Original Research Article

Cytotoxic Synergism between a Proprietary *Commiphora mukul* Gum Extract GU-MCT810, 2-deoxy-D-glucose, and Metformin in Human Alveolar Rhabdomyosarcoma and Hepatoma Cell Lines *In vitro*

ABSTRACT

In this investigation we have analyzed the synergism for cytotoxic effect of a proprietary guggul gum extract (GU), 2-deoxy-D-glucose (2-DG) and metformin (Met) in SJRH30 human alveolar rhabdomyosarcoma and HepG2 hepatoma cell lines *in vitro*. 2-DG and Met as single agents have weak cytotoxic effect s in both cell lines. However, the combination of GU+2DG, GU+Met and 2DG+Met showed synergism for cytotoxic effect by CompuSyn analysis. Therefore, GU can be included in the combination of drugs involving 2DG and Met to have synergistic effect. GU also showed a dose-dependent increase in cellular glucose uptake in HepG2 cells like the antidiabetic drug 2,4-thiozolidine dione (TZ). The demonstration of synergism of anticancer effects between GU, metformin and 2-DG, suggest that their mechanisms are in general complementary, though further studies are required to delineate the mechanism of GU, 2-DG and metformin combinations.

Key Words: *Anticancer effect, guggul, 2-deoxy-D-glucose, metformin, rhabdomyosarcoma, hepatoma, cytotoxicity, synergism, glucose uptake*

1. INTRODUCTION

GU-MCT810 (GU) is a proprietary nutraceutical ingredient complex that includes a *Commiphora mukul* (guggul) extract prepared by a supercritical CO_2-co-solvent extraction with ethanol and dissolved in medium chain triglyceride (MCT) oil composed of C8 and C10 fatty acids. GU was shown to promote hypolipidemic effects *in vitro* as demonstrated by reduction of low-density lipoprotein cholesterol and increased high-density lipoprotein through its direct inhibitory effect on HMG-CoA reductase activity. It was also shown to up regulate expression of LXR, PPAR , BABP and SHP genes associated with the lipid metabolism. Additionally, GU inhibits adipocyte differentiation, increases AMPK phosphorylation and AMPK kinase activity and inhibits phosphorylation of mTOR expression [1].

Previously we reported the anticancer effects of GU in combination with hexokinase inhibitor (2-deoxy D-glucose, 2-DG) through the inhibition of HIF-1α expression in human HepG2 cell line [2]. Additional anticancer effects were described in a subsequent publication that showed synergism between a proprietary supercritical CO2 extract of mango ginger (*Curcuma amada* Roxb.) and the hexokinase inhibitor 2-DG and the lactate dehydrogenase-A inhibitor, sodium oxamate, in the U-87MG glioblastoma cell line [3].

Metformin (N,N-dimethylbiguanide) has been an important first-line drug for treatment of type 2 diabetes (T2D) for decades and is regarded as a generally safe drug [4,5]. It is the most widely used oral antihyperglycemic agent and is currently recommended as first line therapy for all newly diagnosed T2D patients [6]. It belongs to the biguanide class of antidiabetic drugs (containing two linked guanidine rings) originally derived from galegine (isoamylene guanidine), a guanidine derivative found in the French lilac *Galega officinalis*. Besides its glucose-lowering effect, there is interest in its potential relevance to cardiovascular diseases and cancer, although the underlying mechanisms of action remain elusive. Energy metabolism, the target of metformin's mechanism of action in diabetes may also be of importance in cardiovascular diseases and cancer. Accumulating evidence indicates that metformin inhibits growth, survival, and metastasis of different types of tumor cells, including those from breast, liver, bone, pancreas, endometrial, colorectal, kidney, and lung cancers [7].

The anticancer properties of metformin appear to be mediated by AMPK-dependent and -independent pathways. Metformin has been shown to activate AMPK, with compensatory inhibition of mTOR signaling, resulting in suppression of protein synthesis, cell growth and proliferation in neoplastic cells [8]. Suppression of cancer development through AMPK independent activation of autophagy and apoptosis has also been proposed [9] Other potential mechanisms include suppression of crosstalk between G protein-coupled receptors (GPCRs) and insulin receptor signaling systems that may contribute to the inhibition of pancreatic cancer proliferation [10,11]. Metformin has also been shown to indirectly inhibit cancer proliferation through regulation of angiogenesis, fibroblast and tumor-associated macrophages, and other changes in the tumor microenvironment [12] and through decreased plasma glucose levels that has an inhibitory effect on cancer cell proliferation and survival [13].

2-DG, a synthetic glucose analogue, is a glycolytic inhibitor that is phosphorylated by hexokinase upon transport into the cells and is not fully metabolized [14,15]. 2-DG blocks the first step in glycolysis by inhibiting hexokinase, the first rate-limiting enzyme involved in the conversion of glucose to glucose-6 phosphate. It has been shown to inhibit cell growth in several cancer types and enhances the therapeutic efficiency of chemotherapeutic drugs in human xenograft studies [16-19]. In this paper we describe the anticancer effect of GU-MCT810, 2-DG and metformin as well as their combinations in alveolar rhabdomyosarcoma and hepatoma cell lines.

2. MATERIALS AND METHODS

2.1 Cell lines and Culture: Human alveolar rhabdomyosarcoma (SJRH30) and hepatoma (HepG2) cell lines were purchased from American Type Culture Collection, Manassas, VA, USA. SJRH30 and HepG2 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco's Modified Eagle Medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS) and antibiotics in a 5% CO_2 incubator.

2.2 Drugs: Metformin (Met) , 2-deoxy-D-glucose (2-DG) , and 2,4-thiazolidine dione (TZ) and were purchased from Signa Aldrich Chemical Co., St. Louis, MO. GU-MCT810 was prepared by Flavex Naturextrakte, GmbH, Rehlingen, Germany [1,2] which is formulated to contain 2% guggulsterones in it by dissolving in medium chain triglyceride oil.

2.3 Cytotoxicity: SJRH30 and HepG2 cells were treated with increasing concentrations of drugs and/or extracts for 72 h in 96 well plates. MTT assay was used to analyze cytotoxicity of individual drugs/extracts and their combinations [2].

2.4 Glucose uptake: HepG2 cells (4 x 10^6 cells/4ml) were suspended in DMEM medium on multiwell plates and allowed to grow in the CO_2 incubator. Once the cells were attached, the medium was replaced overnight with starving DMEM medium containing 0.5% FBS and antibiotics. On the next day, the medium was replaced with fresh starving medium and treated with increasing concentrations of GU or TZ and incubated in the incubator for 72 h at 37°C. Total cellular protein was extracted with Invitrogen protein extraction buffer containing protease inhibitors. Cellular extracts equivalent to 100 ug protein was analyzed for the glucose content using the Glucose quantitation kit (MBL Laboratories, MA). Cellular glucose concentration was plotted against drug concentrations.

2.5 Data analysis: Mean ICs and standard deviation estimates were calculated using Microsoft Excel software. The fraction of surviving cells at each concentration of drugs/combinations was used for the analysis of synergism/additivity/antagonism between drugs/extracts by the CompuSyn software (ComboSyn Inc, Paramus, NJ). Synergism was evaluated by the combination index (CI) method of Chou and Talalay [20], which is based on the median-effect principle. The CIs at different IC concentrations were calculated by the Chou-Talalay equations for multiple drug effects, which take into consideration both potency (IC values) and shape (slope, m) of dose-effect curve [21].

3. RESULTS

3.1 Cytotoxicity: Cytotoxicity curves of GU, 2-DG, metformin and different drug combinations in SJRH30 alveolar rhabdomyosarcoma and HepG2 hepatoma cell lines are presented in Fig. 1 and 2, respectively. Also, the inhibitory concentrations of GU, 2-DG, metformin and different drug combinations in SJRH30 alveolar rhabdomyosarcoma and HepG2 hepatoma cell lines are presented in Table 1 and 2, respectively. 2-DG and metformin alone do not demonstrate significant cytotoxicity to SJRH30 and HepG2 cells. However, the combination is synergistic with

respect to cytotoxicity. Similarly, when GU is combined with 2-DG or metformin, cytotoxicity was increased in both SJRH30 and HepG2 cells.

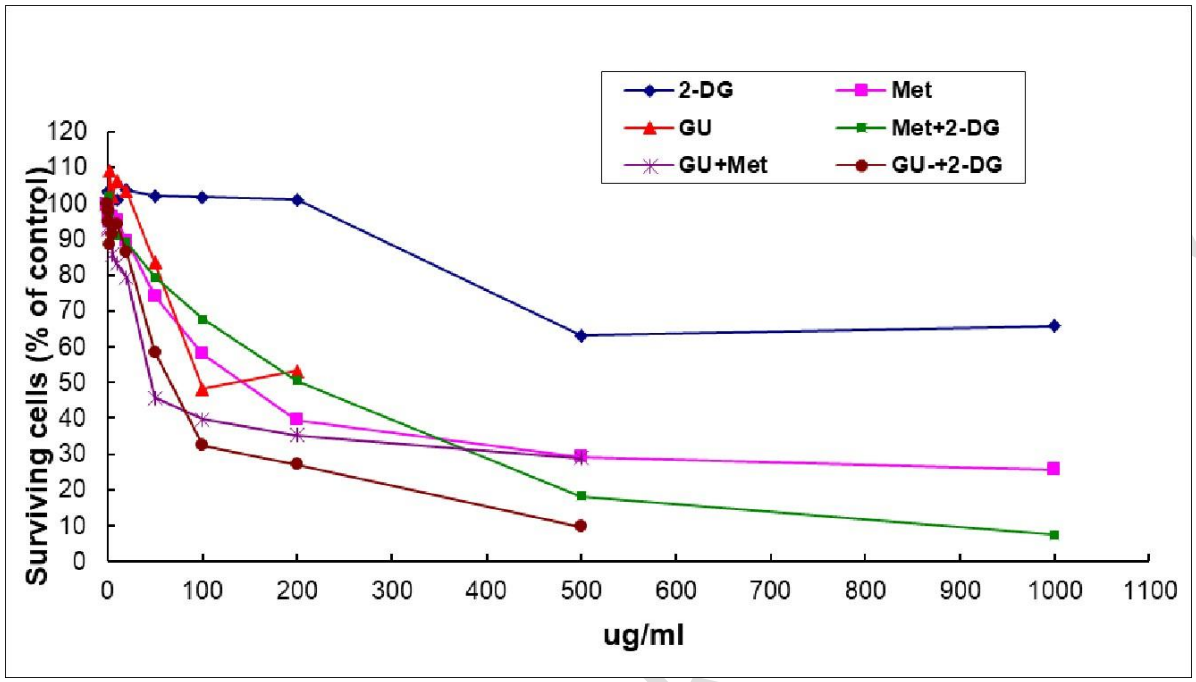


Fig. 1. Cytotoxicity of 2-D-glucose (2-DG), Metformin (Met), GU-MCT810 (GU) and their combination in SJRH30 rhabdomyosarcoma cell line

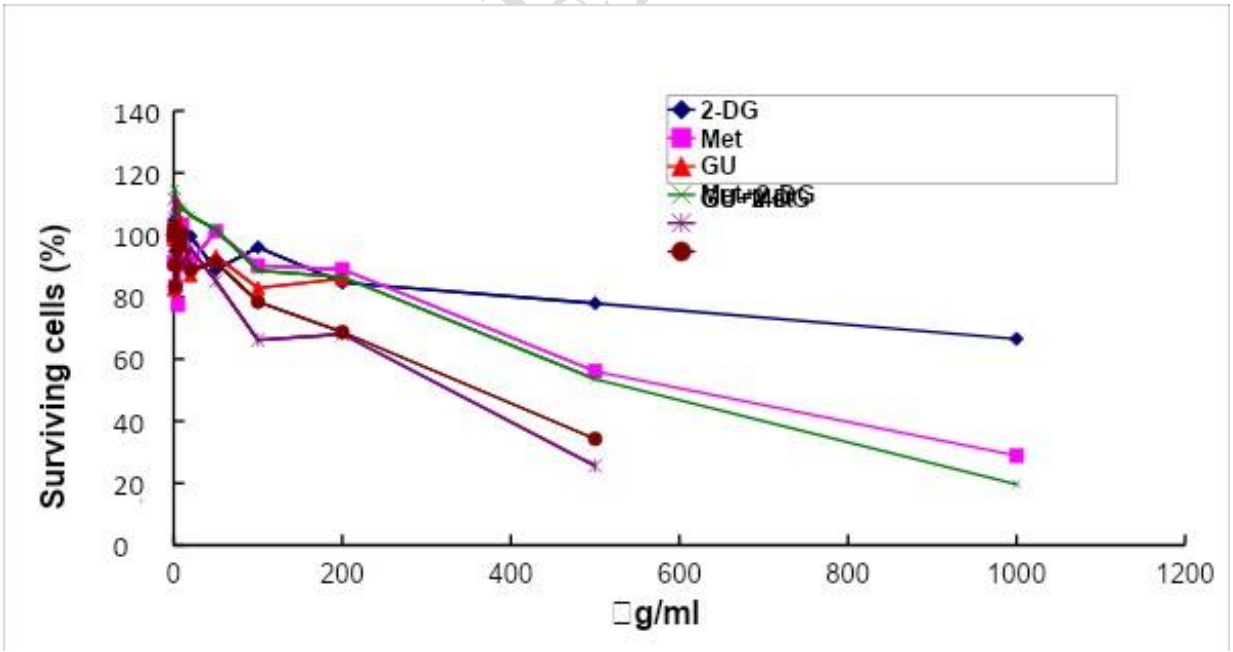


Fig. 2. Cytotoxicity of 2-D-glucose (2-DG), Metformin (Met), GU-MCT810 (GU) and their combinations in HepG2 hepatoma cell line

Table 1. Cytotoxicity of GU-MCT810 (GU), 2-D-glucose (2-DG), metformin (Met) and their combinations in SJRH30 cell line

Drug/combination	IC_{50} (µg/ml)	IC_{75} (µg/ml)	IC_{90} (µg/ml)
GU	50	93	157
2D-Glucose	895	>1000	>1000
Metformin	158	>1000	>1000
GU+2DG	65	>1000	>1000
GU+Met	46	>500	>500
Met+2DG	200	470	900

Table 2. Cytotoxicity of GU-MCT810 (GU), 2-D-glucose (2-DG), metformin (Met) and their combinations in HepG2 hepatoma cell line

Drug	IC_{50} (µg/ml)	IC_{75} (µg/ml)
2-DG	>1000	>1000
Met	605	>1000
GU	>200	>200
Met+2-DG	550	940
GU+2-DG	360	>500
GU+Met	325	498

3.2 CompuSyn Analysis: The median-effect plots of single drugs and drug combinations in SJRH30 cell line is given in Fig. 3A and 3B. Also, the polygonogram indicating the synergism/additivity/antagonism is presented in Fig. 4. The combination index values given in Table 3 indicate that GU+2DG, GU+Met and Met+2DG combinations are synergistic for death of SJRH30 cells. Furthermore, GU+2DG is more synergistic than GU+Met and Met+2DG combinations. Essentially all three drugs can be combined against the alveolar rhabdomyosarcoma.

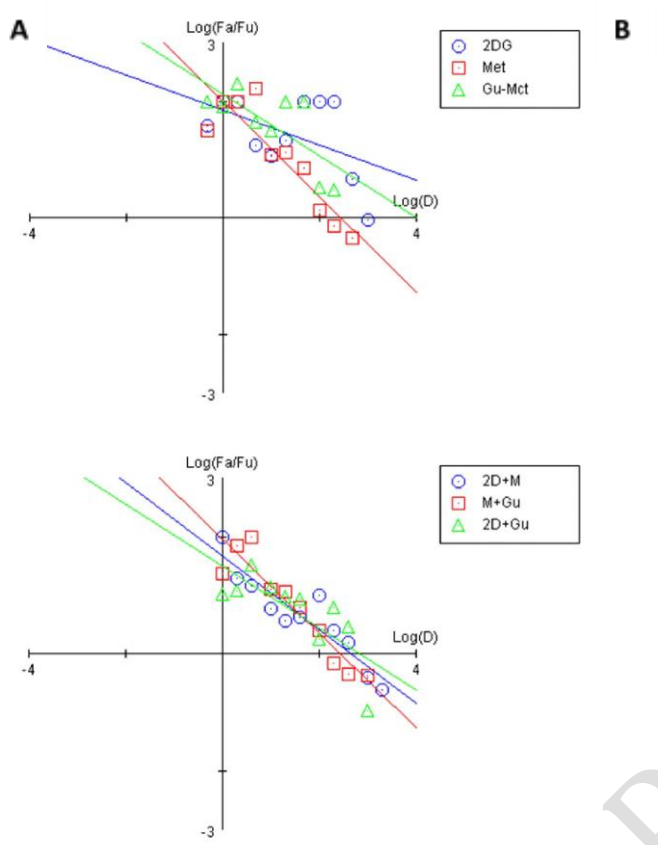


Fig. 3. Median-effect plots of single drug (A) and drug combinations (B) in SJRH30 rhabdomyosarcoma cell line

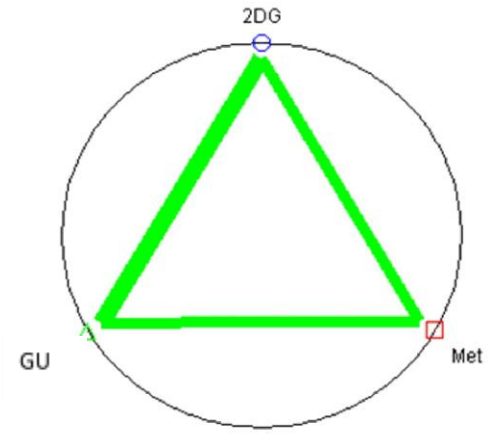


Fig.4. Polygonogram (obtained with CompuSyn analysis) indicating the synergy among GU-MCT810 (GU), 2-deoxy-glucose (2-DG) and metformin (Met) in SJRH30 rhabdomyosarcoma cell line. Thick green line (GU+2-DG) indicates the higher synergism level than thin green line (GU+Met and Met+2-DG).

Table 3. Combination Index (CI) values for drug combinations calculated with CompuSyn software in SJRH30 rhabdomyosarcoma cell line

Drug combination	CI at IC_{50} level	CI at IC_{75} level	CI at IC_{90} level
GU+2DG	0.12	0.14	0.17
GU+Met	0.49	0.49	0.51
Met+2DG	0.79	0.53	0.36

CI <0.1 very strong synergism, 0.1-0.3 strong synergism, 0.3-0.7 synergism, 0.8-0.9 moderate to slight synergism, 0.9-1.1 nearly additive, 1.1-1.45 moderate to slight antagonism, 1.45-3.3 antagonism

Median-effect plots of single drugs and drug combinations in HepG2 cell line is given in Fig. 5A and 5B. Polygonogram indicting the synergism/addictiveness/antagonism is given in Fig. 6. The combination index values given in Table 4 indicate that GU+2DG and GU+Met combinations are synergistic for cytotoxicity. The combination index values between Met+2DG are additive at best at IC_{50} level. Hence, GU must be included in the combination of drugs involving 2DG and Met in HepG2 cells to have synergistic effect.

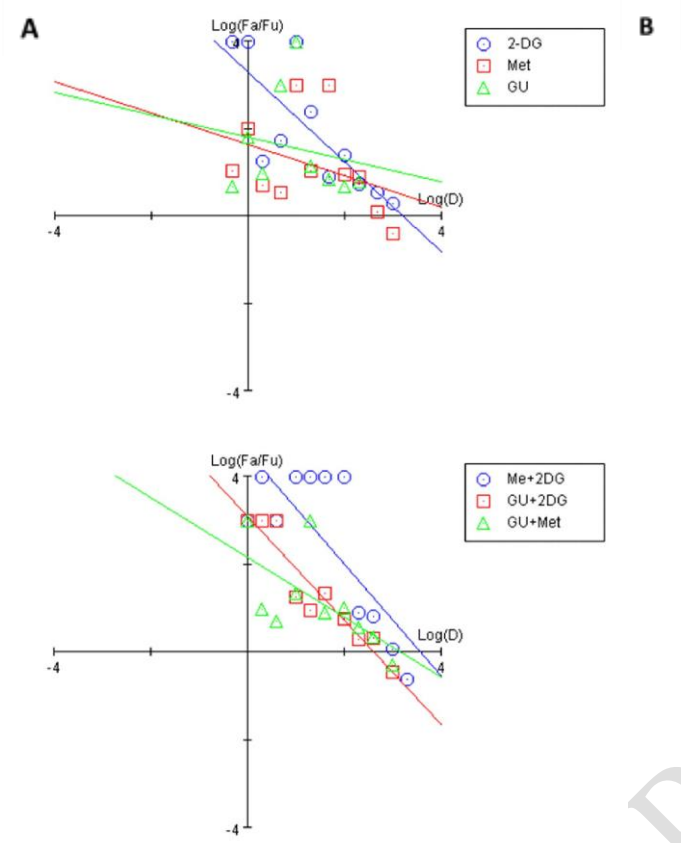


Fig. 5. Medium-effect plot of single drug (A) and drug combinations (B) in HepG2 hepatoma cell line

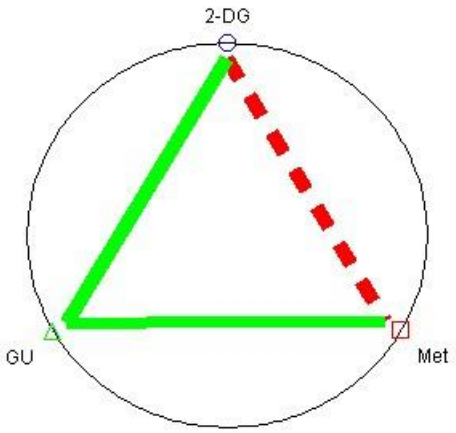


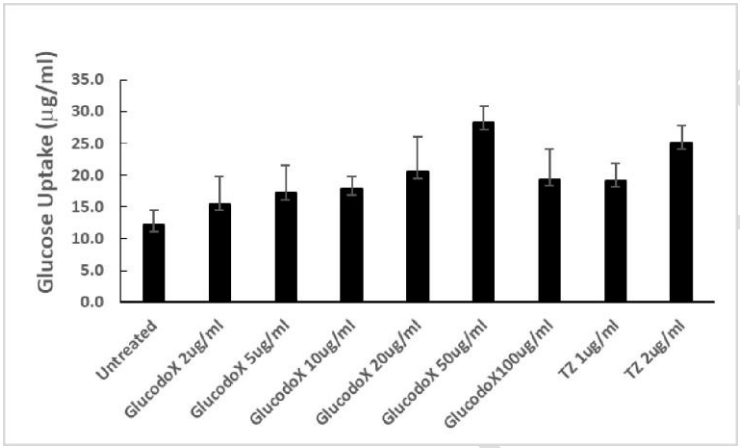
Fig.6. Polygonogram (obtained with CompuSyn analysis) indicating the synergy among GU-MCT810 (GU), 2-deoxy-glucose (2-DG) and metformin (Met) in HepG2 cell line. The green line indicates the synergism and dotted red line indicates partial additivity. Thick green line (GU+2-DG) indicates higher synergism level than thin green line (GU+Met).

Table 4. Combination Index (CI) values for drug combinations calculated with CompuSyn software in HepG2 cell line

Drug	CI at IC_{50} level	CI at IC_{75} level
Met+2-DG	1.11	1.84
GU+2-DG	0.21	0.22
GU+Met	0.35	0.98

CI <0.1 very strong synergism, 0.1-0.3 strong synergism, 0.3-0.7 synergism, 0.8-0.9 moderate to slight synergism, 0.9-1.1 nearly additive, 1.1-1.45 moderate to slight antagonism, 1.45-3.3 antagonism

3.3 Glucose uptake: Fig. 7 shows the effect of GU and the antidiabetic drug (2,4-thiozolidine dione -TZ) on glucose uptake in HepG2 cells. GU and TZ induced a dose-dependent increase in cellular glucose uptake in HepG2 cells. GU at 50 ug/ml almost doubled the cellular glucose concentration of HepG2 cells.



Fig, 7. Effect of GU-MCT810 and 2, 4-thiazide (TZ) on glucose uptake in HepG2 cells.

4. DISCUSSION

GU, a proprietary nutraceutical containing 2% guggulsterones, previously shown to demonstrate hypolipidemic effects through direct inhibition of HMG-CoA reductase activity in a dose-dependent manner [1] has also demonstrated anticancer activity through increased AMPK alpha phosphorylation and AMPK kinase activity and inhibition of mTOR phosphorylation. Given the anticancer activity of metformin [22-24] and 2-DG, via different mechanisms [3,25,26], the object of this study was to determine whether the anticancer effects are synergistic, merely additive or possibly antagonistic. In an earlier publication, we have reported that GU+2-DG combination is useful due to its synergistic antiglycolytic and cytotoxic

effects ([2]. Furthermore, we reported that GU+2-DG combination is useful because of the inhibition of HIF-1alpha pathway genes [2]. In the present investigation, GU, 2-DG and metformin have comparatively higher IC values individually in rhabdomyosarcoma and hepatoma cell lines indicating their weak direct cytotoxic effects. However, when the compounds were combined there was significant reduction in IC values. CompuSyn analysis also showed that these agents work synergistically with respect to cytotoxic effects, indicating a rationale for the combination, compared to single agents. Previously several investigators have shown that 2-DG and metformin can be combined with other front-line cancer drugs for improving chemotherapeutic efficiency (3,18,19,27,28]. The current study suggests that the inclusion of GU could also enhance anticancer efficacy.

Proposing a synergistic combination of these compounds requires several considerations including whether such combinations are safe and practical. 2-DG is a glucose analog that has been shown to act as a competitive inhibitor of glucose metabolism [15]. 2-DG has also been shown to enhance the antitumor activity of Adriamycin and paclitaxel in human xenograft studies [18,25,26]. 2-DG is generally administered intravenously, therefore, in a clinical setting, 2-DG and Metformin or GU could be administered separately as oral preparations. Metformin on the other hand, is administered orally and has been in use for over a half century and is the most widely prescribed anti-diabetic medication in the world [29]. Therefore, it has an excellent safety profile thus making it appealing for repurposing as an anticancer therapy. Several epidemiologic studies have reported the antitumor effect of metformin in different tumors, such as ovarian [30,31] breast [32] prostate [33] and colorectal [34] cancers. It has been shown to have anticancer effects both in vitro and in vivo [35,36] with the underlying mechanism subject to ongoing investigations. Anticancer properties of metformin result from both direct effects on cancer cells particularly through inhibition of the AMPK/mTOR pathway [22] and indirect effects on the host by virtue of its glucose-lowering properties and anti-inflammatory effects [23.24]. Both mechanisms may be important, although their relative contribution may differ according to cancer stage. We have also shown in the present investigation that the cellular glucose uptake is enhanced with GU treatment in HepG2 cells, which will in turn reduce the glucose level in the medium/serum contributing to the anti-diabetic effect. Hence with GU

and metformin treatment it is quite possible to reduce the blood glucose level contributing to the indirect effect of drug combination on the inhibition of cancer cell growth and proliferation.

The concept of repurposing metformin for cancer treatment may be quite appealing [37-39] because it is inexpensive and well-tolerated relative to commonly used antineoplastic agents. However, the antineoplastic activity of metformin requires drug exposure levels considerably higher than those in the serum of metformin treated diabetic patients. Similarly, 2-DG is also expensive and requires significantly higher doses to have the cytotoxic effects on cancer cells [2,3]. Therefore, novel strategies combining these agents with other drugs/extracts exhibiting synergism between them would be preferred over the single agent use for improved efficacy and reduced toxicity. The demonstration of synergism of anticancer effects between GU, metformin and 2-DG, suggest that their mechanisms are in general complementary, though further studies are required to delineate the mechanism of GU, 2-DG and metformin combinations. Nevertheless, this study has demonstrated the potential that combinations of these compounds are practical and appropriate for investigation in clinical trials.

CONSENT

Not applicable

ETHICAL APPROVAL

Since this investigation does not involve lab animals or human subjects, ethical approval is not applicable.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1. Ramachandran C, Nair SM, Quirin K-W and Melnick SJ. Hypolipidemic effects of a proprietary *Commiphora mukul* gum resin extract and medium-chain triglyceride preparation (GU-MCT810). J Evid based Complementary Altern Med. 2013, 18: 248-256.
2. Ramachandran C, Portalatin GM, Quirin K-W, Escalon E and Melnick SJ. Effect of a proprietary *Commiphora mukul* gum resin extract and medium-chain triglyceride preparation (GU-MCT810) on hypoxia-inducible factor-1 pathway in HepG2 cell line. Int J Phytomed. 2015, 7: 324-336.
3. Ramachandran C, Juan A, Quirin K-W, Khatib Z, Schultz Y, Lampidis TJ, Escalon E and Melnick SJ. 2018. Synergistic effect of supercritical CO_2 extract of mango ginger (*Curcuma amada* Roxb.) with glycolytic inhibitors in human glioblastoma cells *in vitro*. Adv Cancer Res Ther. 2018, 2: 1-11.
4. Crowley MJ, Diamantidis CJ, McDuffie JR, et al. Clinical outcomes of metformin use in populations with chronic kidney disease, congestive heart failure, or chronic liver disease. Ann Intern Med. 2017, 166(3):191–200.
5. FDA. Label information: glucophage tablets and glucophage XR extended-release tablets. http://www.accessdata.fda.gov/drugsatfda_docs/label/2017/020357s037s039,021202s021s023lbl.pdf. Accessed April 16, 2019.
6. American Diabetes Association. Standards of medical care in diabetes—2014. Diabetes Care 2014, 37 (Suppl 1): S14–S80.
7. Podhorecka M, Ibanez B, Dmoszynska A. Metformin - its potential anticancer and anti-aging effects. Postepy Hig Med Dosw. (2017) 71:170–5. doi: 10.5604/01.3001.0010.3801
8. Gwinn DM, Shackelford DB, Egan DF, Mihaylova MM, Mery A, Vasquez DS, et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell.,2008, 30:214–26. doi: 10.1016/j.molcel.2008.03.003
9. Ziquan LV, Guo Y. Metformin and its benefits for various diseases. Front. Endocrinol., 16 April 2020 | https://doi.org/10.3389/fendo.2020.00191

10. Thompson MD, Cole DE, Jose PA, Chidiac P. G protein-coupled receptor accessory proteins and signaling: pharmacogenomic insights. Methods Mol Biol. 2014, 1175:121–52. doi: 10.1007/978-1-4939-0956-8_7
11. Kisfalvi K, Eibl G, Sinnett-Smith J, Rozengurt E. Metformin disrupts crosstalk between G protein-coupled receptor and insulin receptor signaling systems and inhibits pancreatic cancer growth. Cancer Res. 2009, 69:6539– 45. doi: 10.1158/0008-5472.CAN-09-0418.
12. Kurelac I, Umesh Ganesh N, Iorio M, Porcelli AM, Gasparre G. The multifaceted effects of metformin on tumor microenvironment. Semin Cell Dev Biol. 2019, 98:90–97. doi: 10.1016/j.semcdb.2019.05.010
13. Sui X, Xu Y, Wang X, Han W, Pan H, Xiao M. Metformin: a novel but controversial drug in cancer prevention and treatment. Mol Pharm. 2015, 12:3783–91. doi: 10.1021/acs.molpharmaceut.5b00577
14. Pelicano H, Martin DS, Xu RH and Huang P. Glycolysis inhibition for anticancer treatment Oncogene 2006, 25: 4633-4646.
15. Brown J. Effects of 2-deoxyglucose on carbohydrate metabolism: review of the literature and studies in the rat. Metabolism 1962, 11: 1098-1112.
16. Liu H, Hu YP, Savaraj N, Priebe W, Lampidis TJ. Hypersensitization of tumor cells to glycolytic inhibitors. Biochemistry 2001, 40:5542-5547.
17. Lampidis TJ, Kurtoglu M, Maher JC, Liu H, Krishan A, Sheft V, et al. Efficacy of 2-halogen substituted D-glucose analogs in blocking glycolysis and killing „hypoxic tumor cells‰. Cancer Chemother Pharmacol 2006, 58:725-734.
18. Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh GF, et al. 2-deoxy-D-glucose increases the efficacy of adriamycin and paclitaxel in human osteosarcoma and non-small cell lung cancers in vivo. Cancer Res 2004, 64:31-34.
19. Liu H, Jiang CC, Lavi CJ, Croft A, Dong L, Tseng H-Y, et al. 3-Deoxy-D-glucose enhances TRAIL-induced apoptosis in human melanoma cells through XBP-1- mediated up-regulation of TRAIL-R2. Mol Cancer 2009, 8:122-139.
20. Chou TC, Talalay P. Analysis of combined drug effects: a new look at an old problem. Trends Pharmacol 1983, 4:450-453.
21. Ramachandran C, Resek AP, Escalon E, Aviram A, Melnick SJ. Potentiation of gemcitabine by Turmeric Force in pancreatic cancer cell lines. Oncol Rep 2010, 23:1529-1535.
22. Dowling RJ, Zakikhani M, Fantus IG et al. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res 2007; 67: 10804–10812.
23. Fidan E, Onder Ersoz H, Yilmaz M et al. The effects of rosiglitazone and metformin on inflammation and endothelial dysfunction in patients with type 2 diabetes mellitus. Acta Diabetol 2011, 48: 297–302.
24. Dowling RJ, Goodwin PJ, Stambolic V. Understanding the benefit of metformin use in cancer treatment. BMC Med. 2011, 9: 33
25. Kurtoglu M, Gao N, Shang J, Maher JC, Lehrman MA, Wangpaichitr M, et al. Under normoxia, 2- deoxy-D-glucose elicits cell death in select tumor types not by inhibition of

glycolysis but by interfering with N-linked glycosylation. Molecular cancer therapeutics. 2007, 6(11):3049-58.
26. Boutrid H, Jockovich ME, Murray TG, Pina Y, Feue WJ, Lampidis TJ, et al. Targeting hypoxia, a novel treatment for advanced retinoblastoma. Investigative ophthalmology & visual science. 2008, 49(7):2799-2805.
27. Zhang H-H, Guo X-L. Combinational strategies of metformin and chemotherapy in cancers. Cancer Chemother Pharmacol. 2016, 78: 13-26.
28. Peng M, Darko KO, Tao T, Huang Y, Su Q, He C, Yin T, Liu Z, Yang X. Combination of metformin with chemotherapeutic drugs via different molecular mechanisms. Cancer Treat Rev. 2017, 54: 24-33.
29. National Institute of Health and Care Excellence. Type 2 diabetes in adults: management. 2015, 1-35
30. Dowling RJ, Niraula S, Stambolic V, Goodwin PJ. Metformin in cancer: translational challenges. J Mol Endocrinol 2012; 48: R31–R43.
31. Tseng CH. Metformin reduces ovarian cancer risk in Taiwanese women with type 2 diabetes mellitus. Diabetes Metab Res Rev. 2015;31(6): 619–626.
32. Jiralerspong S, Palla SL, Giordano SH, et al. Metformin and pathologic complete responses to neoadjuvant chemotherapy in diabetic patients with breast cancer. J Clin Oncol. 2009;27(20):3297–3302.
33. Campagnoli C, Pasanisi P, Abbà C, et al. Effect of different doses of metformin on serum testosterone and insulin in non-diabetic women with breast cancer: a randomized study. Clin Breast Cancer. 2012;12(3):175–182.
34. Tseng CH. Metformin significantly reduces incident prostate cancer risk in Taiwanese men with type 2 diabetes mellitus. Eur J Cancer. 2014;50(16):2831–2837.
35. Sehdev A, Shih YC, Vekhter B, Bissonnette MB, Olopade OI, Polite BN. Metformin for primary colorectal cancer prevention in patients with diabetes: a case-control study in a US population. Cancer. 2015;121(7): 1071–1078.
36. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, and Andreelli F. (2012). Cellular and molecular mechanisms of metformin: an overview. Clin. Sci. 122, 253–270.
37. Faubert B, Boily G, Izreig S, Griss T, Samborska B, Dong Z, Dupuy F, Chambers C, Fuerth BJ, Viollet B, et al. (2013). AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. Cell Metab. 17, 113–124.
38. Pollak M. Investigating metformin for cancer prevention and treatment: the end of the beginning. Cancer Discov. 2012, 2:778–790.
39. Pollak M. Overcoming drug development bottlenecks with repurposing: Repurposing biguanides to target energy metabolism for cancer treatment. Nat. Med. 2014, 20, 591–593