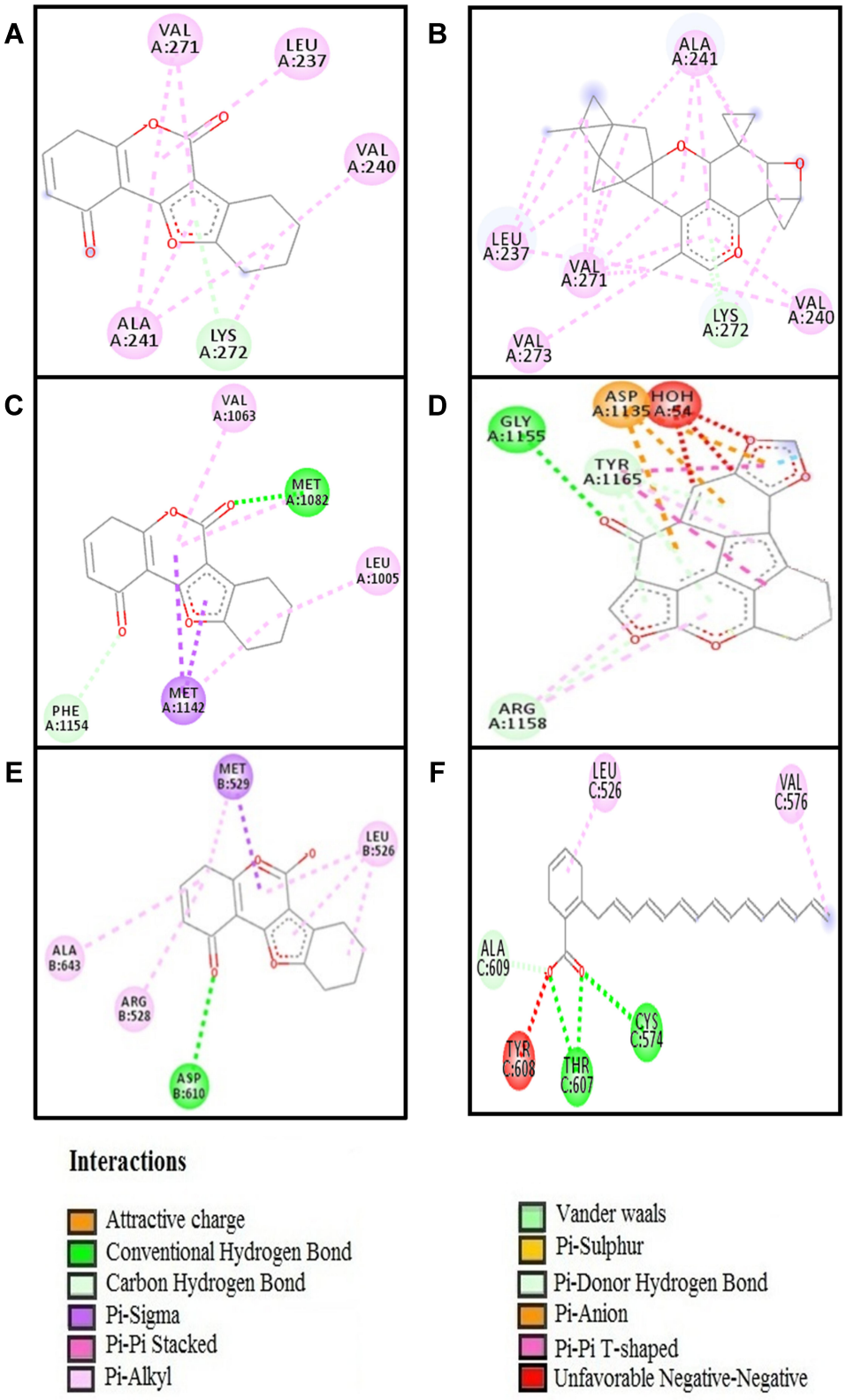

Figure 10 Shows 2D interactions of the best pose of (**A**) heat shock protein 60 (HSP60) with wedelolactone (**B**) heat shock protein 60 (HSP60) with myrtucommulone; (**C**) insulin like growth factor (IGF1) with wedelolactone (**D**) insulin like growth factor (IGF1) with PPP; (**E**) P300/CBP-associated factor (PCAF) with wedelolactone (**F**) P300/CBP-associated factor (PCAF) with anacardic acid.

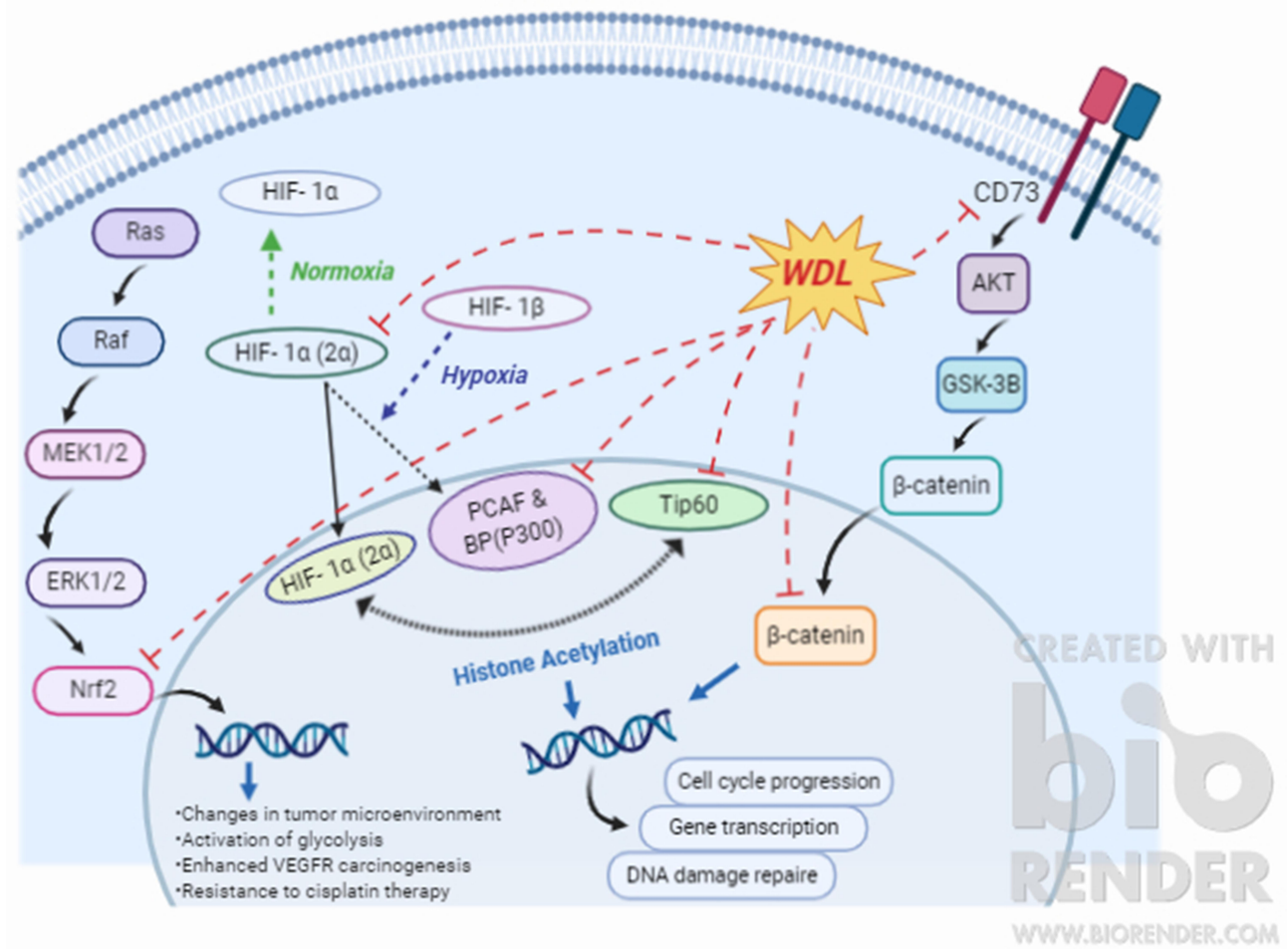

Figure 11 Signaling pathways involved in inactivation of apoptosis and resistance to cisplatin which are possibly inhibited by wedelolactone (WDL). Created with BioRender.com.

migratory properties of these cells, which enables these cells to become mesenchymal, a phenomenon known as epithelial mesenchymal transition. Additionally, TGFBI, TGFBR2, TWIST1, ZEB1, ZEB2, VIM, FN1, CAV1 were up-regulated in A2780cisR.[37] This cluster results in the worst prognosis clinically. Therefore, these findings can guide the further research and treatment options for ovarian cancer subtype of endometrioidal non serous origin.

Conclusion

In this investigation, combination of wedelolactone and cisplatin has been found to be more effective/additive in endometrioidal non serous cisplatin resistant ovarian cancer cell line A2780^{cisR} than in parent cell line A2780, irrespective of any sequence of administration.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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