Original Article

Sensitization to oxaliplatin in HCT116 and HT29 cell lines by metformin and ribavirin and differences in response to mitochondrial glutaminase inhibition

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ABSTRACT

Aim of study: In the present study, we evaluated the effect of ribavirin and metformin on the sensitivity of oxaliplatin and 5-fluorouracil (5-FU) on colon cancer.

Materials and Methods: Cell viability of two commercially available colon cancer cell lines (HT29 and HCT116) were analyzed by sulforhodamine B (SRB) assay.

Results: A clinically achievable and nontoxic concentration of ribavirin and metformin showed a significant synergistic effect on oxaliplatin in HT29 and HCT116 cell lines. Ribavirin showed a synergistic effect on oxaliplatin in HT29 ($R = 2.93$, $P < 0.001$) and HCT116 ($R = 1.71$, $P < 0.001$), while only in HT29 metformin synergized with oxaliplatin by 2.66 (± 0.28, $P < 0.01$). In addition, both cell lines showed significant differences in response to Compound 968, inhibitor of mitochondrial glutaminase activity.

Conclusion: The data suggested that these cell lines not only turn to metabolic different sustainability process after oxaliplatin treatment but that they also have different basal metabolic requirements of glutamine *in vitro* which can be exploits in the future for colorectal cancer (CRC) treatment and further studies are required.

KEY WORDS: 5-FU, colorectal cancer, metformin, oxaliplatin, ribavirin

INTRODUCTION

Compounds or designed drugs to hinder specific cell pathways and interfere with specific targets in the treatment of different diseases and conditions other than cancer seems to be recognized recently as promising anticancer therapeutics. In this regards, the repurposing of the antiviral, ribavirin, as well as metformin, a clinical valuable drug for diabetes type 2, showed recently in cancer therapy some promising results in different types of cancers.[1-4] Ribavirin (1-β-D-ribofuranosyl-1,2,4,-triazole-3-carboxami de) is a well-known antiviral agent against several deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) viruses with more than 40 years of clinical usage.[3,5,6] Ribavirin has been proposed as a specific inhibitor of inositide-5'-monophosphate dehydrogenase (IMPDH), a cellular enzyme catalyzing the rate limiting step of *de novo* guanosine-5'-triphosphate (GTP) synthesis, and a transcription factor.[7] In various animal and human tumor types, IMPDH expression and activity are markedly elevated; hence, it has been associated to transformation and proliferation of malignant cells. Guanine nucleotides are required for several metabolic and signaling pathways and functions of cells.[8] In addition, other studies indicate that ribavirin acts as a physical mimic of the m7G cap and subsequently inhibits the eukaryotic translation initiation factor 4E (eIF4E) activity by binding to it and interferes cap-dependent translation.[3] In consequences, eIF4E is a rate-limiting factor for cap-dependent protein synthesis that is regulated by the phosphoinositide 3-kinase (PI3K)/ AKT/mammalian target of rapamycin (mTOR) signaling pathway as well as mitogen-activated protein kinase (MAPK)-interacting kinase 1 and 2 (MNK1/2)-mediated phosphorylation. eIF4E is overexpressed in many cancers and has been reported to have important roles in the development and progression of hematological malignancies and to be overexpressed in colorectal cancer (CRC).[9]

Metformin is a biguanidine believed to inhibit the mitochondrial respiratory chain complex I

and to interfere with glucose metabolism by activating liver kinase B1 (LKB1)/adenosine monophosphate-activated protein kinase (AMPK) pathway resulting in an inhibitory effect on mTOR pathway.[10]

In the present study, we used two drugs commonly used in CRC therapy; oxaliplatin and 5-fluorouracil (5-FU). Oxaliplatin mechanism of action is mediated by the formation of DNA adducts and consequently it induces DNA lesions such as intrastrand crosslinks by covalently binding the platinum compound to guanine residues.[11] Oxaliplatin-DNA adducts are suggested to exert their cytotoxicity by directly inhibiting DNA and RNA synthesis and inducing apoptosis.[11] A side effect of oxaliplatin is producing reactive oxidative species (ROS) and ROS-dependent endoplasmic reticulum (ER) stress and autophagy.[12] On the other hand, 5-FU is a precursor of deoxythymidine triphosphate (dTTP) and uridine thriphosphate (UTP) during biogenesis; consequently it interferes with both DNA and RNA metabolism affecting DNA repair and DNA or RNA synthesis. A side effect of 5-FU on metabolism is on processes dependent on mTOR such as autophagy and ER stress response in colon cancer cells.[13] It has been reported that compounds which inhibit autophagy or hinder ER stress response can sensitized cancer cells to oxaliplatin or 5-FU.[12-14]

In this study, we examined the effect of two known metabolic stressors compounds, ribavirin and metformin on the sensibility to Oxaliplatin and 5-FU in two commercial CRC cell lines as well as the importance of glutaminolisys in these cell lines by the use of Compound 968, a well-known inhibitor of mitochondrial glutaminase activity.[15]

There are nonoverlapping side effect profiles of Oxaliplatin and 5-FU on metabolic processes this provide a basis for investigating the toxicity of drugs such as ribavirin and metformin in combination with the two primary drugs. We found synergistic interaction on Oxaliplatin as well as differences between the cell lines in the way they use glutamine. On the basis of these findings it is required continue with the validation in different phenotypic and genetic backgrounds of CRC cell lines to support the real use of ribavirin and metformin specially on oxaliplatin treatment in CRC.

MATERIALS AND METHODS

Cell culture and reagents: HT29 and HCT116 colon cancer cell lines were obtained from the American Type Culture Collection and were maintained at 37°C in 5% CO_2 and Roswell Park Memorial Institute medium (RPMI) with 10% fetal bovine serum (Cat # 11875093, Invitrogen, Argentina) and gentamicin (40 µg/ml, Invitrogen, Argentina Cat # 15750078). The chemicals and reagents obtained from Sigma-Aldrich, Buenos Aires, Argentina were: ribavirin (Cat. R9644, 10 mg); metformin (Cat. 53183-1,1-dimethylbiguanide hydrochloride); oxaliplatin (Cat. O9512, 5 mg), and 5-FU (Cat. F6627, 1 g).

Sulforhodamine B (SRB) cytotoxicity assays: SRB assays were performed according to the method of.[16] Briefly, cells were seeded at low density in wells of a 96-well plate (final density of 80% of confluency in wells of nontreated cells in a 96-well plate) and after 24 h incubation they were subsequently treated with oxaliplatin or 5-FU alone, ribavirin or metformin alone, and in combination with oxaliplatin or 5-FU. After three double time post drug treatment, cells were fixed with trichloroacetic acid, stained with SRB (Sigma Aldrich, Argentina, Cat. S1402), and analyzed for percent of survival on a 96-well plate reader. Efficacies of the various drug treatments were determined by calculating 50% inhibitory concentrations (IC_{50}) and synergy values. Synergy values were calculated using the ratio of IC_{50} of primary drug alone (oxaliplatin or 5-FU) divided by combination IC_{50}s as previously used.[4] Using this equation, R values more than 1.6 were indicative of synergy, equal to 1 indicate additive behavior, and less than 1 indicate inhibitory drug interactions.

Analysis

There were at least five replicates for all SRB experiments. Means were calculated and then compared employing the Student's t-test analysis ($P < 0.05$) using SigmaStat software.

RESULTS

We obtained the 50% inhibitory concentrations, IC_{50}, for all the drugs used in this study [Table 1]. Both cell lines showed no significant different values for IC_{50} with the exception of fivefold significantly different response to a mitochondrial glutaminase activity inhibitor, Compound 968. HT29 cell line showed to be more resistant (17.5 µM ± 3) than HCT116 (3.6 µM ± 2.4); suggesting that they have different metabolic requirements of glutamine which can be exploited for future studies ($P < 0.05$) [Table 1].

Our study of drug combination showed that a clinically achievable concentration of ribavirin (10µM), which was not cytotoxic to the cells in culture, resulted in a significantly synergistic effect on oxaliplatin in both cell lines tested with the sensitization index (R) of 2.93 ± 0.3 for HT29 and 1.71 ± 0.2 for HCT116, respectively (IC_{50} [Figure 1] and $P < 0.001$ [Table 1]); while ribavirin did not affect the response to 5-FU. Metformin sensitized significantly HT29 cell line to oxaliplatin (R = 2.66 ± 0.28), while it did not show synergy in HCT116 or in combination with 5-FU in both the cell lines [Table 1].

Interestingly, Compound 968 showed a differential effect alone between cell lines [Table 1], though neither did it sensitize HT29 nor HCT116 to oxaliplatin or to 5-FU (data not shown).

DISCUSSION

Genomic studies reported that PI3K/AKT pathway is highly deregulated in CRC.[17] AKT activates downstream mTOR,

Table 1: IC_{50} in HT29 and HCT116

HT29	IC_{50}		HCT116	IC_{50}	
Ribavirin	38.6 µM±1.1		Ribavirin	27.5 µM±1.8	
Metformin	5.9 mM±0.09		Metformin	6.5 mM±1,7	
Comp968	17.5 µM±3.6		Comp968	3.6 µM±2.4	
Oxaliplatin	0.88 µM±0.2		Oxaliplatin	0.41 µM±0.02 µM±0.01	
5-FU	4.20 µM±0.3		5-FU	3.30 µM±0.6	
HT29	**IC_{50}**	**R**	**HCT116**	**IC_{50}**	**R**
Oxaliplatin			Oxaliplatin		
Plus 5 µM ribavirin	0.30 µM±0.01*	R=2.93, $P<0.001$	Plus 5 µM ribavirin	0.24 µM±0.02*	R=1.71, $P<0.001$
Plus 1 mM metformin	0.33 µM±0.02*	R=2.66, $P<0.010$	Plus 1 mM metformin	0.37 µM±0.1	R=1.6, $P>0.05$
5-FU			5-FU		
Plus 5µM ribavirin	4.7 µM±0.7		Plus 5 µM ribavirin	2.8 µM±0.7	
Plus 1 mM metformin	4.5 µM±0.5		Plus 1 mM metformin	2.4 µM±0.6	

*Significant synergy. IC_{50}=50% inhibitory concentration, 5-FU=5-fluorouracil. Top part of the table shows. IC_{50} concentrations for the drug individually; and the second part shows IC_{50} for different combinations for each of the primary drugs, oxaliplatin and 5-FU plus ribavirin and metformin with their corresponding R values
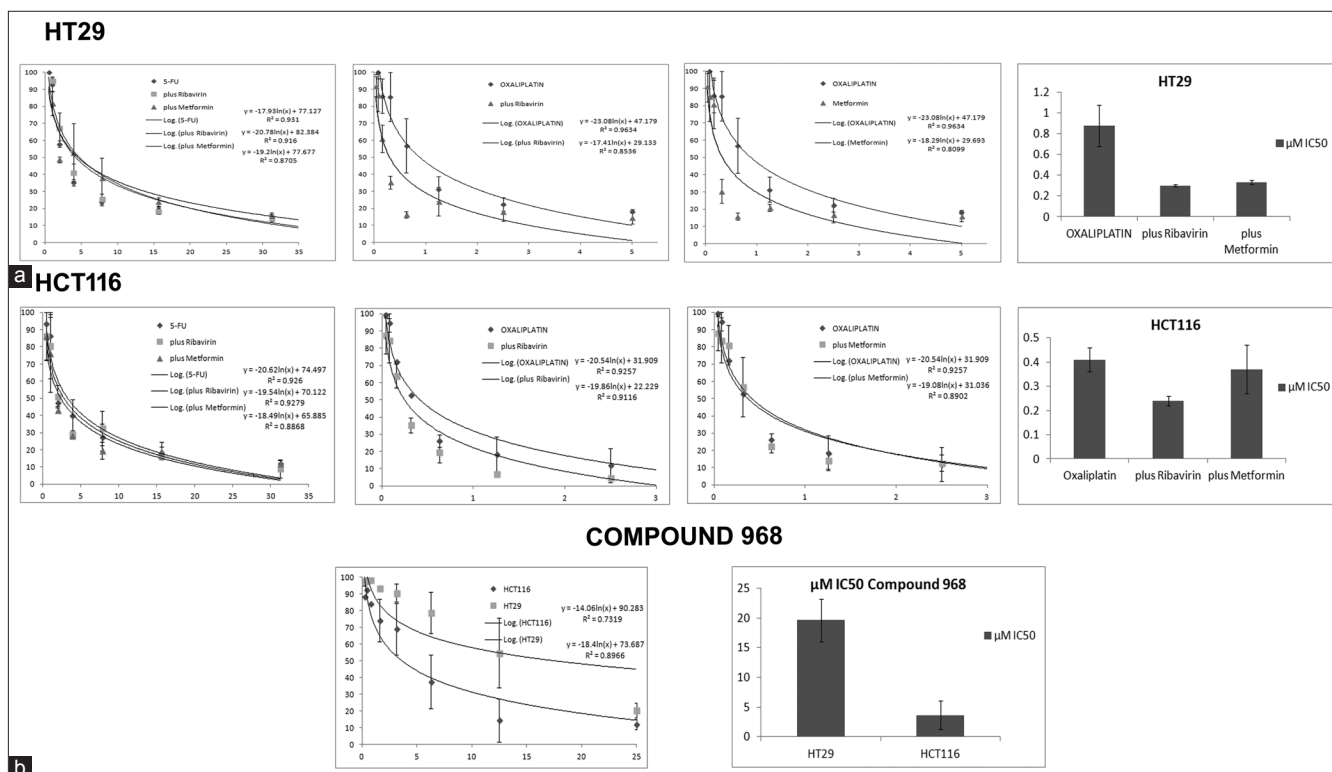
Figure 1: Survival curves and histogram showing IC_{50}. (a) Metformin and ribavirin Neither Metformin nor ribavirin showed synergy with 5-FU in HT29 or HCT116 when combined in nontoxic concentrations when combined in nontoxic concentrations. Metformin and ribavirin significantly sensitized HT29 to oxaliplatin and only ribavirin synergized with oxaliplatin in HCT116 cell line. (b) HT29 and HCT116 differed significantly in their response to Compound 968 ($P < 0.05$). IC_{50} = 50% inhibitory concentration, 5-FU = 5-fluorouracil

a serine/threonine kinase that can be found in two types of complex mTorc1 and mTorc2. In tumorigenic development and cancer progression, mTorc1 is critical[18] as well as it is deregulated. mTorc1 is also involved in the activation of eIF4E and eIF4E recognition of mRNA 5'cap important in the regulation of translation in eukaryotes.[4] In cancer and CRC as mTorc1 is deregulated, eIF4E is generally upregulated.[9] In consequences, ribavirin as an inhibitor of cap-dependent translation would target naturally a pathway that is overexpressed in CRC cells.[9] Our results showed that this pathway seems to become essential only in both cell lines, HT29 and HCT116, after the treatment with oxaliplatin and not with 5-FU. It is inferred that CRC cells turn to cap-dependent translation after Oxaliplatin treatment as we found a significant synergy resulted in combined treatment with ribavirin. In summary, the data suggest that at low dose ribavirin combined treatment with oxaliplatin can be an effective therapeutic approach for CRC, which needs further testing.

On the other hand, in animal studies metformin have shown to inhibit the proliferation of colon epithelium as well as the aberrant crypt foci;[18-21] summing up metformin seems to inhibit tumors growth in colon of animal models.[18,19] Here,

metformin significantly synergized with oxaliplatin only in HT29 cell line, suggesting that cell response to oxaliplatin treatment rely more on mTOR, and downstream of PI3K/AKT, as well as on mitochondrial complex I and AMPK, in a heterogeneous genetic or metabolic manner.[22]

Regarding the heterogeneous genetic and metabolic baseline between the two cell lines analyzed here, we reported that HCT116 on the contrary to HT29 depends on glutamine metabolism as Compound 968 showed to be more toxic in this cell line. Previously other reports also suggested that the commercial CRC cell lines show differences in the use of nutrients and the metabolic process which sustain their survival *in vitro* and in response to stressors.[23,24] Furthermore, though we showed basal differences between HT29 and HCT116 in how they responded to Compound 968; Compound 968 did not show synergy to oxaliplatin or 5-FU (data not showed), suggesting that nonetheless mitochondrial glutaminase activity is highly active and overexpressed in CRC,[25] the cells did not turn to this metabolic pathway for survival after the treatments.

Here we showed sensitization to oxaliplatin by blocking the cap-dependent translation using ribavirin and interfering mTOR/mitochondrial complex I by the use of metformin. These findings suggested that the metabolic changes induced by oxaliplatin were dependent on downstream of PI3K/AKT pathway,[23] in particular mTOR/eIF4E pathway in HT29 and HCT116 cell lines and LKB1/AMPK pathway and mitochondrial complex I in HT29.

HT29 and HCT116 are two of the commonly employed colon cancer cell lines in research. They differ in their gender origin and mutation status, and probably as well in their metabolic requirements as expressed above. HT29 have been reported to use glucose preferentially through the pentose phosphate pathway,[24] while higher metabolic requirement of glutamine by HCT116 could be inferred by our and[26] results. In addition, HCT116 has mutations in PI3KCA and K-RAS genes which confer constitutive activation of PI3K/AKT and K-RAS pathways,[27] while HT29 has a deficient p53 gene expression.[28] In the present work how this can affect the metabolic reorganization of these cell line after oxaliplatin treatment could be only suggested. While ribavirin showed a synergistic effect on oxaliplatin in both cell lines, metformin showed only synergistic effect in HT29. Both drugs hind PI3K/AKT pathways; however, metformin also activates AMPK, a metabolic stress sensor that regulates several downstream effects on metabolic adaptation; such effect include AMPK-dependent activation of p53 which stimulates a stress response. It has been reported that metformin has a strong effect on p53 deficient cell lines;[29] though the scope of the present work was not to elucidate the differential mechanism by which HT29 and not HCT116 respond synergistically to metformin and oxaliplatin, we can suggest that the sensitivity to metformin can be accounted to deficiency in the AMPK/p53 axis.[29,30]

In summary, our results implied that it is indispensable to analyze further the changes undergone by CRC cells and described the metabolic pathways they turn to maintain their survival and cell homeostasis after oxaliplatin. We suggest that such studies could help to predict the mechanism involved in developing recurrence and resistance to drugs. Finally, the way CRC cells adapt to metabolic stress induced by drugs commonly used in CRC treatment may inform the different ways patients answer to therapy.

ACKNOWLEDGEMENTS

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