Anti-parasitic Drug Ivermectin Exhibits Potent Anticancer Activity Against Gemcitabine-resistant Cholangiocarcinoma *In Vitro*

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Abstract. *Background/aim: The antiparasitic drug, ivermectin (IVM), exerts anticancer activities in diverse cancer types. However, its anticancer activity against cholangiocarcinoma (CCA), especially the drug-resistant phenotype, has not yet been explored. Materials and Methods: IVM was tested for its anticancer activity against gemcitabine-sensitive (KKU214) and gemcitabine-resistant (KKU214^{GemR}) CCA cell lines in vitro using the sulforhodamine B and clonogenic assays as well as cell-cycle analysis. Results: IVM treatment inhibited cell proliferation and colony formation of both KKU214 and KKU214^{GemR} in a dose- and time-dependent manner. KKU214^{GemR} cells were more sensitive than KKU214 to IVM treatment. IVM treatment caused S-phase cell-cycle arrest and also cell death as indicated by an increase of sub-G_0/G_1 population in KKU214^{GemR} cells treated with IVM for 48 h. Conclusion: IVM exerts anti-CCA activities and gemcitabine-resistant KKU214^{GemR} cells are more sensitive to IVM treatment. Thus, IVM might be useful as an alternative treatment for CCA, especially in patients who do not respond to gemcitabine.*

Chemotherapeutic drugs have been developed and used as first-line treatment of several cancers. Although satisfactory outcomes are usually achieved at the beginning of treatment, the efficacy any drug tends to decline over time. Cancer cells can adapt to the toxic effects of chemotherapy and eventually become tolerant of the drug (1, 2). Drug resistance is the major impediment to effective cancer therapy and has been observed in several cancers including cholangiocarcinoma (CCA), a bile-duct cancer originating from bile-duct epithelial cells. Although surgical resection can be effective for CCA treatment, approximately two-thirds of CCA patients are inoperable (3). Hence, chemotherapy is a treatment of choice and has been used to control disease progression and improve survival and quality of life for unresectable CCA patients (4). Gemcitabine (2', 2'-difluorodeoxycytidine) is a deoxycytidine analogue which has been widely used for treatment of CCA (3). However, several clinical studies have indicated that response rates and overall survival rates are relatively low (4), likely due to acquired drug resistance. Therefore, it is necessary to identify other agents or drugs that can be used against drug-resistant CCA.

Ivermectin (IVM) is a derivative of avermectin and was discovered in 1970. IVM is one of the most effective anti-parasitic drugs and is widely used in human and veterinary medicine (5). Apart from its anti-parasitic activity, IVM has been shown to exert anti-cancer activities including inhibition of proliferation as well as induction of apoptosis and autophagy (6-8). The anticancer activities of IVM have been observed both *in vitro* and *in vivo* in a number of solid tumors such as colon cancer (9), glioblastoma (10), ovarian cancer (11) and in hematological malignancies such as leukemia (12). IVM has also been shown to reverse the drug resistance of cancer cells (13-15). Therefore, IVM is a promising anticancer agent and could possibly be used against drug-resistant cancers.

It is not known whether IVM can exert anticancer activity against CCA, and especially against drug-resistant CCA. Herein, we explored the anti-CCA activity of IVM and

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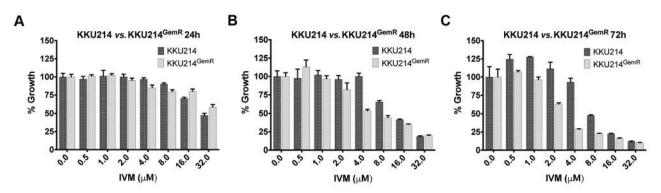
Figure 1. *IVM treatment inhibited cell proliferation of KKU214 and KKU214^GemR CCA cell lines. The anti-proliferative activity of IVM was evaluated using the SRB assay. Both KKU214 and KKU214^GemR CCA cell lines were treated with different concentrations of IVM for (A) 24 h, (B) 48 h and (C) 72 h. DMSO-treated cells were used as controls. The experiment was performed in triplicate. IVM: Ivermectin.*

demonstrated that IVM inhibits CCA cell growth and clonogenicity in dose- and time-dependent manner in both gemcitabine-sensitive and gemcitabine-resistant CCA cell lines *in vitro*. Interestingly, the anticancer properties of IVM were more prominent in gemcitabine-resistant CCA cells. These results suggest that IVM is a promising agent for treatment of CCA.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), sterile phosphate-buffered saline (PBS), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA solution and flat-bottomed 96-well plates were obtained from Corning (Corning, NY, USA). FxCycle PI/RNase staining solution was obtained from Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA, USA). Ivermectin (IVM), dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA) and sulforhodamine B (SRB) were purchased from Sigma Aldrich (St. Louis, MO, USA). Gemcitabine (Gemzar) was purchased from Eli Lilly (Indianapolis, IN, USA).

Human CCA cell lines. Gemcitabine-sensitive CCA KKU214 (16) and gemcitabine-resistant KKU214 (KKU214^GemR) cell lines (17) were established from Thai CCA patients as described previously. Both cell lines were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C with 10% CO_2 in a humidified incubator. KKU214^GemR was maintained in the presence of gemcitabine. Before use in an experiment, this cell line was cultured in a drug-free medium for one passage.

Assessment of cell proliferation. CCA cells seeded in flat-bottomed 96-well plates (2,000 cells/well) were treated with either DMSO (control) or different concentrations of IVM dissolved in DMSO for 24, 48 and 72 h. Thereafter, the cells were fixed with cold 40% TCA for 1 h in a refrigerator and washed 3 times with running tap water. Then, the fixed cells were stained with 0.4% (w/v) SRB solution in 1% acetic acid for 1 h at room temperature. Excess SRB solution was removed by washing with 1% acetic acid and SRB dye was

dissolved with 10 mM Tris buffer pH 10.5. Absorbance at 492 nM was measured using an ELISA reader (Tecan group ltd., Männedorf, Switzerland). Absorbance obtained from DMSO-treated cells was used as control.

Clonogenic assay. Approximately 1,000 cells per well of CCA cell lines were grown in 6-well plates and treated with different concentrations of either gemcitabine or IVM. The culture medium was changed every 2 days and cells were grown for approximately 2 weeks. The cells were then fixed with 4% paraformaldehyde, stained with 0.5% crystal violet and dissolved with 33% acetic acid. The absorbance at 620 nM was measured using an ELISA reader (Tecan group ltd.).

Cell cycle analysis. One million CCA cells treated with either DMSO (control) or 4 μM IVM for 48 h were washed with ice-cold PBS and fixed with 70% ethanol overnight at –20˚C. After washing twice with ice-cold PBS, 500 μl of FxCycle PI/RNase staining solution were added and the cells were incubated at room temperature in the dark for 30 min. Finally, stained cells were detected using a BD FACSCanto II flow cytometer (BD biosciences, San Jose, CA, USA) and data analyzed using BD FACSDiva software (BD biosciences).

Statistical analysis. Data are expressed as mean±SD. Student's *t*-test was used to test differences between experimental groups. A value of $p < 0.05$ was considered statistically significant. Non-linear regression analysis was carried out to calculate IC_{50}. All statistical analyses were performed using Graphpad Prism 7.0 for Mac (GraphPad Software, Inc., CA, USA).

Results

IVM treatment suppressed proliferation of CCA cells and displayed potent growth inhibitory activity against gemcitabine-resistant CCA cells. Both gemcitabine-sensitive (KKU214) and gemcitabine-resistant (KKU214^GemR) CCA cell lines (17) were treated with different concentrations of IVM for up to 72 h. The SRB assay showed that cell proliferation in both cell lines
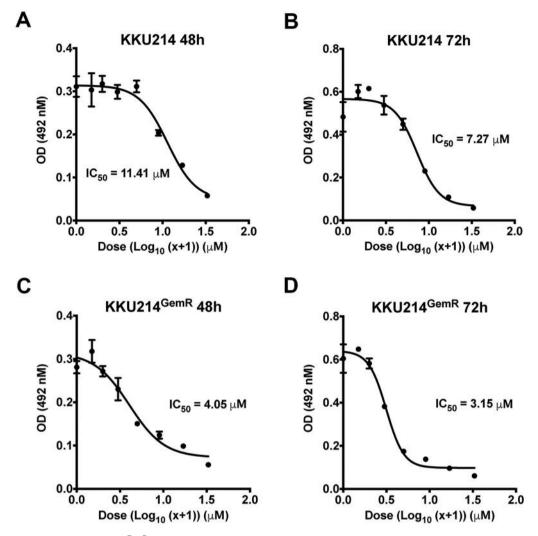
Figure 2. *Gemcitabine-resistant KKU214GemR cells were more sensitive to IVM treatment than gemcitabine-sensitive KKU214. Non-linear regression analysis was performed to calculate the half-maximal inhibitory concentration 50 (IC_{50}) of IVM at 48 h and 72 h for (A, B) KKU214 and (C, D) KKU214GemR CCA cell lines. Data generated from the SRB assay were used for calculation.*

decreased in dose- and time-dependent manner (Figure 1). Potent cell proliferation inhibition (more than 75%) was achieved in both CCA cell lines treated with 32 μM of IVM for 48 h or 72 h (Figure 1B and C). Interestingly, KKU214GemR cells, which are extremely resistant to gemcitabine, were more sensitive to IVM treatment than the parental KKU214 cell line. This was particularly apparent at concentrations of IVM of 4 μM or higher, at all time points. Non-linear regression analysis showed that the IC_{50} concentrations of IVM at 48 h and 72 h for KKU214 cells were 11.41 μM and 7.27 μM, respectively. Corresponding values for KKU214GemR cells were 4.05 μM and 3.15 μM, respectively (Figure 2). These findings indicated that IVM exhibited anti-CCA potential, especially for gemcitabine-resistant CCA cells.

IVM treatment inhibited clonogenicity of CCA cells. A clonogenic assay was employed to test the ability of IVM to inhibit unlimited division and colony formation of CCA cell lines (18). In line with the SRB assay, IVM treatment significantly inhibited colony formation of KKU214 cells in a dose-dependent manner compared to untreated controls ($p<0.0001$), but was less effective than a similar dosage of gemcitabine (Figure 3A-C). In contrast, gemcitabine treatment was ineffective against the KKU214GemR CCA cell line (Figure 3A and D), whereas IVM treatment significantly suppressed colony formation of KKU214GemR cells compared to untreated controls ($p<0.0001$, Figure 3B and D). Once again, the inhibitory effect of IVM on colony formation was more potent in KKU214GemR cells compared to KKU214 cells. These results suggest that IVM exerts its
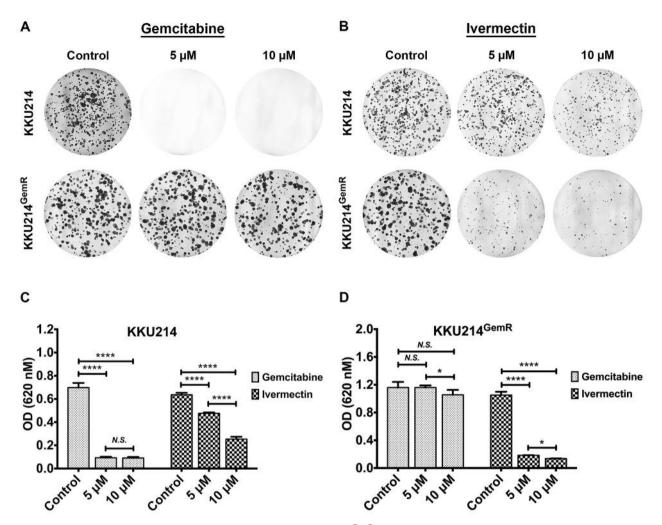
Figure 3. *IVM treatment inhibited colony formation of KKU214 and KKU214^GemR CCA cell lines. The effect of (A) gemcitabine treatment and (B) IVM treatment on colony formation of both CCA cell lines was investigated using a clonogenic assay. Relative differences in colony formation of (C) KKU214 and (D) KKU214^GemR CCA cell lines treated with either gemcitabine or IVM compared to controls was investigated by measuring absorbance at optical density (OD) 620 nM. The experiment was performed in triplicate. * and **** indicate a significant difference at $p<0.05$ and $p<0.0001$, respectively. N.S.: No significant difference.*

anti-CCA activity partly through inhibition of unlimited division and colony formation.

IVM treatment induced cell-cycle arrest of gemcitabine-resistant KKU214^GemR. Since the SRB assay demonstrated the growth inhibition properties of IVM against CCA cell lines, we then examined whether induction of cell-cycle arrest contributed to this phenomenon. Flow cytometry demonstrated that the cell-cycle profiles of the IVM-treated KKU214 cells did not differ from those of controls (Figure 4A-C). However, treatment with 4 μM of IVM for 48 h significantly induced cell-cycle arrest at the S phase in KKU214^GemR cells compared to controls ($p<0.01$, Figure 4D-F). Furthermore, the population of sub-G_0/G_1 of IVM-treated KKU214^GemR cells, an indicator of apoptotic cell

death (19), was significantly higher compared to controls (2.93±0.83 *vs.* 0.67±0.15, $p<0.05$, Figure 4D-F). Therefore, in addition to inhibition of growth and colony formation, IVM treatment can induce apoptotic cell death of KKU214^GemR cells.

Discussion

Resistance to conventional chemotherapy has been the most important obstacle for effective treatment of many cancer types including CCA. In addition to the increasing chance of relapse, the development of cancer drug resistance could possibly make treatment nearly impossible (20). A number of therapeutic approaches to circumvent this problem have been proposed such as targeted therapy (21), nanomedicine
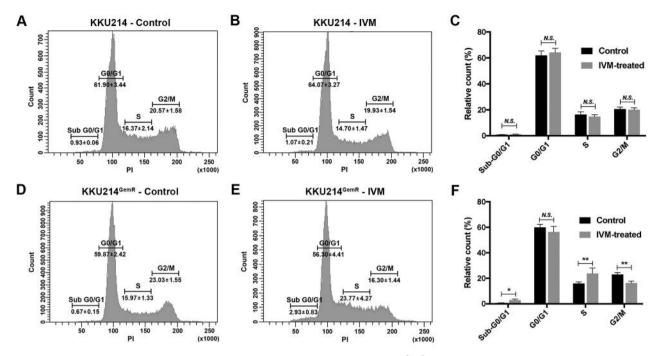
Figure 4. *IVM treatment caused S-phase and sub-G$_0$/G$_1$ cell-cycle arrest of KKU214^{GemR}. Cell-cycle analysis of (A-C) KKU214 and (D-F) KKU214^{GemR} CCA cell lines after treatment with either DMSO (controls) or 4 μM of IVM for 48 h, was performed by flow cytometry. Cell-cycle distribution was visualized using BD FACSDiva software. The experiment was performed in triplicate. * and ** indicate a significant difference at p<0.05 and p<0.01, respectively. N.S.: No significant difference.*

(22), drug combination therapy using drugs with different actions (23), *etc*. However, the efficacy of these approaches for treatment of drug-resistant cancer has been questioned. Herein, we demonstrated that the anti-parasitic drug IVM exerts anticancer activity against CCA cells. Importantly, IVM also exerted a potent anticancer activity against gemcitabine-resistant CCA cells.

Due to its safety and high efficacy, IVM has been used for treating both endo- and ectoparasites of animals and humans for decades (24). IVM is also known to act against several cancer types. A number of mechanisms have been found to be associated with the anticancer activity of IVM. However, an antiproliferative function has been widely reported (8). We found that IVM treatment effectively inhibited cell proliferation and colony formation of both gemcitabine-sensitive and gemcitabine resistant CCA cell lines. Our findings are in general agreement with a previous report investigating colon cancer (9). Notably, non-linear regression analysis found that the IC$_{50}$ of IVM against KKU214 cells was higher than that in KKU214^{GemR} cells. Increased expression levels of chloride channels (12), which are usually observed in drug resistant cancer cells (25-27), might be responsible for the difference in susceptibility to IVM between these cell lines. Furthermore, there might be a difference in transcriptomic profile between gemcitabine-

sensitive and gemcitabine-resistant CCA cell lines (28). Cell-cycle analysis found that IVM treatment induced cell death of KKU214^{GemR} cells as demonstrated by the significant increase in the sub-G$_0$/G$_1$ population. The induction of death of KKU214^{GemR} cells by IVM may be mediated by the induction of mitochondrial dysfunction and oxidative DNA damage as demonstrated in renal cancer cells (29) and glioblastoma (10). However, significant cell-cycle arrest was not observed in the KKU214 CCA cell line treated with 4 μM of IVM for 48 h, consistent with the cell proliferation assay in which treatment with 4 μM of IVM did not cause a significant decrease in cell-proliferation rate compared to controls. These results indicate that drug resistant cells are more susceptible to IVM-induced cytostasis and cell death. However, the precise mechanism by which IVM exerts anticancer activity against CCA cells, especially drug resistant cancer cells, remains to be elucidated.

Conclusion

IVM treatment exhibited anti-CCA potential as shown by inhibition of cell growth and colony formation of CCA cell lines. The anticancer activities of IVM were more potent in gemcitabine-resistant KKU214^{GemR} cells. Furthermore, IVM treatment also induced S-phase and sub-G$_0$/G$_1$ cell-cycle

arrest in gemcitabine-resistant KKU214^{GemR} cells. Our results highlight the therapeutic value of IVM: this drug could be used for treatment of CCA, especially in patients who do not respond to gemcitabine treatment.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Author's Contributions

KI, CH, PP, and SP designed the experiments. KI performed the experiments. KI, CH, PP, and SP performed the data analysis. KI and SP drafted the manuscript. All Authors approved the final version of manuscript for publication.

Acknowledgements

References

1 Holohan C, Van Schaeybroeck S, Longley DB and Johnston PG: Cancer drug resistance: An evolving paradigm. Nat Rev Cancer *13(10)*: 714-726, 2013. PMID: 24060863. DOI: 10.1038/nrc3599

2 Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N and Sarkar S: Drug resistance in cancer: An overview. Cancers (Basel) *6(3)*: 1769-1792, 2014. PMID: 25198391. DOI: 10.3390/cancers6031769

3 Khan SA, Davidson BR, Goldin R, Pereira SP, Rosenberg WM, Taylor-Robinson SD, Thillainayagam AV, Thomas HC, Thursz MR and Wasan H: Guidelines for the diagnosis and treatment of cholangiocarcinoma: Consensus document. Gut *51*: VI1-9, 2002. PMID: 12376491. DOI: 10.1136/gut.51.suppl_6.vi1

4 Thongprasert S: The role of chemotherapy in cholangio-carcinoma. Ann Oncol *16*: ii93-96, 2005. PMID: 15958484. DOI: 10.1093/annonc/mdi712

5 Laing R, Gillan V and Devaney E: Ivermectin - old drug, new tricks? Trends Parasitol *33(6)*: 463-472, 2017. PMID: 28285851. DOI: 10.1016/j.pt.2017.02.004

6 Norenberg W, Sobottka H, Hempel C, Plotz T, Fischer W, Schmalzing G and Schaefer M: Positive allosteric modulation by ivermectin of human but not murine p2x7 receptors. Br J Pharmacol *167(1)*: 48-66, 2012. PMID: 22506590. DOI: 10.1111/j.1476-5381.2012.01987.x

7 Polakis P: Wnt signaling and cancer. Genes Dev *14(15)*: 1837-1851, 2000. PMID: 10921899.

8 Juarez M, Schcolnik-Cabrera A and Duenas-Gonzalez A: The multitargeted drug ivermectin: From an antiparasitic agent to a repositioned cancer drug. Am J Cancer Res *8(2)*: 317-331, 2018. PMID: 29511601.

9 Melotti A, Mas C, Kuciak M, Lorente-Trigos A, Borges I and Ruiz i Altaba A: The river blindness drug ivermectin and related macrocyclic lactones inhibit wnt-tcf pathway responses in human cancer. EMBO Mol Med *6(10)*: 1263-1278, 2014. PMID: 25143352. DOI: 10.15252/emmm.201404084

10 Liu Y, Fang S, Sun Q and Liu B: Anthelmintic drug ivermectin inhibits angiogenesis, growth and survival of glioblastoma through inducing mitochondrial dysfunction and oxidative stress. Biochem Biophys Res Commun *480(3)*: 415-421, 2016. PMID: 27771251. DOI: 10.1016/j.bbrc.2016.10.064

11 Hashimoto H, Messerli SM, Sudo T and Maruta H: Ivermectin inactivates the kinase pak1 and blocks the pak1-dependent growth of human ovarian cancer and nf2 tumor cell lines. Drug Discov Ther *3(6)*: 243-246, 2009. PMID: 22495656.

12 Sharmeen S, Skrtic M, Sukhai MA, Hurren R, Gronda M, Wang X, Fonseca SB, Sun H, Wood TE, Ward R, Minden MD, Batey RA, Datti A, Wrana J, Kelley SO and Schimmer AD: The antiparasitic agent ivermectin induces chloride-dependent membrane hyperpolarization and cell death in leukemia cells. Blood *116(18)*: 3593-3603, 2010. PMID: 20644115. DOI: 10.1182/blood-2010-01-262675

13 Jiang L, Wang P, Sun YJ and Wu YJ: Ivermectin reverses the drug resistance in cancer cells through egfr/erk/akt/nf-kappab pathway. J Exp Clin Cancer Res *38(1)*: 265, 2019. PMID: 31215501. DOI: 10.1186/s13046-019-1251-7

14 Pouliot JF, L'Heureux F, Liu Z, Prichard RK and Georges E: Reversal of p-glycoprotein-associated multidrug resistance by ivermectin. Biochem Pharmacol *53(1)*: 17-25, 1997. PMID: 8960059. DOI: 10.1016/s0006-2952(96)00656-9

15 Furusawa S, Shibata H, Nishimura H, Nemoto S, Takayanagi M, Takayanagi Y and Sasaki KI: Potentiation of doxorubicin-induced apoptosis of resistant mouse leukaemia cells by ivermectin. Pharm Pharmacol Comm *6(3)*: 129-134, 2000. DOI: 10.1211/146080800128735764

16 Tepsiri N, Chaturat L, Sripa B, Namwat W, Wongkham S, Bhudhisawasdi V and Tassaneeyakul W: Drug sensitivity and drug resistance profiles of human intrahepatic cholangio-carcinoma cell lines. World J Gastroenterol *11(18)*: 2748-2753, 2005. PMID: 15884115.

17 Wattanawongdon W, Hahnvajanawong C, Namwat N, Kanchanawat S, Boonmars T, Jearanaikoon P, Leelayuwat C, Techasen A and Seubwai W: Establishment and characterization of gemcitabine-resistant human cholangiocarcinoma cell lines with multidrug resistance and enhanced invasiveness. Int J Oncol *47(1)*: 398-410, 2015. PMID: 25998688. DOI: 10.3892/ijo.2015.3019

18 Franken NA, Rodermond HM, Stap J, Haveman J and van Bree C: Clonogenic assay of cells *in vitro*. Nat Protoc *1(5)*: 2315-2319, 2006. PMID: 17406473. DOI: 10.1038/nprot.2006.339

19 Darzynkiewicz Z, Halicka HD and Zhao H: Analysis of cellular DNA content by flow and laser scanning cytometry. Adv Exp Med Biol *676*: 137-147, 2010. PMID: 20687474. DOI: 10.1007/978-1-4419-6199-0_9

20 Leary M, Heerboth S, Lapinska K and Sarkar S: Sensitization of drug resistant cancer cells: A matter of combination therapy. Cancers (Basel) 10(12), 2018. PMID: 30518036. DOI: 10.3390/cancers10120483

21 Gao Y, Shen JK, Milane L, Hornicek FJ, Amiji MM and Duan Z: Targeted cancer therapy; nanotechnology approaches for overcoming drug resistance. Curr Med Chem *22(11)*: 1335-1347, 2015. PMID: 25666804.

22 Markman JL, Rekechenetskiy A, Holler E and Ljubimova JY: Nanomedicine therapeutic approaches to overcome cancer drug resistance. Adv Drug Deliv Rev *65(13-14)*: 1866-1879, 2013. PMID: 24120656. DOI: 10.1016/j.addr.2013.09.019

23 Al-Lazikani B, Banerji U and Workman P: Combinatorial drug therapy for cancer in the post-genomic era. Nat Biotechnol 30(7): 679-692, 2012. PMID: 22781697. DOI: 10.1038/nbt.2284

24 Omura S: Ivermectin: 25 years and still going strong. Int J Antimicrob Agents *31(2)*: 91-98, 2008. PMID: 18037274. DOI: 10.1016/j.ijantimicag.2007.08.023

25 Chen Q, Liu X, Luo Z, Wang S, Lin J, Xie Z, Li M, Li C, Cao H, Huang Q, Mao J and Xu B: Chloride channel-3 mediates multidrug resistance of cancer by upregulating p-glycoprotein expression. J Cell Physiol *234(5)*: 6611-6623, 2019. PMID: 30230544. DOI: 10.1002/jcp.27402

26 Shimizu T, Lee EL, Ise T and Okada Y: Volume-sensitive cl(–) channel as a regulator of acquired cisplatin resistance. Anticancer Res *28(1A)*: 75-83, 2008. PMID: 18383827.

27 Kischel P, Girault A, Rodat-Despoix L, Chamlali M, Radoslavova S, Abou Daya H, Lefebvre T, Foulon A, Rybarczyk P, Hague F, Dhennin-Duthille I, Gautier M and Ouadid-Ahidouch H: Ion channels: New actors playing in chemotherapeutic resistance. Cancers (Basel) 11(3), 2019. PMID: 30884858. DOI: 10.3390/cancers11030376

28 Varamo C, Peraldo-Neia C, Ostano P, Basirico M, Raggi C, Bernabei P, Venesio T, Berrino E, Aglietta M, Leone F and Cavalloni G: Establishment and characterization of a new intrahepatic cholangiocarcinoma cell line resistant to gemcitabine. Cancers (Basel) 11(4), 2019. PMID: 30979003. DOI: 10.3390/cancers11040519

29 Zhu M, Li Y and Zhou Z: Antibiotic ivermectin preferentially targets renal cancer through inducing mitochondrial dysfunction and oxidative damage. Biochem Biophys Res Commun *492(3)*: 373-378, 2017. PMID: 28847725. DOI: 10.1016/j.bbrc.2017.08.097