School of Biomedical Sciences

Impact of heavy metal/pesticide mixtures on colorectal cell function

David R. Wallace, Ph.D.

Department of Pharmacology, Oklahoma State University Center for Health Sciences, Tulsa OK

CENTER FOR HEALTH SCIENCES

INTRODUCTION

Cancer of the colon and rectum (colorectal; CRC) is the fourth most prevalent cancer type in 2018. Information from the National Cancer Institute estimates over 140,000 new cases in 2018 with nearly 51,000 death from CRC (both approximately 8% of new cancer cases and deaths). Information on environmental factors that may be involved in CRC development is sparse. A recent publication suggests that heavy metal concentration is elevated in CRC compared to control CR tissue (Sohrabi et al., 2018). We have recently shown that cadmium content in pancreatic tumors is elevated compared to surrounding non-cancerous pancreatic tissue Buha et al., 2017). Additional work is needed to examine the mechanism of heavy metal action on CRC development. Recent studies have begun to examine the actions of cadmium on the pancreas and its ability to alter apoptotic pathways or cellular function.

OBJECTIVES

To determine the effects of cadmium, glyphosate and subtoxic mixtures of cadmium and glyphosate on CR cell function, and apoptotic pathway activity. These studies address needed areas of investigation; environmental toxicant impact on CRC and the toxicology of chemical mixtures on this cancerous development.

METHODS

Cell Lines and Cell Culture Maintenance: Cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Control CR cells, CCD-18Co (CRL-1459™) and CRC cells, DLD-1 (ATCC® CCL-221™) were grown and maintained as described in the ATCC-suggested protocols. Cells were maintained in recommended optimum growth media except when cytotoxicity assays were performed (Assay media: MEM, 1% fetal bovine serum, 2 mM glutamine). Growth media for CCD-18Co cells was MEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin. DLD-1 cells were maintained in RPMI-1640 with the same supplements as the CCD-18Co growth media. Cells were kept at 37°C in 5% CO_2 humidified air.

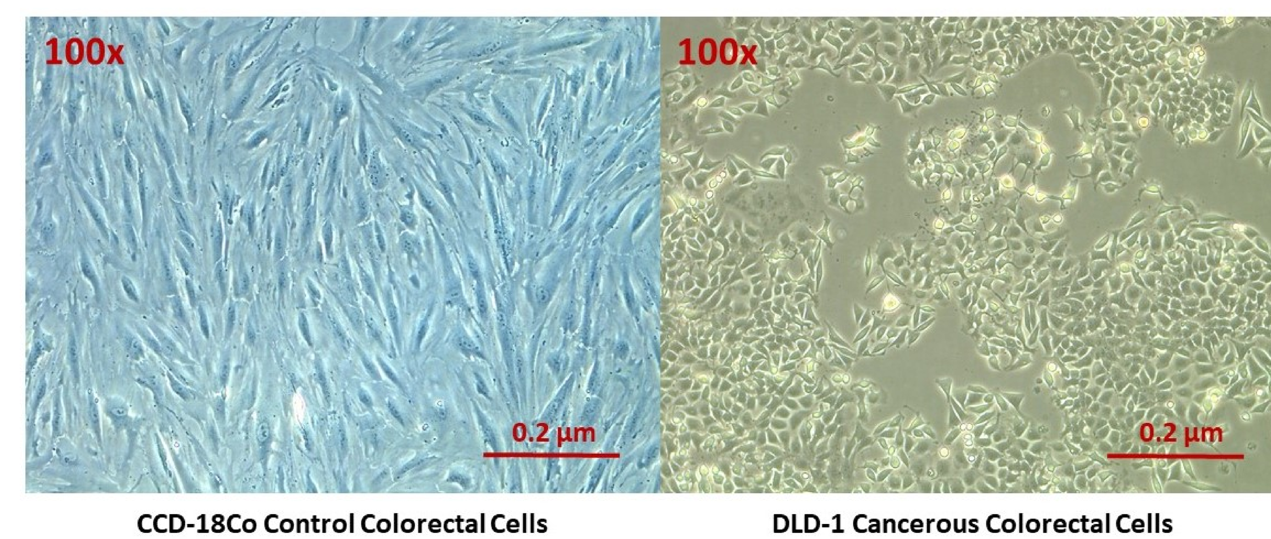
Cell Viability & Cell Growth Assays: Both CCD-18Co and DLD-1 cell lines were plated at a final density of 2-5 x 10^4 cells/well and allowed to adhere for 24 hours before LDH assays were performed to determine 1) the number of viable cells within the culture and 2) the percentage of the total cell number that are viable. LDH activity was measured using the Cytotox-ONE™ Assay kit (Promega; Madison, WI) according to the manufacturer's protocol. Fluorescence was quantified using a Biotek plate reader set to $485nm_{ex}$ /$530nm_{em}$.

Apoptosis Assays: The Apo-ONE™ Homogeneous Caspase-3/7 (Promega; Madison, WI) assay was performed according to the manufacture's protocol. Caspase reagent (1:1 with the quantity of media used) is added to each well and plates were incubated for 2 hours. Fluorescence was then measured using a Biotek plate reader set at an excitation of 499nm and emission of 521nm. Data was then expressed as the mean ± SEM of N=6 assayed in triplicate.

Data Analysis: All data were analyzed using GraphPad Prism (v 7.04; San Diego, CA). Mitochondrial toxicity assays; the data were fit by nonlinear single-site inhibition curve to determine the IC_{50} value for cadmium in each media. If there was a three-fold or great leftward shift in IC_{50} values in the galactose group, this was indicative of mitochondrial toxicity. One-way analysis of variance was performed with the specialized Dunnett's test for posthoc comparison to control values. In the oxidative stress assays, data were analyzed by two-way ANOVA (2 x 3 or Cell type x Concentration) using Holm-Sidak for multiple comparisons. All data are expressed as the mean ± SEM of 4 assays (N=4) performed in either duplicate or triplicate. A significance level was set at α = 0.05.

RESULTS

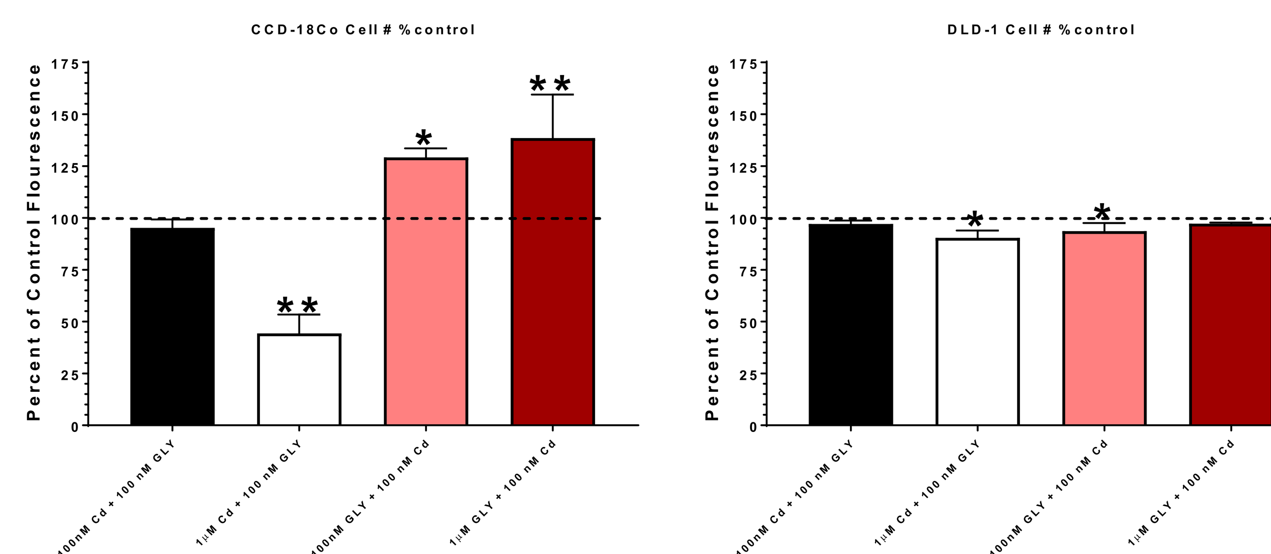
Figure 1: CCD-18Co and DLD-1 cells in culture



CCD-18Co Control Colorectal Cells

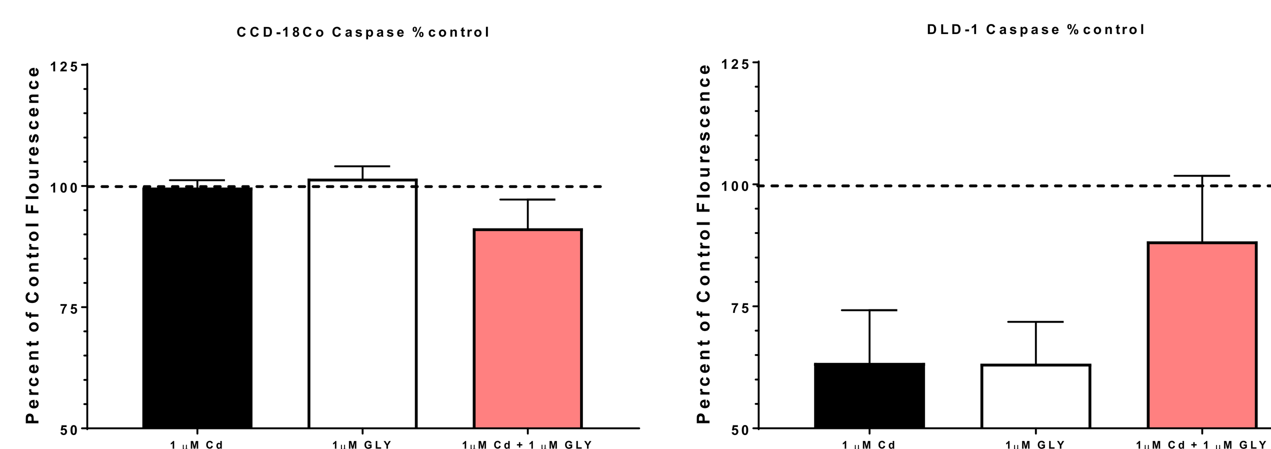
DLD-1 Cancerous Colorectal Cells

Figure 3: Cd/GLY Mixture exposure alters cell number



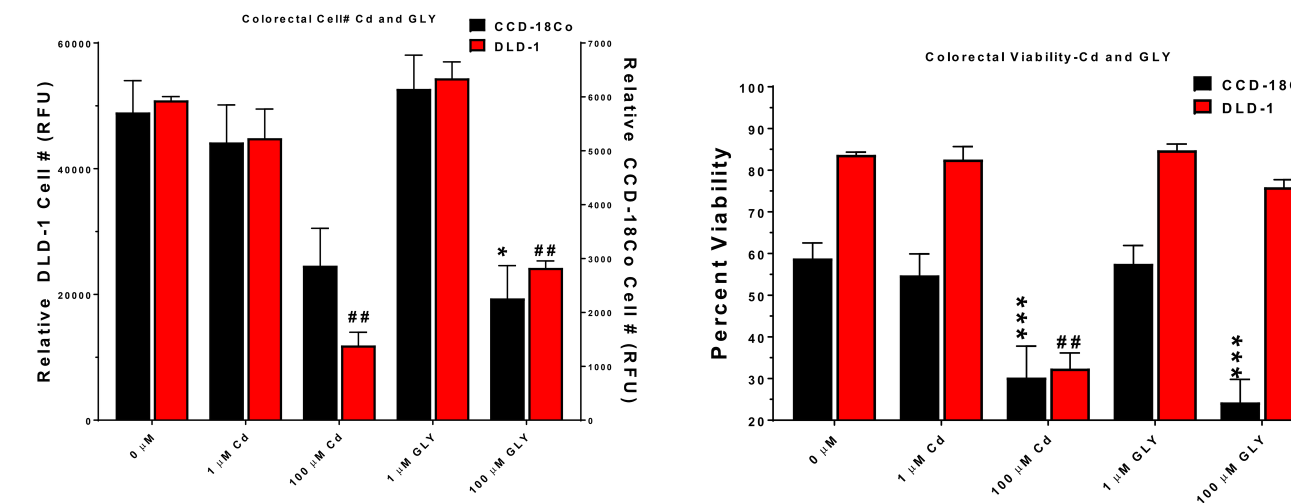
Effect of Cd-GLY mixtures on cell number. CCD-18Co and DLD-1 cells were incubated for 48 h in the presence of Cd and GLY mixtures using subtoxic concentrations determined in Figure 1. Cell number was determined using the LDH assay and data are expressed as percent of control values. There was a significant effect of treatment in both CCD-18Co ($F_{5,12}$=25.27; p<0.0001) and DLD-1 ($F_{5,12}$=15.88; p<0.0001) cells. Mixture group values where compared to control cell number for each cell line. Data are expressed as the mean ± SEM, N=4 in duplicate. *****:p<0.05 and ******:p<0.01 using Dunnett's test to compare treatment group to control.

Figure 5: Cd/GLY effect on Caspase 3/7 activity



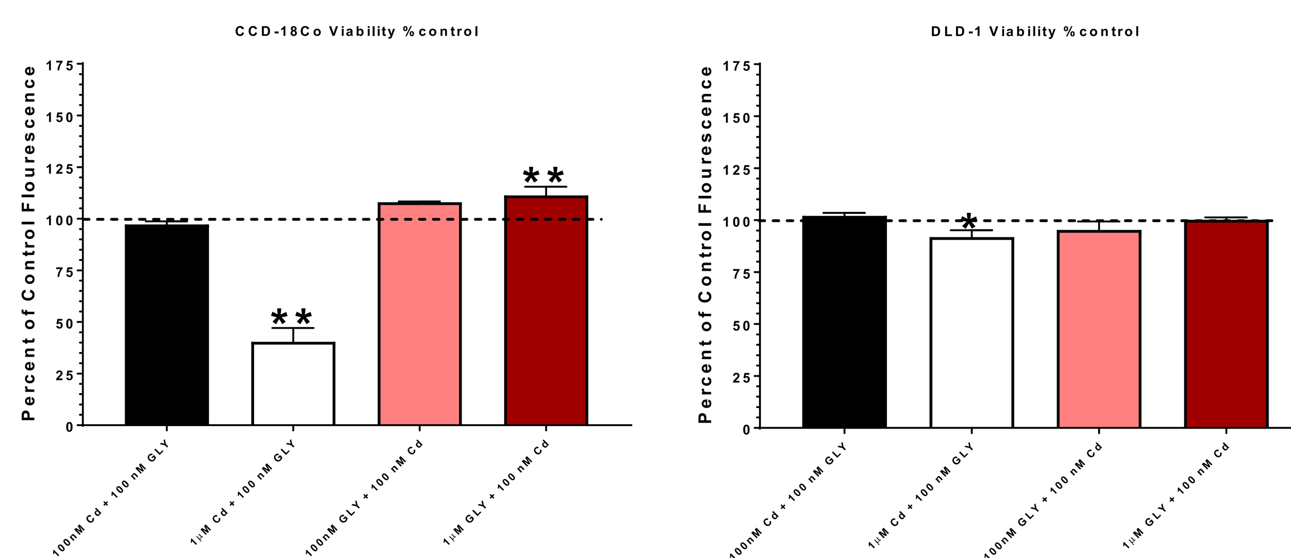
Effect of Cd-GLY on caspase 3/7 activity. CCD-18Co and DLD-1 cells were incubated for 48 h in the presence of 1µM Cd, 1µM GLY alone, or a mixture of 1µM. Caspase activity determined using the Promega Apo-Tox caspase 3/7 assay and data are expressed as percent of control values. Control of basal caspase activity for each cell line was 5005 RFU (CCD-18Co) and 41,858 RFU (DLD-1). There were no effect of treatment on caspase activity compared to control values in either CCD-18Co ($F_{3,20}$=1.3; p=0.304) or DLD-1 ($F_{3,19}$=1.5: p=0.258). Data are expressed as the mean ± SEM, N=6 in duplicate.

Figure 2: Effect of Cd or GLY on CCD-18Co and DLD-1 cell number and viability



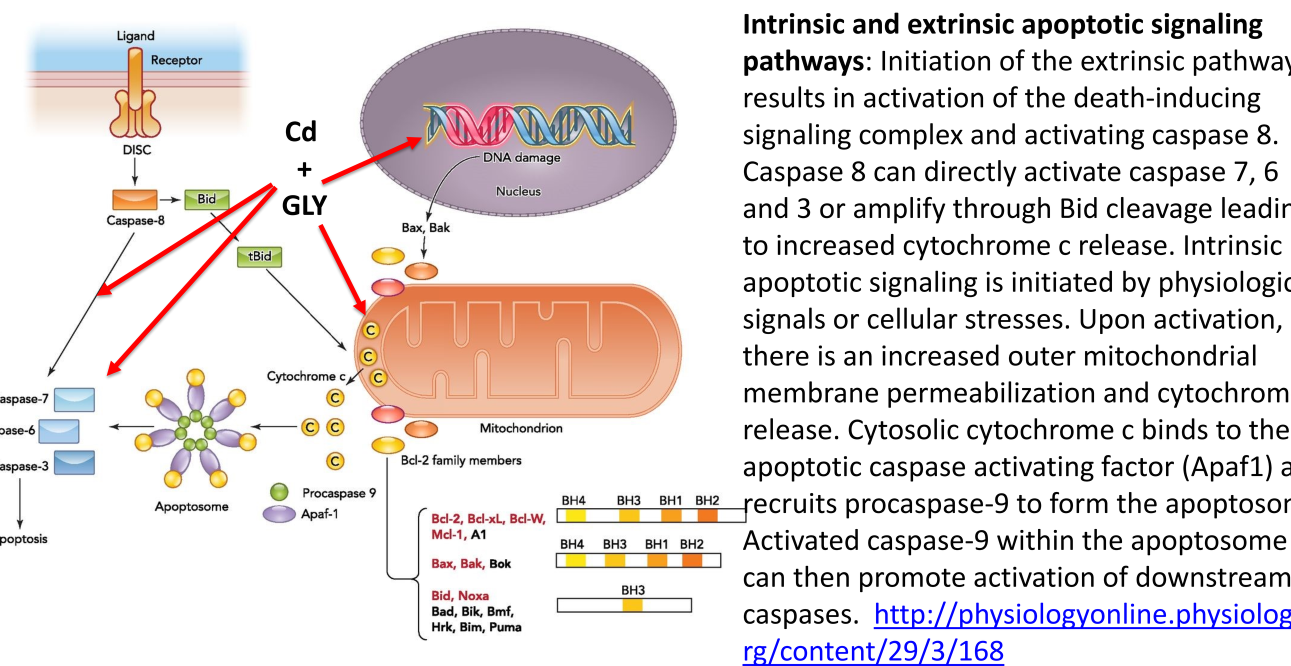
Cd- and GLY-mediated changes cell number and viability. CCD-18Co and DLD-1 cells were incubated for 48 h in the presence Cd or GLY (0-100µM) and cell viability and number were determined using a LDH assay. There was a significant effect of both treatment ($F_{4,30}$=220; p<0.0001) and cell type ($F_{1,30}$=2957; p<0.0001) on cell number. Analysis of the viability data revealed a significant effect of both treatment ($F_{4,30}$=140.4; p<0.0001) and cell type ($F_{1,30}$=406.3; p<0.0001) . Data are expressed as Relative Fluorescent Units (RFU), and the mean of N=4 performed in duplicate. *****: p<0.05 and *******:p<0.0001 compared to CCD-18Co control, **##**: p<0.001 compared to DLD-1 control values.

Figure 4: Cd/GLY Mixture exposure effects on Cell Viability



Effect of Cd-GLY mixtures on cell viability. CCD-18Co and DLD-1 cells were incubated for 48 h in the presence of Cd and GLY mixtures using subtoxic concentrations determined in Figure 1. % Viability was determined using the LDH and data are expressed as percent of control values. There was a significant effect of treatment in both CCD-18Co ($F_{5,12}$=7.82; p=0.0018) and DLD-1 ($F_{5,12}$=168; p<0.0001) cells. Mixture group values where compared to control cell number for each cell line. Data are expressed as the mean ± SEM, N=4 in duplicate. *****:p<0.05 and ******:p<0.01 using Dunnett's test to compare treatment group to control.

Figure 6: Apoptotic Pathways



Intrinsic and extrinsic apoptotic signaling pathways: Initiation of the extrinsic pathway results in activation of the death-inducing signaling complex and activating caspase 8. Caspase 8 can directly activate caspase 7, 6 and 3 or amplify through Bid cleavage leading to increased cytochrome c release. Intrinsic apoptotic signaling is initiated by physiological signals or cellular stresses. Upon activation, there is an increased outer mitochondrial membrane permeabilization and cytochrome c release. Cytosolic cytochrome c binds to the apoptotic caspase activating factor (Apaf1) and recruits procaspase-9 to form the apoptosome. Activated caspase-9 within the apoptosome can then promote activation of downstream caspases. http://physiologyonline.physiology.org/content/29/3/168

SUMMARY/CONCLUSIONS

- ❑ CCD-18Co control colorectal cells displayed a slightly greater sensitivity to the action of both Cd and GLY. When incubated alone, potency order was GLY > Cd in CCD-18Co cells and Cd > GLY in DLD-1 cells
- ❑ To assess the effects of a mixture of Cd and GLY, subtoxic concentrations were determined from figure 1. Interestingly the effects on cell number in CCD-18Co cells was robust and biphasic. Again the GLY groups exhibiting the greater effects of increased cell number. For DLD-1 cells, there was only a very slight decrease in cell number.
- ❑ The values for viability mirrored what we observed in cell number, although not as robust increase in the GLY-Cd group. A similar reduction in viability was seen in the CCD-18Co group (1 µM Cd + 100 nM GLY) compared to the reduction in cell number.
- ❑ The effects of Cd, GLY and Cd/GLY mixtures does not appear to arrest either CCD-18Co or DLD-1 cell growth. Changes in cell number tend to mirror the effects on viability. Interestingly, GLY in the presence of 100 nM Cd results in a slight increase in cell viability, but a robust increase in cell number. This would suggest that in control cells, these treatment combinations are actually facilitating growth of the control cells.
- ❑ These findings suggest that a transformation occurred in the CCD-18Co cells – possibly a foundation for cancer development
- ❑ Exposure to 1 µM Cd, GLY or a combination of Cd and GLY did not alter caspase activity in either cell line. This was somewhat surprisingly considering the 25-30% reduction observed in the DLD-1 cells.
- ❑ Collectively, exposure to Cd, GLY or a mixture of the two toxicants had little effect on DLD-1 cell function. CCD-18Co cells exposed to the two toxicant showed elevated cell growth, but no elevation in caspase activity. This may lay the foundation for the transition of CCD-18Co cells into a cancerous cell

REFERENCES

- National Cancer Institution, https://www.cancer.gov/types/colorectal (*accessed Jan 3, 2019*)
- A Buha, DR Wallace, V Matovic, A Schweitzer, et al., (2017) Cadmium exposure as a putative risk factor for the development of pancreatic cancer: three different lines of evidence. Biomed Res International, Article ID 1981837, 1-8; DOI: 10.1155/2017/1981837
- M Sohrabi, A Gholami, MH Azar, M Yaghoobi, et al. (2018) Trace element and heavy metal levels in colorectal cancer: comparison between cancerous and non-cancerous tissues. Biol Trace Elem Res, 183(1):1-8 DOI: 10.1007/s12011-017-1099-7
- D.R. Wallace, A. Schweitzer, V. Djordjevic, A. Buha-Djordjevic (2019) Cadmium Interaction with Pancreatic Mitochondria: Implications for Pancreatic Cancer. International Journal of Environmental Research and Public Health (Accepted pending major revisions)
- M. Anđelković, A. Buha-Đorđević, E. Antonijević, B. Antonijević, M. Stanić, J. Kotur-Stevuljević, V. Spasojević-Kalimanovka, M. Jovanović, N. Boričić, D.R. Wallace, Z. Bulat (2018) Toxic effects of acute cadmium and lead exposure on in rat blood, liver and kidney. International Journal of Environmental Research and Public Health (Accepted with minor revisions)

ACKNOWLEDGEMENTS

We would like to acknowledge or collaborators at the University of Belgrade and the outstanding input from Dr. Aleksandra Buha. Funding was obtained from intramural funds (OSU-CHS; #1-54333) and through funding by Eyra Biotechnology, LLC