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THE ANTIGLYCATION EFFECT OF MONOMETHYL BRANCHED CHAINED FATTY ACID AND PHYTOCHEMICAL COMPOUNDS AND THEIR SYNERGISTIC EFFECT ON OBESITY RELATED COLORECTAL CANCER CELL PANEL

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Abstract

Background and aims: *monomethyl branched chain fatty acids (mmBCFA) and phytochemicals including: Hydroxycitric Acid, Chlorogenic Acid and Piperine have been considered as an interesting agent for researches due to their role in diabetes and cancer. The present study examines the antiglycation effect of mmBCFA and phytochemicals and investigate their Synergistic effect on different colorectal cancer cell lines.* ***Methods:*** *This study was carried out by using murine monocyte-macrophage cell line and Methylglyoxal (MGO) to enhance the glycation process, furthermore to examine the antiproliferative effect of both the mmBCFA and phytochemicals we use Sulforhodamine B (SRB) assay against obesity related-colorectal cancer cell line panel.* ***Results:*** *Both phytochemicals and mmBCFA have a higher antiglycation effect than Aminoguanidine (AMG) significantly, moreover, all of the phytochemicals and mmBCFA have antiproliferative against SW620, CACO2 and SW480, nevertheless none of these agents was equipotent to Cisplatin, furthermore, the synergetic effect observed only when we co-incubate Piperine with mmBCFA.* ***Conclusions****: phytochemicals such as Hydroxycitric Acid, Chlorogenic Acid and Piperine and mmBCFA could be used as treatment to prevents the accumulation of advanced glycation end-products (AGEs) in diabetes. Furthermore, the co-incubation between these compounds can inhibit cancer growth, as alternative therapeutic strategy against obesity related-colorectal cancer.*

key words: *phytochemicals, mmBCFA, antiglycation, antiproliferative, co-incubation*

Background and aims

The most common mark of diabetes and the cause of diabetes complications, is building up the AGEs, the formation of these products can occur by the so-called Maillard reaction, between the reducing sugar and the amino group in nucleic acid, protein as well as in lipid [1] _ENREF_1. The AGEs can accumulate in the tissue causing oxidative stress, as result for modification of the protein structure and function during aging, and could lead eventually to tumor progression [2]. Although the association between AGEs and cancer still

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unclear yet [3]. Recently, a growing body of evidence has indicated that alteration of the extracellular matrix (ECM) induced by the production of AGEs has been considered a significant factor that enhanced crosslinking and promote stiffening of the ECM, which could stimulate growth, invasion and migration of the cancer cells [4]. Therefore, it was an important to discover antiglycation compounds to inhibit the harmful effect of the AGEs, thus many compounds have been bringing to light as antiglycation a gents, however the majority of these compounds is not identified as safe agents [5]. In the other hand, it was reported, that mmBCFA could be involved in obesity-related insulin resistance, and it was indicated that mmBCFA correlates positively with insulin sensitivity [6]. In cancer, however, the mmBCFA have been proven to induce the apoptosis in different human cancer cell lines [7]. On the other side, phytochemicals has a remarkable value due to their activity against different type of cancer and as antioxidant agents [8].

So in this study we have investigated the possible antiglycation effect of mmBCFA and phytochemicals using RAW 264.7 macrophage cell line and the antiproliferative effect of both mmBCFA and phytochemical compounds using different obesity related-colorectal cancer cell lines panel.

Materials and methods

RAW 264.7 cell line culture

RAW 264.7 cell line (murine monocyte-macrophage cell), were maintained in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS, penicillin (100 µg/mL), streptomycin (100 µg/mL), and L- glutamine (100 µg/mL) in a 37 oC humidified atmosphere with 95% air and 5% CO2. The culture medium was changed every 48 -72 h [9,10]. All the material in tissue culture was obtained from Invitrogen (USA). Unless stated otherwise, all other chemicals and solvents used in this article were purchased from Sigma-Aldrich (St. Luis, MO, USA). Unless stated otherwise.

The glycation assay

The RAW 264.7 cell line (murine monocyte-macrophage cell), were seeded at 10^4 cell/ well in 96- well tissue culture plates and incubated at 37°C and 5% CO2 overnight. After 12 h incubation, the cells were treated with the tested compounds as well as with the Methylglyoxal (MGO) at several concentrations. The tested mmBCFA include: 10-methyl- tetradecanoic acid (10MTD), 12-Methyl tetradecanoic acid (12 MTD), 13-methyl-tetradecanoic acid (13 MTD) and 14-methyl-pentadecanoic acid (14 MTD) and the tested phytochemical compounds include: Hydroxycitric Acid, Chlorogenic Acid and Piperine as well as the Aminoguanidine (AMG) as a positive control, which were procured from Santa Cruz (USA), these agents were added 20 minutes prior to Methylglyoxal (MGO). The cells were then incubated for 48 h and assessed for viability using Sulforhodamine B (SRB) dye. Which was purchased from Promega (USA). The absorbance is measured at 570 nm [11]. The absorbance is proportional to cell viability.

Cancer cell lines culture

Human obesity related-colorectal cancer cell lines; namely SW620 (ATCC CCL-227), SW480 (ATCC CCL-228), and CACO2 (ATCC HTB-37) were cultured in DMEM containing 10% FBS, HEPES Buffer (10 mM), L- glutamine (100 µg/mL), gentamicin (50 µg/mL), penicillin (100 µg/mL), and streptomycin (100 mg/mL) [10].

The viability assay

The cytotoxicity measurements were determined using SRB colorimetric assay. Colorectal cell lines (SW480, SW620 and CACO2), were seeded in 96-well plates at a density of 5000 cells/well and cultured for 24 h, then cultured in the medium containing different phytochemical compounds such as: Hydroxycitric Acid, Chlorogenic Acid and Piperine at concentrations (200-1 µM). After 72 h, the SRB assay was performed [10,12] .All of the assays were performed in triplicate and the calculated IC50 antiproliferative activities were reported as the mean values ± SD (n=3).

The antagonism and Synergism analysis

In combination experiments, mmBCFA were added with the phytochemical compounds at ½ of their IC50 value. To assess synergy and antagonism, experimentally and a combination index (CI) was determined according to the method of Mertens–Talcott [13]. The CI values consider as synergism if (CI < 1), additive effect if (CI = 1) and antagonism if (CI > 1).

Statistical analysis

The results were presented as means± standard deviation (SD) of three independent experiments. Statistical differences were measured between control and different treatment groups using GraphPad Prism ANOVA followed by Tukey test. For all statistical analysis, a p-value of less than 0.05 was considered statistically significant. p values of less than 0.0001 were considered of a highly significant statistical difference.

Result

Effect of MGO on cell toxicity

Table 1 shows the effect of different concentrations (100, 200, 300, 400 and 500 µM) of MGO on cell toxicity. The effects of different concentrations of MGO were significantly different from each other ($p<0.01$).

Table 1. The effect of different concentrations of MGO on cell toxicity.

	The effect of different concentrations of MGO on cell toxicity				
(µM)	100	200	300	400	500
	25.27±1.42	39.26±1.71	72.05±2.28	89.02±1.04	98.25 ± 2.19

The results represent percentages of cytotoxity of treated cells, expressed as means of the three measurements ± SD (n = 3-4 independent replicates) measurements.

The antiglycation effect of mmBCFA and phytochemical compounds

Table 2 display the antiglycation effect and the viability against RAW 264.7 macrophage cell line of both mmBCFA and phytochemical compounds. All the mmBCFA as well as the phytochemicals show significant antiglycation effect compared to positive control AMG.

In cell viability test, only 12MTD and Hydroxycitric Acid showed no significant reduction in cell viability after 48 h of incubation.

Modulation of viability of colorectal cancer cell lines by mmBCFA and phytochemical compounds

Table 3 show the antiproliferative effect of phytochemicals, including: Hydroxycitric Acid, Chlorogenic Acid and Piperine, as well as the mmBCFA against obesity related-colorectal cancer cell lines such as: SW620, CACO2 and SW480, it also shows the effect of co-incubation of these agents, as well as the antiproliferative efficacies of Cisplatin against these cell lines.

All the phytochemicals as well as the mmBCFA show cytotoxity against SW620,

CACO2 and SW480 cell lines after 72h of incubations. However, none of the investigated phytochemicals and the mmBCFA could be identified as equally affective as Cisplatin.

In combination experiment, most of the combination result show antagonistic effect against cancer cell lines. Nevertheless, the synergistic effect observed when Piperin co-incubated with mmBCFA, especially 13 MTD and 14 MTD in SW620 and SW480 cell lines. In fibroblasts most of these treatments showed antagonistic effect when co-incubated with each other, except when Piperin co-incubated with 14 MTD.

Table 2. Antiglycation effect of mmBCFA and phytochemical compounds, using RAW 264.7 macrophage cell line

	antiglycation (as of %Control) IC50 value					
(μM)	10 MTD	12 MTD	13 MTD	14 MTD	AMG (positive control)	
	135.56±2.32	106.18±7.12	10.67±1.57	12.34±2.97	225± 1.59	
p-value	<0.0001	<0.0001	<0.0001	<0.0001		
	RAW 264.7 cell line viability (as % control)					
Treatment (μM)	20	40	60	100	200	400
12 MTD	112.15±3.47	102.90±9.70	97.78±2.97	92.49±0.97	76.52±4.08	74.73 ±3.07
13 MTD	99.53±11.51	98.90±8.25	97.32±3.07	81.15±3.56	66.60±1.79	47.17±3.67
14 MTD	137.83±3.01	123.11±2.56	111.33±6.02	109.61±3.41	71.66±2.92	53.44±4.44
10 MTD	103.09±9.93	95.13±6.98	72.19±3.94	68.10±2.35	61.96±3.44	57.43±0.34
antiglycation (as of %Control) IC50 value						
(μM)	Hydroxycitric Acid	Chlorogenic Acid		Piperine	AMG (positive control)	
	140.7±0.84	41.35±0.31		65.31±2.97	225± 1.59	
p-value	<0.0001	<0.0001		<0.0001		
RAW 264.7 cell line viability (as % control)						
(μM)	5	10	25	50	100	200
Hydroxycitric Acid	99.38±1.24	98.95±1.04	98.19±1.22	89.35±2.43	87.92±2.12	75.53±1.53
Chlorogenic Acid	91.39±1.09	88.29±2.03	79.21±3.16	72.82±1.12	55.71±2.84	36.53±1.24
Piperin	95.91±1.81	94.73±3.92	88.62±2.82	82.81±1.31	71.82±2.19	41.41±2.14

Results are mean ± SD of three independent replicates. IC50 values (concentration at which 50% of glycated cell took place in comparison to non-glycated cells basal 48 h incubations). NS non-significant compared to AMG.

Table 3. Modulatory effect of phytochemical compounds and mmBCFA, on the viability of obesity related-colorectal cancer cell lines measured by SRB dye

	Cytotoxicity (as of %Control) IC25 value μM			
	SW620	CACO2	SW480	Fibroblasts
10 MTD	157.8±2.26	145.33±1.42	83.3±1.23	NI
12 MTD	63.4±1.14	70.72±19.35	84.25±1.94	153.3±8.25
13 MTD	67.2±2.54	124.44±15.24	45.4±2.43	135.4±3.54
14 MTD	114.34±13	124.35±2.62	132.6±8.22	112.5±6.63
Cisplatin	4.71±0.14	1.46±0.28	4.56±0.11	4.13±0.62
	The p-value compared to Cisplatin treatment			
	SW620	CACO2	SW480	Fibroblasts
10 MTD	NS	NS	NS	NS
12 MTD	NS	NS	NS	NS
13 MTD	NS	NS	NS	NS
14 MTD	NS	NS	NS	NS

Table 3. *Continued.*

	Cytotoxicity (as of %Control) IC25 value µM			
	SW620	CACO2	SW480	Fibroblasts
Hydroxycitric Acid	NI	510.98±7.79	469.41±10.64	516.08±5.78
Chlorogenic Acid	240.86±2.78	226.14±7.32	220.49±1.25	196.14±2.01
Piperine	76.62±11.75	268.82±4.63	120.39±2.77	103.38±0.49
Cisplatin	4.71±0.14	1.46±0.28	4.56±0.11	4.13±0.62
	The p-value compared to Cisplatin treatment			
	SW620	CACO2	SW480	Fibroblasts
Hydroxycitric Acid	NS	NS	NS	NS
Chlorogenic Acid	NS	NS	NS	NS
Piperine	NS	NS	NS	NS
	CI value			
	SW620			
	10 MTD	12 MTD	13 MTD	14 MTD
Hydroxycitric Acid	NI	NI	NI	NI
Chlorogenic Acid	1.54±0.15	1.31±0.41	1.82±0.27	1.42±0.53
Piperine	1.09±0.23	1.02±0.23	0.91±0.11	0.52±0.14
	CACO2			
	10 MTD	12 MTD	13 MTD	14 MTD
Hydroxycitric Acid	1.53±0.27	1.24±0.43	1.24±0.36	1.23±0.23
Chlorogenic Acid	1.64±0.85	1.45±0.23	1.39±0.41	1.21±0.34
Piperine	1.01±0.21	1.05±0.38	1.04±0.21	0.41±0.18
	SW480			
	10 MTD	12 MTD	13 MTD	14 MTD
Hydroxycitric Acid	1.45±0.35	2.45±0.53	1.33±0.16	2.03±0.56
Chlorogenic Acid	1.56±0.20	1.56±0.67	1.56±0.63	1.49±0.75
Piperine	1.04±0.42	0.45±0.04	0.34±0.05	0.59±0.08
	Fibroblasts			
	10 MTD	12 MTD	13 MTD	14 MTD
Hydroxycitric Acid	1.98±0.52	1.58±0.28	1.68±0.36	1.36±0.83
Chlorogenic Acid	1.49±0.16	1.35±0.42	1.53±0.52	1.96±0.48
Piperine	1.16±0.18	1.02±0.06	1.09±0.11	0.22±0.01

Results are mean ± SD of three independent replicates. IC25 values (concentration at which 25% inhibition of cell proliferation took place in comparison to non-induced basal 72h incubations). NI is non inhibitory. NS non-significant compared to cisplatin.

Discussion

Diabetes, the disease that is closely related to glycation, can lead to a diverse number of serious complications including nerve damage and vision loss. it was reported that hyperglycemia responsible for 2.2 million deaths [14]. Therefore, a large number of studies were aims to discover antidiabetic agents that increase insulin secretion and enhancing the beta cell proliferation [15-18]. Furthermore, one of the alternative therapeutic approaches proposed so far for the diabetes is based on the decrease in the AGEs production. due to the role of AGEs in disturbed function on the cellular, tissue and organ level over time [19]. Moreover, different studies indicated the role of AGEs in activating receptor on the surface of tumor cells which stimulate growth and invasiveness of the cancer cells [20].

Most of the phytochemicals have antioxidant ability, so it was proposed that they have antiglycation effect [21].

In the present study all the tested phytochemicals including: Hydroxycitric Acid, Chlorogenic Acid and Piperine show significant increase in antiglycation effect more than the AGE inhibitor AMG, consistent with our results Wu and Yen demonstrated the antiglycation

effect of different phtyochemicals, including: catechin, epicatechin, luteolin, and kaempferol [22].

Hydroxycitric acid is the active ingredient of *Garcinia cambogia, Garcinia indica*, and *Garcinia atroviridis*, it was documented that this component used for weight loss [23]. Consistent with our results it was indicated that *Garcinia indica*, inhibit the glycation process as well as the cross-link formation significantly compared to AMG [24].

Chlorogenic Acid, which is the active compound in many dicotyledonous plants, and Piperine, which is the active component of pepper belonging to the *Piperaceae* family. According to our results both of these compounds show more antiglycation effect than Hydroxycitric Acid, which it may occur due to the presence of the active site of the aromatic ring, which is the major site for trapping MGO, gives the ability for these compounds to inhibits the generation of AGEs [25-27]. Furthermore, in this study we also investigated the antiglycation effects of mmBCFA, interestingly our findings indicated that mmBCFA have higher antiglycation effect than MGO significantly, according to the structure of the mmBCFA, we can suggest that the position of the methyl group play an important role in the activity of these acids.

Preventing the formation of AGEs considered as indirect approached to prevent growth of the cancer cells, however in this study our results show direct antiproliferative effect of phytochemicals including: Hydroxycitric Acid, Chlorogenic Acid and Piperine against certain type of obesity related-colorectal cancer cell lines such as: SW480, SW620, and CACO2, nevertheless none of these tested compounds was equipotent to Cisplatin on the same cell lines. In agreements with our result studies was reported the antiproliferative activity of the several phytochemicals against different cancer cell lines [28-30]. Parallel with our antiproliferative results regarding Hydroxycitric Acid, it was observed the antiproliferative activity of citric acid in skin cancer by inducing apoptotic [31], and the antiproliferative activity of hydroxycitrate against lung cancer, bladder cancer, and melanoma [32]. Furthermore, it was reported that Chlorogenic Acid can inhibit the growth of colon cancer and leukemia by inducing cell cycle arrest at S-phase and activating caspase-3 [33].

However, Piperine has been extensively investigated against different cancer cell lines, indicated the antiproliferative activity of this compound, which was consistent with our results [34]. Several studies have reported that Piperine can inhibit the tumor growth by activated caspase 3 and stimulated cell cycle arrest [35]. Piperine suppressed the growth prostate cancer via decrease the expression of nuclear factor-κB (NF-κB) [36]. Furthermore, Piperine can affect the metastatic action of cancer cells by modify the expression of matrix metalloproteinase (MMPs) [37]. Moreover, Piperine can inhibit the extracellular signal-regulated kinase (ERK), NF-κB, p38 mitogen-activated protein kinase (MAPK), and Akt pathway in cancer cells [38,39].

Moreover, this study reported the viability of mmBCFA on different colorectal cancer cell lines using SRB assay. Our finding indicates that mmBCFA including: 10MTD, 12 MTD, 13 MTD and 14 MTD exerted antiproliferative efficacies against SW620, CACO2 and SW480 over 72h incubations. Consistent with our result it was reported that 13 MTD can exert inhibitory effect on fatty acid synthetase in breast cancer, which reduce the activity of glucose-6-phosphate dehydrogenase leading to inhibits the formation of NADPH in breast cancer [40]. Furthermore, it was observed that 13 MTD have cytotoxity

effect on different cancer cell lines including: K-562, MCF7, DU 145, NCI-SNU-1, SNU-423, NCI-H1688, BxPC3, and HCT 116 [7]. Although, the mechanism of antitumor activity has not been clear yet, it was suggested that mmBCFA especially 13 MTD have antiproliferative effect on human cancer by inhibiting the activity of AKT and NF-κB pathways [41] as well as the activity of MAPK pathway [42].

In our study we revealed the co-incubation effect of phytochemicals with mmBCFA on SW620, CACO2 and SW480 cell lines cell, and our result show the synergistic effect occur when mmBCFA especially: 13 MTD and 14 MTD co-incubated with only Piperine, however Hydroxycitric Acid and Chlorogenic Acid show antagonistic effect in SW620, CACO2 and SW480 cell lines when co-incubated with mmBCFA. Suggesting that significant inhibition occur to AKT, NF-κB and MAPK pathways when 13 MTD and 14 MTD co-incubate to Piperine. And a significant activation to AKT and MAPK pathways when 10 MTD and 12 MTD co-incubated to either Hydroxycitric Acid or Chlorogenic Acid. we suggested that the location of the methyl group in mmBCFA could be responsible for such behavior. However Further studies are necessary in order to determine the modulatory effect of several pathways when different type of mmBCFA co-incubated with phytochemical compounds on obesity related-colorectal cancer cell lines.

Conclusions

This study has demonstrated the effect of mmBCFA as well as phytochemical compounds, including Hydroxycitric Acid, Chlorogenic Acid and Piperine as antiglycation agent in vitro, Furthermore, in this study we tested the cytotoxicity of these compound separately and combined on obesity related-colorectal cancer cell lines, and it was clear that these compounds has higher antiglycation effect than AMG. Moreover, it was also clear that Piperine and mmBCFA especially: 13 MTD and 14 MTD can reach higher cytotoxity effect against obesity related-colorectal cancer cell lines when we co-incubated these agents with each other. However, Further studies are necessary in order to determine their mechanism of action. Nevertheless, we suggested that phytochemicals as well as mmBCFA could be used as treatment for diabetes, and the co-incubation between the phytochemical compounds and the mmBCFA considered as different therapeutic strategy against diabetes and obesity related-colorectal cancer.

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