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Untargeted Metabolomic Profiling of Rolipram-Stimulated Immune Cells

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Abstract

Inflammation is a significant component of neurological diseases such as multiple sclerosis (MS). One potential anti-inflammatory agent, rolipram, was tested for efficacy in MS; however, despite showing promise in early trials, the drug has been largely sidelined due to side effects. However, it is still of interest to elucidate the anti-inflammatory mechanisms of rolipram in order to identify more targeted inhibitors of inflammatory signaling. In order to achieve this end, we have utilized liquid chromatography-mass spectrometry-based metabolomics to determine metabolic pathways that are altered by rolipram treatment in human THP-1 monocytic cells and primary mouse microglial cultures. Amino acids and related metabolites as well as steroid species were identified as being significantly dysregulated. Shotgun lipidomics showed upregulation of glycerophospholipids, including phosphatidylcholine and phosphatidylserine after rolipram treatment. Taken together, these results suggest that rolipram inhibition of cAMP breakdown leads to significant alterations on the metabolome of monocytic immune cells.

Introduction

Rolipram is a phosphodiesterase-4 (PDE-4) inhibitor originally developed as anti-inflammatory agent(Zhu et al., 2001). This drug acts to prevent the breakdown of 3', 5'-cyclic adenosine monophosphate (cAMP). cAMP is a second messenger in a number of different intracellular signaling cascades induced by hormonal, neurotransmitter, and cytokine stimulation(Hurley et al., 1999). In line with this role, studies have shown that cAMP inhibition by rolipram has a potent anti-inflammatory action due to its ability to attenuate production of nitric oxide (NO) and other pro-inflammatory cytokines in immune cells(Yang et al., 2008).

Microglial cells are the resident macrophages of the central nervous system and play both beneficial and harmful roles in neurodegenerative processes. Microglial cells enter into an activated state after tissue injury and phagocytose damaged cells to promote healing(Rock et al., 2004). However, these cells also release pro-inflammatory mediators such as tumor necrosis factor alpha (TNFα) and NO that may result in additional pathology after brain insults(Guadagno et al., 2013). Along with microglia, macrophages from the peripheral tissues also migrate into the damaged nervous system and these cells are an additional target for rolipram(Zhang et al. 2002).

Initial preclinical testing of rolipram in autoimmune disorders such as multiple sclerosis were successful and the drug advanced to clinical trials. Unfortunately, these trials were discontinued when it was discovered that rolipram administration precipitated potent gastrointestinal side effects, namely vomiting(Robichaud, 2001). Though early enthusiasm has faded, rolipram, as well as other PDE-4 inhibitors, remain of interest as potential immunomodulatory drugs if side effects can be overcome. Several potential modifications could facilitate the use of these compounds, including targeted delivery to lesions in the CNS to avoid the gut or pairing rolipram with another drug(Glueckert, 2015). In order to design more robust

inhibitors of PDE-4 with fewer side effects; it is necessary to better understand the mechanisms by which this class of compounds could act.

Several *in vitro* cell lines share the characteristics of primary tissue microglia and macrophages. An immortalized monocytic line, THP-1, is easily cultured and phenotypically similar to tissue macrophages. These cells respond to inflammatory mediators such as bacterial lipopolysaccharide and release cytokines, including interleukins 1, 6, and 8, TNFα, and others(Chanput, 2014.). This tissue culture model of tissue macrophages provides an opportunity to evaluate anti-inflammatory mechanisms induced by rolipram.

Untargeted metabolomic profiling is a powerful tool for interrogating the therapeutic actions and off-target effects of compounds (Patti et al., 2015). Global profiling measures changes to the entire metabolome and this technology allows the detection of alterations in biologically important species such as amino acids, nucleic acids, neurotransmitters, and
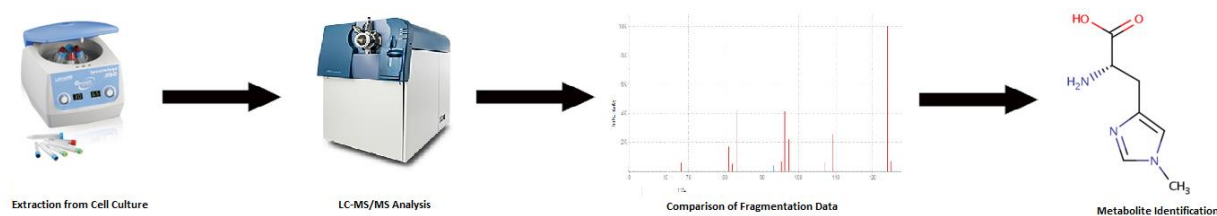

Figure 1: Untargeted Metabolomics platform to identify drug mechanisms

signaling molecules in response to drug administration or tissue pathology(Kaddurah-Daouk et al., 2008). Currently, the most popular analytical technique to measure metabolites is liquid chromatography-mass spectrometry (LC-MS)(Patti et al., 2015). In this study, we applied a platform that combines hydrophilic chromatography-mass spectrometry (HILIC-MS) with shotgun lipidomics to determine changes in polar species and lipids associated with rolipram treatment(Han et al., 2005).

Materials and Methods

Chemicals

Rolipram (>99.9%) was purchased from Enzo Biosciences (Farmingdale, NY,USA). Lipopolysaccharide was purchased from Sigma Aldrich (St. Louis. MO, USA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Fischer Scientific (Fair Lawn, NJ, USa). Phosphate buffer saline (PBS), Dulbecco's Modification of Eagle's Medium (DMEM), and penicillin-streptomycin were purchased from Corning (Manassas, VA, USA.) HPLC-grade water, ethanol, methanol, and acetonitrile were purchased from Fischer Scientific (Fair Lawn, NJ, USA.) All MTT materials, including thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA.)

Cell Culture

The THP-1 cell line was a gift from the Paruchuri lab. These cells were cultured in DMEM media with 10% FBS and 1% penicillin-streptomycin and grown at 37°C in 5% CO_2. For experimental procedures, the cells were plated at approximately 1.0×10^6 cells per well in six well plates in a total volume of 3 mL per well for 24 hours. Cells were subsequently treated with 100 nM phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, for a period of 72 hours to induce their differentiation from a monocytic phenotype into macrophage-like cellls. After this period, rolipram was added to the experimental group at a concentration of 5 μM dissolved in DMSO for an additional 24 hours.

Primary glial cultures were prepared from C57BL/6 mice according to a protocol approved by the institutional care and use committee (IACUC) at the University of Akron (protocol # 14-03-03). Cortices from neonatal mice were isolated and placed in in a falcon tube

with 5 mL of HBSS. Tissue was minced and 0.1 mL DNAse I (0.2 mg/mL) was added to each tube, along with 0.1 mL trypsin (1mg/mL.) After 15 minutes of incubation at 37 °C, 2 mL of media (DMEM +10% FBS and 1% Penicillin/Streptomycin) was added to each sample to halt trypsinization, and
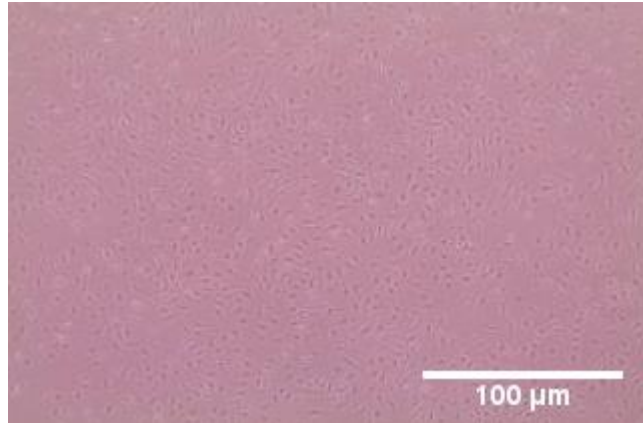

Figure 2: Brightfield image of primary mouse-isolated microglia cells at 200X magnification.

centrifuged at 100 x g for 10 min. The supernatant was discarded and 5 mL of plating media was added to each tube to dissociate the pellet. The sample was then run through a 40 µm diameter strainer and the flow-through was collected. After counting, the cells were plated at approximately 1×10^6 cells/mL in 15 mL of DMEM in a poly-D-lysine coated T75 flask. Cells were incubated at 5% CO_2 and 37 °C for approximately ten days to reach confluency. The flasks were subsequently shaken on an orbital shaker under incubation at 37 °C for a period of three hours to allow microglia to detach from the adherent astrocytes. The supernatant, containing the free-floating microglia, was separated into a 50 mL conical flask and centrifuged at 168 x g for 10 min at 4 °C. The pellet, containing the microglia, was resuspended in DMEM. The cells were counted and plated at a density of 1.0×10^5 cells per well in six well plates

with 3 mL of media (DMEM) in each well, and allowed to adhere for one day. In some experiments, cultures consisted of both astrocytes and microglial cells (i.e. mixed glial cultures).

Metabolite Extraction

Metabolites were isolated from rolipram and control-treated cells by using a modified Bligh-Dyer Extraction(Bligh and Dyer, 1959; Fei et al., 2014). After drug treatment, tissue

culture medium was aspirated away and 180 µL HPLC grade water with 20 µL HPLC methanol

was added to each well prior to removing cells from the bottom of the plates by scraping.

Isolated cells were subsequently resuspended into 200 µL of cold HPLC-grade methanol.

Samples were subjected to three rounds of freezing in liquid nitrogen, thawing, and sonication to

lyse the cell membrane. Solvents consisting of 750 µL of 1:2(v:v) chloroform: methanol and 125

µL chloroform were added to each sample along with 250 µL of water. Cells were then

incubated for one hour at -20°C, followed by centrifugation at 100 x g for 10 minutes at 4°C in

order to facilitate the formation of an upper aqueous phase, a lower organic phase and a protein

disk between the two phases. Care was taken to separate both aqueous and organic phases into

centrifuge tubes, leaving the protein disk undisturbed in the original tube. All samples were then

dried in a CentriVap concentrator and stored at -80°C until MS analysis. Storage varied

according to the trial and instrument availability.

Mass Spectrometry

A bicinchoninic acid assay(BCA) was performed on protein pellets to normalize between

samples. Subsequently, samples were resuspended in 35% acetonitrile in water at a volume

commensurate with protein concentration. A 5 µL volume was injected into the Micro200

LC(Eksigent, Redwood, CA, USA) equipped with a hydrophilic interaction liquid

chromatography(HILIC) column(Luna 3µ NH2 100Å, 150mm×1.0mm, Phenomenex, Torrance,

CA, USA). Water and acetonitrile, each with 5 mM ammonium acetate, were used for mobile

phase A and B, respectively(Taraboletti et al., 2016). The HPLC gradient ran at a flow rate of 30

µL/ min as follows: 0 min 98% B, 1 min 95% B, 5 min 80% B, 6 min 46% B, 13 min 14.7% B,

17 min 0% B, 17.1 min 100% B, 23 min 100% B(Taraboletti et al., 2016).

Following separation, samples were injected into an AB SCIEX 5600+ TripleTOF mass spectrometer. The ion source nebulizer gas set to 15 psi, heater gas was set to 20 psi, and curtain gas was set to 25 psi. Positive mode samples were collected with an ionspray voltage of +5000 V and a +100 volt declusteirng potential(Taraboletti et al., 2016). A collision energy spread of +25-40V was used to collect fragmentation data on all candidate ions(Taraboletti et al., 2016).

Shotgun Lipidomics

Extracted organic phase metabolites were resuspended in a methanol:chloroform:water(v:v:v, 45:45:10, 5 mM ammonia acetate) mixture and then placed in an LC vial. Lipid samples were injected at a flow rate of 10 µL/min. An MS/MS^{ALL} method was employed for the lipid analysis using a scan range of 200-1,200 Da with an accumulation time of 300 ms(Simons et al., 2012). The method was run once in positive mode and once in negative mode(Taraboletti et al., 2016).

MTT Assay

An MTT assay was performed on mouse mixed glial cultures to determine the effect of rolipram on cell viability. Glial cells were plated at a concentration of approximately 1.0×10^4 cells per well for a period of three days to allow the cells to adhere and populate. Cells were then treated with i) rolipram alone, ii) 5 µM rolipram and 100 ng/mL LPS, iii) 100 ng/mL LPS, or iv) 100 ng/mL LPS with varying concentrations of rolipram. For the last treatment group containing varying concentrations of rolipram with constant LPS, rolipram was added at concentrations ranging from 20 µM to 40 nM. The plate was then left to incubate for an additional 24 hours after which 5 mg/mL MTT was added. Samples were incubated with MTT for 4 hours, lysed in buffer containing 4 mM HCl, 0.1% NP40 in isopropanol, and the plate was read at 590 nanometers with a Spectromax M2 plate-reader(Molecular Devices, Sunnyvale, CA, USA).

Bioinfomatics

Peak picking and retention time alignment was performed by using Markerview software(AB SCIEX). After importing data from wiff files generated from the mass spectrometer, PCA analysis was performed to identify differences between groups. Elements software(Proteome Software, Inc) was used to compare vehicle-treated versus rolipram-treated cells, identify metabolites based on MS and MS/MS data, and determine features that were significantly different between the two. Identification was based on matching MS and MS/MS data to entries in the publicly-available metabolite databases, the Human Metabolome Database(HMDB, http://www.hmdb.ca/), Metlin(https://metlin.scripps.edu/), and the National Institute for Standards and Technology(nist.gov). Differences in the relative levels of metabolites were determined based an adjusted p value < 0.05 and Log2 fold change of greater than 2 or less than -2. Pathway analysis was performed by using the online software package,
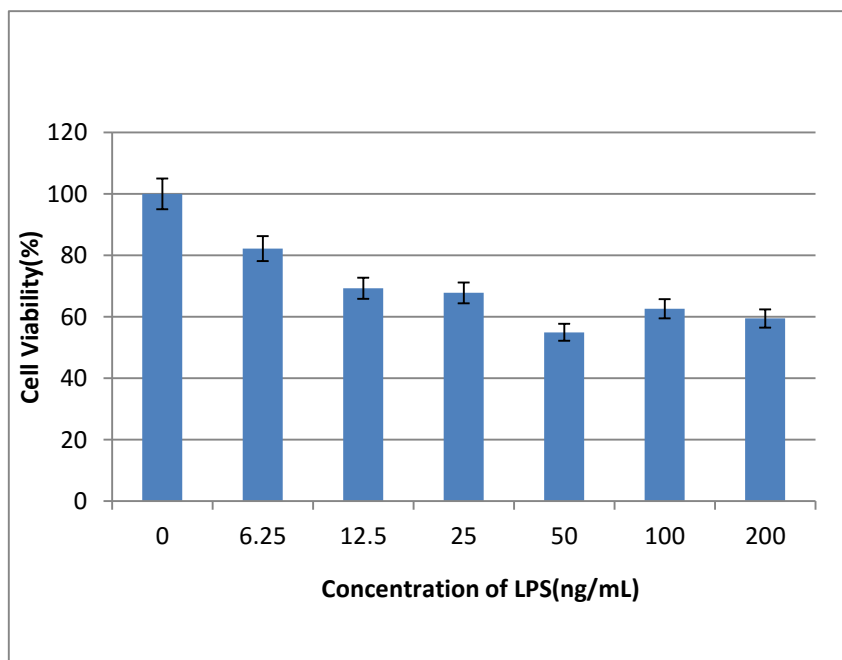

Figure 3: Determination of the viability of mixed glial cells treated with LPS. Mixed glial cells were treated with LPS at increasing concentrations and viability was determined with MTT assay. N=3 cell cultures for each concentration. Kruskal-Wallis(One Way ANOVA) analysis demonstrated the difference in viability to be significant between the treatment groups and the zero LPS concentration treatment.

MetaboAnalyst(www.metaboanalyst.ca). Lipidomic data was analyzed with LipidView software

(AB Sciex).

Results

This project focused on studying the effect of the anti-inflammatory agent, rolipram, on the global metabolic responses of peripheral macrophages and CNS resident microglial cells. We first wanted confirm that our drug concentrations were within a sufficient range and so we performed an
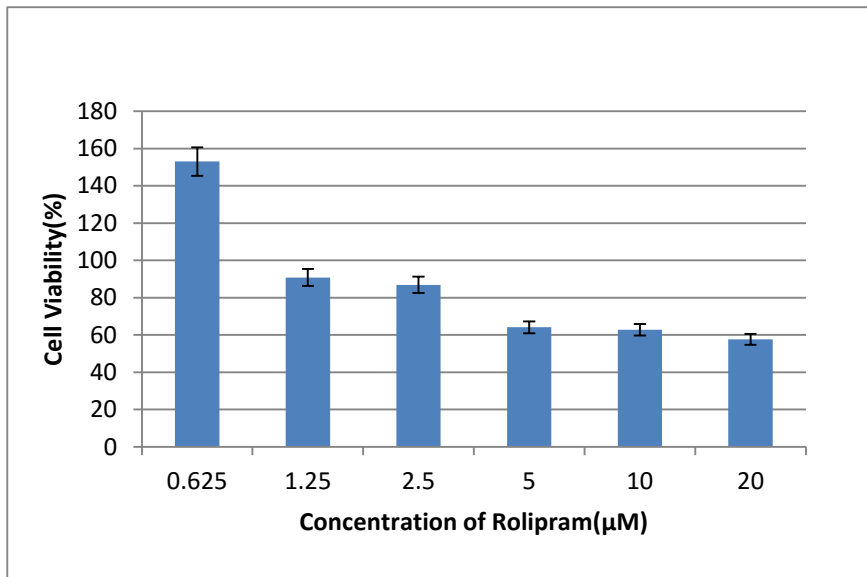

Figure 4: Determination of the viability of mixed glial cells treated with rolipram. Mixed glial cells were treated with rolipram at increasing concentrations and viability was determined with MTT assay. N=3 cell cultures for each concentration. Kruskal-Wallis(One Way ANOVA) analysis demonstrated the difference in viability to be significant between the 0.625 μM concentration and the higher concentration groups.

MTT assay to verify that treatment of primary glial cells did not influence viability

substantially(Figure 3). We also examined whether rolipram differentially influenced viability

following stimulation of the cells with the inflammatory molecule LPS. LPS acts through the

toll-like receptor 4 pathway leading to cellular proliferation and release of inflammatory

cytokines by immune cells.(Chow et al., 1999). In these experiments we used LPS as our

prototypic inflammatory stimulus. We treated the primary glial cells with concentrations of LPS

ranging from 6.25 ng/mL to 200 ng/mL and performed MTT to examine cell viability(Figure 3).

There was a drop in viability after LPS treatment at the higher concentrations that was surprising

since 100 ng/mL is commonly used to activate glial cells in other studies(Chen et al., 2012). Rolipram treatment alone also induced a decrease in cell viability at concentrations of 5 μM and higher(Figure 4). However, activation of glial cells with LPS, followed by rolipram
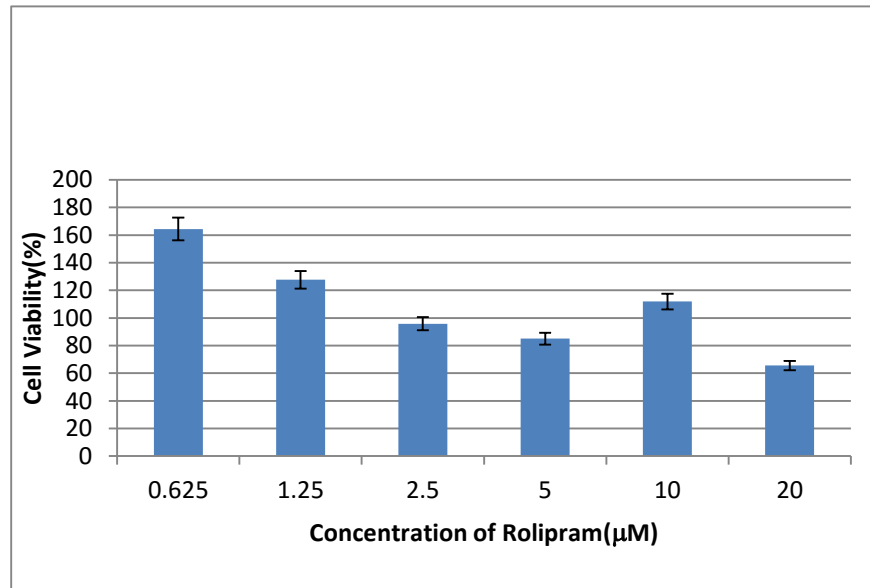

Figure 5*: Determination of the viability of mixed glial cells treated with rolipram at constant LPs concentration(100ng/mL). Mixed glial cells were treated with LPS at a constant concentration and rolipram at increasing concentrations and viability was determined with MTT assay. N=3 cell cultures for each concentration. Kruskal-Wallis(One Way ANOVA) analysis demonstrated the difference in viability to be significant between the 0.625 μM concentration group and the 2.5, 5, and 20 μM concentration groups.*

treatment showed cell loss only at the highest concentration(20 μM) while the lowest concentrations(0.625μM) showed some evidence of proliferation. The combination of 100 ng/mL of LPS with 5 μM rolipram had viability measurements close to 100% and this indicated that rolipram was perhaps suppressing the proliferation seen at the lower concentrations. Based on this data, we chose the combination of 100 ng/mL of LPS with 5 μM rolipram for our metabolomic studies.

In order to identify potential anti-inflammatory pathways activated by rolipram treatment, we performed a global metabolomic screen of rolipram-treated versus vehicle-control THP-1 cells and glial cells. THP-1 cells would serve as our model of peripheral macrophages while testing primary glial cultures would allow us to determine any CNS-specific effects. We

performed HILIC-MS on THP-1 cells treated with rolipram or vehicle(DMSO) followed by principal component analysis(PCA) to identify global perturbations in metabolism induced by this drug. PCA showed significant separation of the feature detection profiles between the two groups(Figure 6). 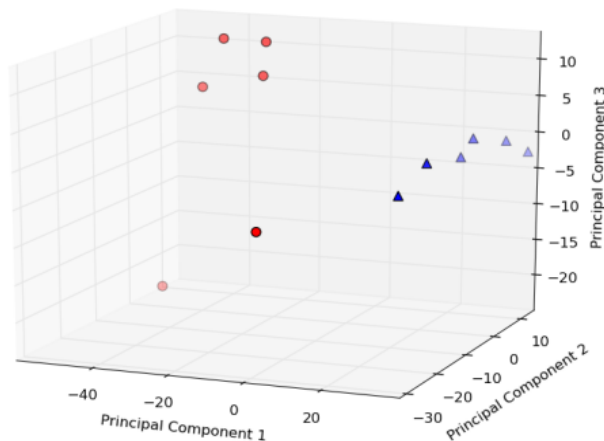The results of this test indicate that metabolic perturbations may play a role in cellular responses to rolipram. In fact, we detected approximately 818 features

Figure 6: *Principal Component Analysis(PCA) comparing rolipram-treated cells (blue) versus vehicle controls (red). Separation of the two groups is observed after PCA indicating that rolipram induced alterations in the metabolome of THP-1 cells.*

Table 2: *A summary of feature and metabolite identification for THP-1 trial 2.*

Total features found	Total significant and dysregulated features	Total Positive IDs with fold change >2 or <-2	Total Positive IDs with fold change of ∞ or -∞
6777	524	37	7

that were significantly upregulated or downregulated by rolipram treatment in the THP-1 cells out of 8094 detected features($p < 0.05$, Table 1). Of these changes, we could positively identify

18 metabolites with fold changes greater than or less than 2 and -2 as well as a set of metabolites that were only in one cell population(either control or rolipram-treated with infinity fold change).

Based on the first trial with THP-1 cells, we performed a second experiment in order to determine whether the metabolic changes observed were reproducible. In this second experiment we identified 6777 features in THP-1 cells by HILIC-MS(Table 3). Of those detected peaks, statistical analysis identified approximately 524 peaks that were significantly altered by rolipram treatment compared with vehicle-control cells. HMDB searches identified approximately 44 metabolites, which were confirmed by matching MS/MS fragmentation data to database standards(Table 4).

We next investigated pathways that were affected by this anti-inflammatory compound. The bioinformatics software, Metabolyzer, was used to make putative identifications based on m/z values obtained in the two replicate profiling experiments. These identifications were used to create pathway maps for related metabolites. Arginine and proline metabolism yielded the greatest number of dysregulated metabolites by putative identification with
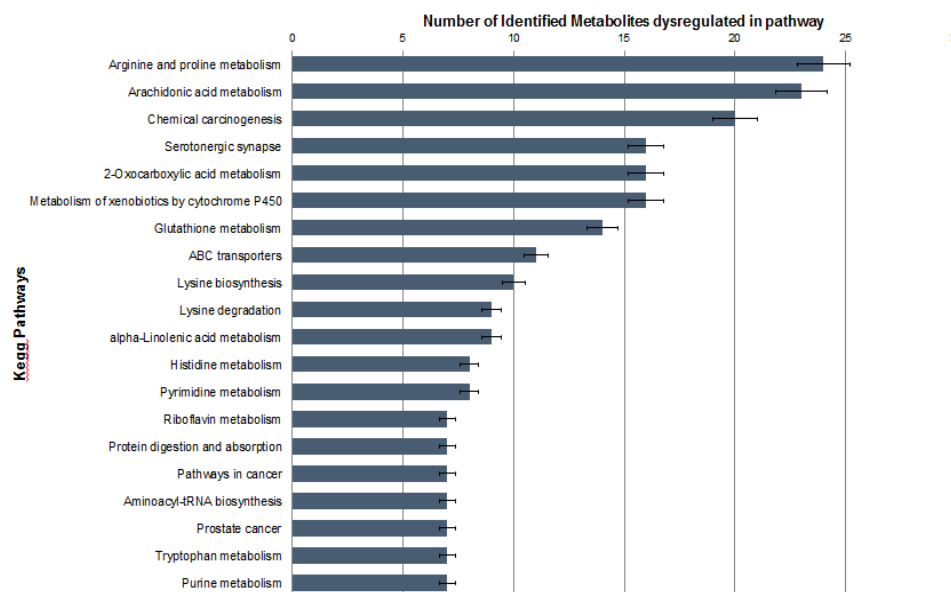

Figure 7: *Pathway analysis on putative identifications, matched by Kegg ID using Metabolyzer, after inputting retention time and mass to charge ratios.*

smaller numbers of metabolites identified in arachidonic acid metabolism, chemical

carcinogenesis, and serotonergic synapse response(Figure 7).

We sought to validate our pathway analysis by confirming the metabolite identifications

performed by Metabolyzer. Metabolite identification involves searching publicly available

databases for matches based on m/z values. These matches are then confirmed by comparing

fragmentation patterns obtained experimentally to those obtained with standards. We focused on the metabolites contained in the Human Metabolome Database(HMDB) and National Institue for Standards and Technology(NIST) to provide putative identifications since this database contains a relatively comprehensive catalog

Table 3: *Metabolites altered by rolipram treatment in THP-1*

Metabolite	P-Value	Fold Change(Log2)
13'-hydroxy-gamma-tocotrienol	0.0380	6.81
4alpha-carboxy-5alpha-cholesta-8-en-3beta-ol	0.0040	2.53
Adenosine	0.0030	-4.86
2-Pyrrolidone-5-carboxylic acid	0.0260	∞

Table 4: I*dentified metabolites regulated by THP-1 treatment replicate.*

Metabolite	P-value	Fold Change
Betaine	0.0010	3.09
Valerylcarnitine	0.0160	2.96
Niacinamide	0.0002	3.02
Carnitine	0.0100	3.11
Creatinine	0.0010	3.15
Butyrylcarnitine	0.0300	2.58
Cytidine	0.0001	5.02
L-Arginine	0.014	∞

of mammalian metabolites(Wishart et al., 2007).   These searches identified approximately 20 metabolites that were confirmed by matching MS/MS fragmentation patterns to database standards(Table 2).   Metabolites that were xenobiotic or plant-derived were excluded. Positively identified metabolites found in the human metabolome are listed in Tables 3 and 4 alongside their respective p-values and fold changes.

In the first experiment examining rolipram-treated THP-1 cells, 3'-hydroxy-gamma-tocotrienol and 4-alpha-carboxy-5alpha-cholesta-8-en-3beta-ol were found to be upregulated but identified only putatively, while adenosine was found to be downregulated and positively identified by spectra matching. 13'-hydroxy-gamma-tocotrienol is a vitamin E derivative and 4alpha-carboxy-5alpha-cholesta-8-en-3beta-ol functions as an intermediate in cholesterol biosynthesis. Additionally, 2-Pyrrolidone-5-carboxylic acid, a metabolite identified as an amino acid derivative that is a component of glutamine/glutamate metabolism was only present in the rolipram treated group and positively identified by MS/MS matching.

Several metabolites increased by rolipram treatment in the second trial were amino acid or amino acid derivatives, including arginine and the osmolyte betaine(Figure 8 and Table 4). In addition, metabolites involved in the β-oxidation of fatty acids and branched chain amino acids, carnitine, valerylcarnitine, and butyrylcarnitine are significantly increased in THP-1 cells after incubation with rolipram. Finally, we observed alterations in the nucleoside, cytidine, which increased upon rolipram treatment.

Figure 8: *The osmolyte betaine is increased by rolipram treatment. Box and whisker plot of betaine from the global profiling experiment. N= 6 were analyzed after 24 hours of treatment.*

We compared both trials to identify metabolites that are shared among both sample sets in order to determine the reproducibility of the metabolite changes. L-arginine was identified in both sets of experiments; however, it did not make the p-value cutoff in the first experiment.

Vitamin E and cholesterol both play a role in membrane biology; therefore, we used shotgun lipidomics to determine whether rolipram might mediate changes in lipid metabolism that could influence proliferation or inflammatory responses.
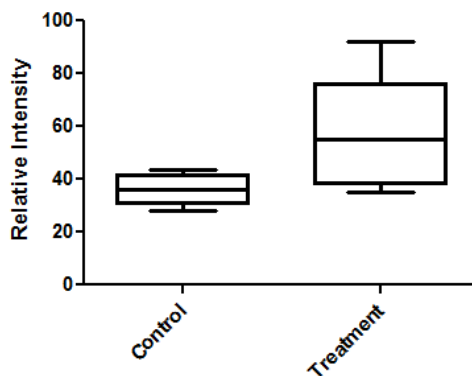
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Shotgun lipidomics was used to profile multiple lipid classes obtained after the Bligh-Dyer extraction and a number of lipid classes were found to be dysregulated (Figure 9). Specific classes upregulated in the experiment were phosphatidylcholines (PC), phosphoglycerols(PG), and phosphatidylserines(PS) In contrast, phosphatidic acid(PA) was downregulated in the treatment group.

Our metabolomic data indicate that rolipram could induce alterations in a macrophage cell line; however, microglial cells are also a significant component of the innate immune response in the CNS. Although these cells display many phenotypic similarities to macrophages, they also are thought to have a

Lipid Class Profiles

Figure 7: Shotgun lipidomic profiling of THP-1 cells. THP-1 cultures were treated with rolipram (5 μM) or DMSO vehicle for 24 hours followed by lipid extraction. Lipids were detected by an MS^{all} method based on accurate mass and fragmentation data. Lipid Classes were graphed by ratio of intensity in treatment vs control group, and each class was graphed only in the mode (positive or negative) in which it was shown to ionize best in. N= 6 cultures examined for each treatment condition.

unique response to inflammatory stimuli such as differential upregulation of immune markers like MHC(Xu, Ling, 1994). To identify microglial-specific responses to rolipram, we cultured primary mouse microglia, treated them with rolipram for 24 hours, and performed global metabolomic profiling followed by bioinformatics analysis with the Elements program.
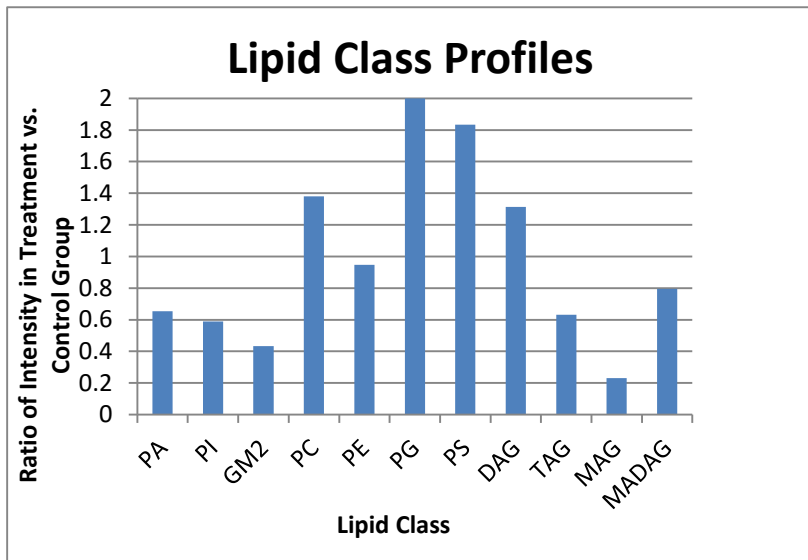
We identified 6946 features in the microglia cells by HILIC-MS. Of those detected peaks, statistical analysis identified approximately 322 peaks that were significantly altered by rolipram treatment compared with vehicle control cells(Table 5). Those m/z values were matched to metabolites in the Human Metabolome Database(HMDB) to provide putative identifications. No positive identifications were matched by spectra, but putatively identified metabolites found in the human metabolome are listed in Table 6, listed alongside their respective p-values and fold changes.

Table 5: *Metabolic perturbations induced by rolipram in primary microglial cell cultures*

Total features found	Total significant and dysregulated features	Total Positive IDs with fold change >2 or <-2	Total Positive IDs with fold change of ∞ or -∞
6946	322	17	5

Table 6: *A chart displaying all putatively identified endogenous metabolites for microglia trial 1 that are found in the human metabolome, alongside fold change and p-value.*

Metabolite	P-Value	Fold Change
S-(3-Methylbutanoyl)-dihydrolipoamide-E	0.0060	-4.99
11 beta,18,21-Trihydroxypregn-4-ene-3,20-dione(18-Hydroxycorticosterone) fold changes	0.0050	-3.04

S-(3-Methylbutanoyl)-dihydrolipoamide-E is an intermediate in valine, leucine, and isoleucine degradation. 11 beta,18,21-Trihydroxypregn-4-ene-3,20-dione(18-Hydroxycorticosterone) is a steroid of the 21-hydroxysteroid class. Both these metabolites were significantly downregulated by rolipram treatment, but their identifications were only putative, as no spectra could be matched in a database. Our previous results indicated that rolipram treatment perturbed multiple classes of lipids in THP-1 cells. We sought to determine if microglial cells also altered their lipid profile after incubation with rolipram. We found several classes of lipids differentially regulated by rolipram, including sphingomyelins(SM,) phosphatidylserines(PS,) diacylglycerols(DAG,) phopsphatidylglycerols(PG,) ceramides(Cer,)

phosphatidlycholines(PC,) and phosphatidylserines(PS) For most of the lipid classes rolipram induced upregulation; however, Cer and PE species were downregulated. Of note, PS were found to be higher in the rolipram-treated versus control groups in both the microglial and THP-1 groups, showing an intensity ratio of 1.83 and 4.48 in THP-1 and microglia trials, respectively(Figures 7,9).
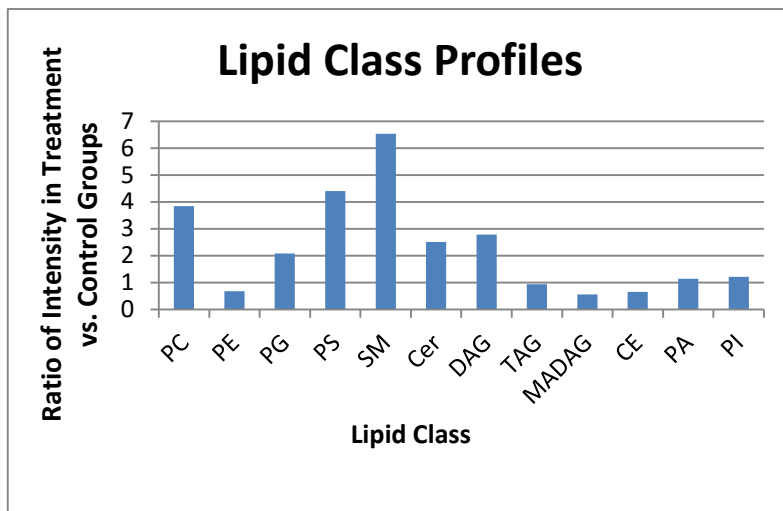

Figure 9: *Shotgun lipidomic profiling of microglial cells. Microglia1 cultures were treated with rolipram (5 μM) or DMSO vehicle for 24 hours followed by lipid extraction. Lipids were detected by an MS[all] method based on accurate mass and fragmentation data. Lipid Classes were graphed as a ratio intensity between treatment and control groups, and each class was graphed only in the mode (positive or negative) in which it was shown to ionize best in. N= 6 cultures examined for each treatment condition.*

Discussion

Rolipram is the prototypic PDE-4 inhibitor and treatment of cells with this compound leads to increases in cAMP, a pleiotropic signaling molecule. It is likely that the anti-inflammatory effects of this drug could result from the activation or suppression of multiple signaling pathways; therefore, we used global metabolite profiling to identify biochemical alterations induced by this drug. In order to capture metabolite alterations induced by rolipram, we first had to determine a concentration range of the drug that would maintain cell viability in the presence and absence of inflammatory mediators like LPS. Our MTT assays indicated that rolipram had some influence on viability at high concentrations. Though no other studies could be found investigating the effect of rolipram on macrophage viability, previous studies have tested cell viability after 24 hours of rolipram treatment. Treatment of mixed glial cells with

concentrations ranging from 10^{-7}–10^{-3} M did not have any effect on cell viability(Leseca-Espinose et al., 2003). This somewhat conflicts with our findings; however, these differential responses may be due to cell phenotype.

Since monocytic cells are one target for rolipram treatment, we examined the metabolic response of macrophage cell line, THP-1, to this drug. PCA analysis indicates that rolipram treatment perturbed global metabolism as seen by the separation of treated versus control groups on the plot. We then identified the metabolic pathways that contributed to these differences. Overall, 818 features were significantly changed by treatment, but we could only positively identify 20 metabolites based on accurate mass and fragmentation data. The other features detected by the mass spectrometry could be true unknowns, metabolites not contained in Metlin or HMBD, or metabolites that did not have sufficient fragmentation data to identify. Pathway analysis that identified metabolites based on accurate mass suggested several metabolic pathways targeted by rolipram. Chief among these was arginine and proline metabolism. Arginine was also positively identified as being dysregulated in both sets of THP-1 trials. Arginine is one of the primary reactants in NO synthesis, and thus its dysregulation may indicate that rolipram not only suppresses cytokine signaling leading to reduced NO production, but may also alter the precursors to NO synthesis. Future directions for the project include targeted metabolomics to quantify levels of arginine as well as citrulline and other urea cycle intermediates.

In THP-1 cells, betaine was upregulated with a Log2 fold change of 3.09, which is not only significant but of considerable interest to this project. Betaine has been previously characterized as an osmolyte and is able to inhibit NO release by LPS-activated microglia through an unknown mechanism(Amiraslani et al., 2012). While this compound may influence

NO production directly, betaine is also involved in the regeneration of homocysteine to methionine. Homocysteine is implicated in promoting an NO-secreting M1 phenotype in macrophages(Winchester et al., 2015). Therefore, upregulation of betaine may directly influence the activity of NO synthase or alternatively prevent activation of this enzyme by decreasing levels of homocysteine through its regeneration to methionine.

We identified fewer numbers of metabolites in microglial cells due to the limited amount of MS/MS spectrum collected on the primary cells, and both of our identifications were only putative, since there were no spectra to match to in databases for both putative identifications. However, matches were obtained for two metabolites, which could possibly later be confirmed by matching to standards. 18-Hydroxycorticosterone and S-(3-Methylbutanoyl)-dihydrolipoamide-E. 18-hydroxycorticosterone is an intermediate in aldosterone synthesis, which is mainly performed by the adrenal gland. Aldosterone can have pro-inflammatory effects; however, it is unclear whether this metabolite may be used in another pathway in microglial cells(Gilbert, Brown, 2014). Increases in S-(3-Methylbutanoyl)-dihydrolipoamide-E has been previously linked with the development of sepsis(Su et al., 2014). It remains to be determined whether a decrease in this metabolite in microglial cells is associated with a reduction in inflammatory responses.
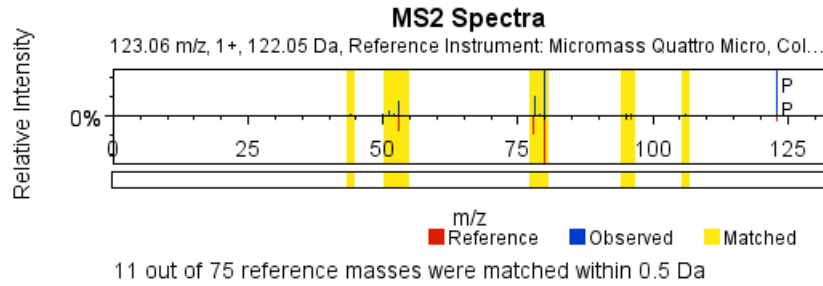
Shotgun lipidomics produced varying results by trial, but several lipid classes were upregulated in rolipram-treated THP-1 cells compared to controls. Phosphatidylserines(PS) were found to be upregulated in rolipram-treatment groups. Interestingly, PS has been shown to be an activator of the rolipram-sensitive PDE-4(Nemoz et al., 1997). It may be that the upregulation of PS is an attempt to overcome the rolipram-induced inhibition of this enzyme. Ceramide species also show increases after rolipram treatment in microglial cells. Increases in ceramide levels in

microglia occur after LPS treatment(Akundi et al., 2005). It has been shown that upregulation of certain ceramides(C2,C8) catalyzes anti-inflammatory effects on LPS-activated mouse microglial cells, which include inhibition of NO synthase and the inhibition of the release of other pro-inflammatory cytokines(Jung et al., 2013). This result indicates that rolipram has some potential to influence levels of these lipids during inflammation. While multiple lipid species change, there appears to be some specificity in lipid alterations. PC and PG species increase while DAG and MAG species do not change. How this influences cell function remains to be determined. It has been previously shown that inhibition of cAMP breakdown upregulates other activators of PDE-4, such as PA in lymphocytes(Nemoz et al., 1997). However, upregulation of PA was not seen in this experiment, potentially due differences in cell responses to this drug(macrophages versus lymphocytes). This could be an area of future investigation.

Our metabolomic studies have identified several putative metabolic pathways that promote the anti-inflammatory function. The inhibition of NO release from macrophages by rolipram has been shown to result from inhibition of cAMP breakdown and reduced transcription of NO synthase(Beshay et al., 2001). Our results suggest that rolipram might also influence the availability of amino acid precursors such as arginine used to synthesize NO. Additionally, we saw a decrease in metabolites associated with inflammatory responses, including branched chain amino acid derivatives and lipids such as ceramides. This study highlights the power of metabolomics to identify pathways important for drug action. Future work will focus on quantifying metabolite changes as well as investigating the downstream effects of these metabolite alterations.

Appendix

Supplemental Figures

MS2 Spectra
123.06 m/z, 1+, 122.05 Da, Reference Instrument: Micromass Quattro Micro, Col...

11 out of 75 reference masses were matched within 0.5 Da

Supplemental Figure 1- *MS/MS spectra match for metabolite identification. The workflow for metabolite identification involves matching m/z values detected in the mass spectrometry with standards deposited in the Human Metabolome Database. Metabolite identifications that match the experimental m/z value are subsequently validated by matching MS/MS patterns with the standards.*

Tumor Necrosis Factor-Alpha ELISA

Mixed Glial TNF-α
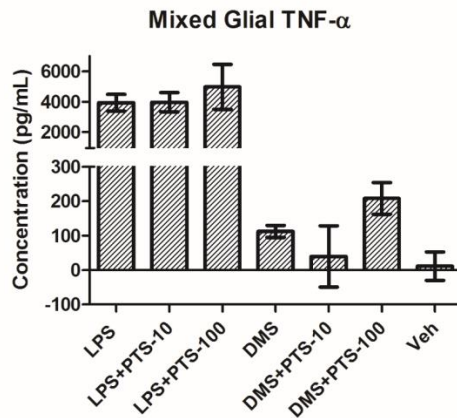
Supplemental Figure 2- *TNF release from glial cultures in response to inflammatory mediators.*

We sought to determine whether rolipram treatment could alter the release of TNF from glial cells in order to confirm that our drug treatment was sufficient to inhibit cAMP breakdown. In order to test the TNF ELISA kit we first examined TNF release after stimulation of the cells with known inflammatory molecules LPS and the lipid dimethylsphingosine (DMS). We treated mixed glial cultures with LPS and DMS in the presence or absence of the GPCR signaling inhibitor pertussis toxin. Both LPS and DMS induced TNF release compared to vehicle control, consistant with the roles of these inflammatory mediators. However, when cells were treated with LPS or DMS plus pertussis toxin, TNF release was maintained. Additional DMS + pertussis toxin increased TNF release. These results indicate that TNF measurements by ELISA would be sufficient to detect inflammatory activation. Due to time limitations, I was unable to test rolipram-treated cultures.

Safety

All procedures were conducted under the supervision of Dr. Shriver or one of the several graduate students in the laboratory workspace- at least one of these persons was present any time work was being conducted to advise on proper safety and experimental procedure. Nitrile gloves were worn at all times in the laboratory. Lab aprons, and protective eye and face-wear were worn when preparing and handling paraformaldehyde. DMSO and other organic solutions were disposed of in the properly marked waste containers. Biohazard materials such as pipette tips, flasks, and plates were disposed of in the properly marked biohazard waste disposal containers. Biohazard liquids were properly neutralized, with application of bleach or ethanol, before disposed of.

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