16th International Online Mini-Symposium
Of The Protein Society of Thailand

Investigation of multidrug resistance protein expression profile in HepG2 cells and microparticles induced by hypoxia

Kanlaya Khounkaew[1], Pornthip Chaichompoo[1], Kriengsak Lirdprapamongkol[2], Chantragan Srisomsap[2], Jisnuson Svasti[2], Saovaros Svasti[3], Titipatima Sakulterdkiat[1,*]

[1] Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, 10400 Thailand
[2] Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok, 10210 Thailand
[3] Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, 73170 Thailand

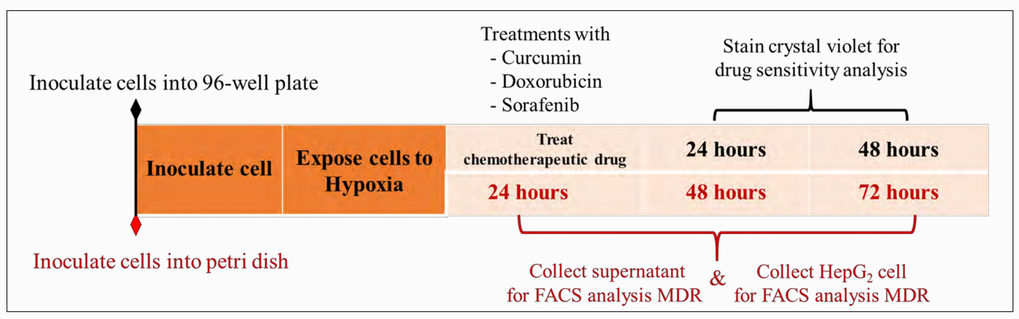
INTRODUCTION

Tumor microenvironment such as hypoxia impact tumor aggressiveness and treatment outcome. Hepatocellular carcinoma, one of the most challenging cancers to treat, has high mortality and recurrence rate due to increase multidrug resistance (MDR). Hypoxia induce MDR through increase expression of ABC transporter proteins. Microparticles (MP), medium extracellular vesicles, plays an important role in intercellular communication. We have shown increased survival rate of HepG2 cells treated with curcumin, doxorubicin and sorafenib under hypoxic condition (1% O_2) compared with that of normoxic (20% O_2) conditions. Interestingly, hypoxic conditioned media was able to increase survival rate of normoxic cells by roughly 20%. We hypothesize that hypoxic induced MDR in hepatocellular carcinoma can convey drug resistance phenotype to normoxic cancer cells via MP transfer of ABC transporter proteins.

MATERIALS AND METHODS

Cell culture

HepG2 cells obtained from the American Type Culture Collection were cultured in a 5% CO_2 humidified incubator at 37°C. Cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Sub-passage of cells were done by trypsinization with 0.5% Trypsin-EDTA and flushed with a 21-gauge needle to separate HepG2 into single cells. For experimentation, cells were exposed to either hypoxic (1% O_2) or normoxic (20% O_2) conditions for 18-24 hours prior to drug sensitivity testing.



Cytotoxicity testing

Cell survival in HepG2 cells exposed to hypoxia/normoxia are determined using 0.4% crystal violet special stain solution. Briefly, HepG2 cells are washed by phosphate buffer saline (PBS) and then fixed with 95% ethanol. Then, crystal violet solution are added to stain remaining living cells that have not been washed away by PBS. Extraction buffer is added to solubilize crystal violet stain and absorbance reading at 540 nm was done to quantify viable cells in hypoxic and normoxic cells treated with curcumin.

Isolation and detected MDR of microparticles and HepG2 cell line

MPs were isolated from HepG2 cell cultures supernatant exposed to hypoxia and normoxia. Collected supernatants and cell are centrifuged to separate each fraction. The supernatant is centrifuged at 14,000 rcf for 45 min at 4 °C to isolate MP , and HepG2 cell are centrifuged at 12,000 rcf for 2 min at room temperature to isolate cellular. And then add antibody multidrug resistance-associated protein 3 (MRP3), P- glycoprotein (MDR-1) and multidrug resistance associated protein (MRP-1) in MP and cell. Which Flow cytometric assay was performed for measure MDR protein expression. Remaining isolated MP is stored at -80 °C.

Figure 1.

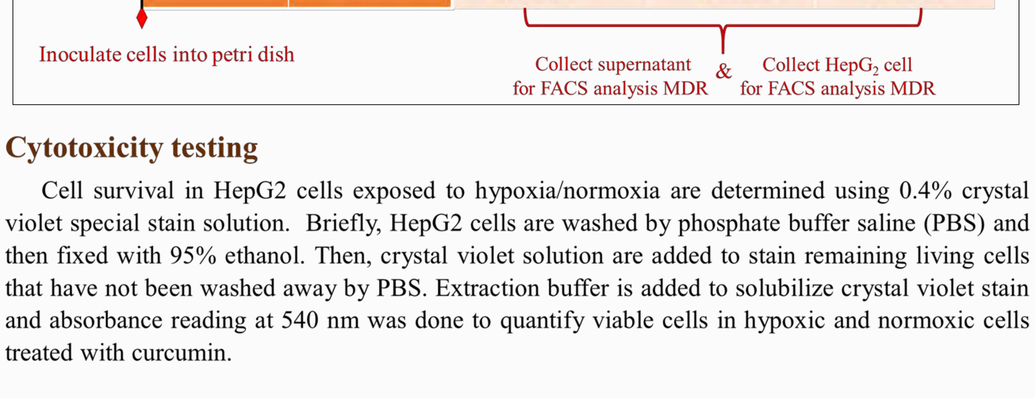


Figure 1. MPs from normoxic and hypoxic HepG2 cells were isolated. A) 1 uM sized beads using BD TruCount ™ was used as indicator of approximate size of MPs population in R3 window. B) Events of all the particles isolated is further separated into 3 subpopulations with R3, R4 and R5 representing medium-sized EV or MP, large-sized EV and cellular debris respectively. C) Detection of normal population of HepG2 cells.

Hypoxic condition

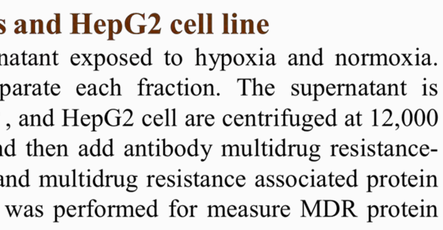


Figure 2. Western blot detection of HIF-1α microenvironment for HepG2 cells in this experiment. N = Normoxic, N+DFX = Normoxic with Deferasirox as positive control of HIF-1α expression and H = hypoxic.

ACKNOWLEDGEMENT
This research project is supported by Mahidol University

RESULTS

Flow Cytometric assessment of MDR

HepG2 Cell — Supernatant

Figure 3.

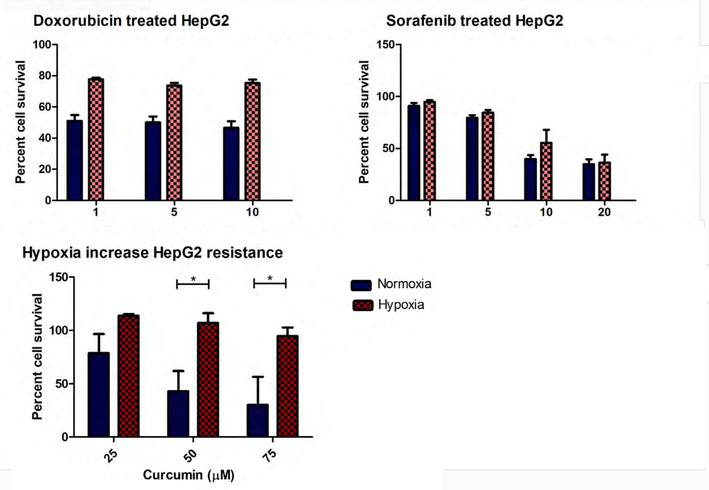


Figure 3. Cytotoxicity testing of HepG2 cells under normoxic and hypoxic condition treated were treated with 1, 5 and 10 µM doxorubicin and 1, 5, 10 and 20 µM sorafenib for 24 and 48 h are shown. Results were obtained from two independent experiments. HepG2 cells in normoxic and hypoxic condition treated with doxorubicin and curcumin both showed decrease cell survival in a dose-dependent manner with hypoxic treated cells expressing higher resistance. Hypoxic condition, however, did not affect treatment with sorafenib.

Figure 4.

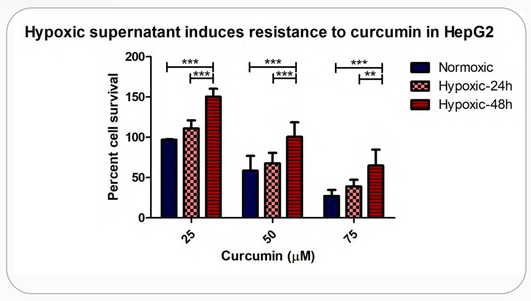


Figure 4. Normoxic cells were treated with conditioned media obtained from hypoxic culture supernatants and curcumin for 24 h. Result is obtained from three independent experiments. **, *** Significant difference between groups at P<0.01 and P<0.001.

Figure 5.

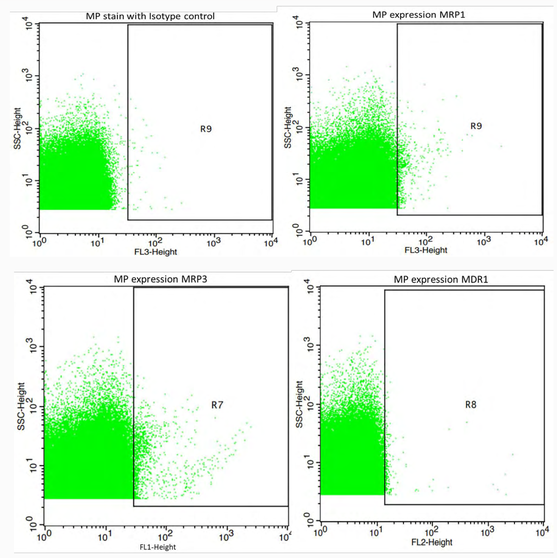


Figure 5. ABC transporter proteins (MDR1, MRP1 and MRP3), expressed on surface of HepG2 MPs in normoxic and hypoxic condition were examined by flow cytometry. Here we demonstrate successful detection of surface expression of MRP3, MDR1 and MRP1 on HepG2 cells using fluorescent tagged antibodies to each respective proteins in panel R7, R8 and R9.

Figure 6.

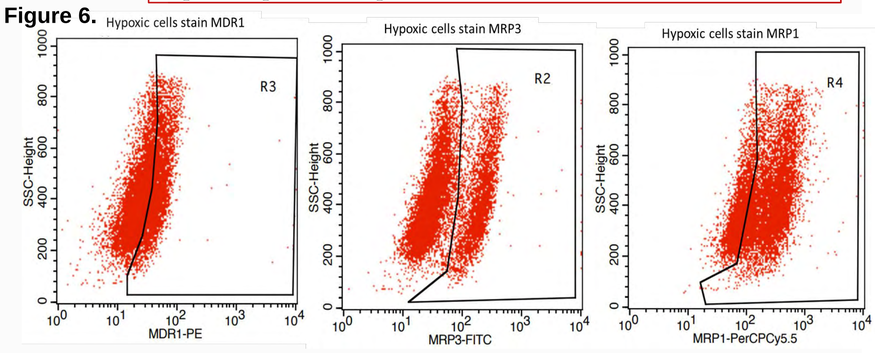


Figure 6. ABC transporter proteins (MDR1, MRP1 and MRP3) on HepG2 cells in hypoxic and normoxic condition were examined by flow cytometry. We successfully detected expression of these three ABC transporter proteins on HepG2 cell surface in hypoxic condition as shown in this figure.

Table 1: Summary of ABC transporter expressions on MP surface of HepG2 cells in normoxic and hypoxic conditions

% MDR Protein expression on MP			
	Condition/ incubation time		Mean ± SD
%MDR1	Normoxic	24 h	0.22 ± 0.02
		48 h	0.2 ± 0.01
		72 h	0.2 ± 0.08
	Hypoxic	24 h	0.62 ± 0.69
		48 h	0.17 ± 0.01
		72 h	0.24 ± 0.07
%MRP1	Normoxic	24 h	0.62 ± 0.45
		48 h	0.28 ± 0.03
		72 h	0.68 ± 0.18
	Hypoxic	24 h	0.46 ± 0.15
		48 h	0.30 ± 0.09
		72 h	0.62 ± 0.10
%MRP3	Normoxic	24 h	0.68 ± 0.08
		48 h	0.54 ± 0.16
		72 h	0.83 ± 0.11
	Hypoxic	24 h	1.38 ± 0.81
		48 h	0.43 ± 0.33
		72 h	0.85 ± 0.18

Analysis of MP with positive expression for each ABC transporter proteins in hypoxic and normoxic conditions were analyzed and were found to show no difference. Data were taken from 2 independent experiments.

Table 2: Summary of ABC transporter expressions on surface of HepG2 cells in normoxic and hypoxic conditions

% MDR Protein expression on HepG2 cell			
	Condition/ incubation time		Mean ± SD
%MDR1	Normoxic	24 h	91.05 ± 5.21
		48 h	92.01 ± 6.72
		72 h	65.98 ± 7.18
	Hypoxic	24 h	91.71 ± 2.18
		48 h	92.79 ± 6.22
		72 h	83.01 ± 9.36
%MRP1	Normoxic	24 h	9.58 ± 0.29
		48 h	99.30 ± 0.48
		72 h	97.90 ± 0.75
	Hypoxic	24 h	92.95 ± 8.41
		48 h	99.68± 0.36
		72 h	99.48 ± 0.08
%MRP3	Normoxic	24 h	60.85 ± 32.22
		48 h	72.54 ± 18.73
		72 h	20.31 ± 5.21
	Hypoxic	24 h	55.44 ± 10.25
		48 h	71.30 ± 14.98
		72 h	35.35 ± 1.73

Analysis of HepG2 cells with positive expression for each ABC transporter proteins in hypoxic and normoxic conditions were analyzed and were found to show no difference. Their percent MDR protein expression or values of mean fluorescent intensity on HepG2 cell surface on a 24, 48 and 72 h time course show not different. Result were obtained from two independent experiments. Data were taken from 2 independent experiments.

DISCUSSION & CONCLUSION

Hypoxia increased resistance in HepG2 cells when treated with curcumin, doxorubicin and sorafenib. Hypoxic supernatant co-incubated with curcumin also increased cell survival by approximately 20%. Here, we investigated time-course of MDR protein expression in both HepG2 cell membrane and MP in normoxic and hypoxia condition at 24-72 hours.

Preliminary analysis showed no difference in expression level of these MDR protein expressions in hypoxia compared with normoxia. As hypoxic treatment alone did not yield significant results at this time, next we plan to determine if resistance observed in hypoxic supernatant study could be attributed to combination of hypoxic conditioning together with treatment using curcumin or chemotherapeutic drug.