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14. ABSTRACT
This project aims to develop synthetic multifunctional compounds as therapeutics for polycystic kidney disease (PKD). In collaboration with the Somlo group at Yale University, we have already shown that two parent compounds, 11β-dichloro and 11β-dipropyl, are effective at preventing and delaying cystic growth in two different mouse models of PKD. One arm of the project focuses on the synthesis of new molecules from the 11β family, which will inform, through a structure-activity study, the key molecular features required for activity and provide additional hints about the mechanism of action. A second arm of the project focuses on the development of a cell culture model that can be used to screen the new molecules for improved efficacy and selectivity; such molecules will be then validated in the established PKD mouse models and pave the way towards their preclinical and clinical development. During the last funding period, we continued the work on validating cell culture models that recapitulate the biological consequences of 11β compounds. We focused on the mouse kidney line IMCD3, which showed good promise for toxicity assays. However, to capture the selectivity of 11β compound for PKD1 null cells, a ratiometric assay was necessary (MultiTox-Glo), which measures both the fraction of live and dead cells. We also investigated aspects of the mechanism of toxicity in the IMCD3 cell culture model and showed that 11β toxicity can be attenuated by the antioxidant vitamin E. Together with our collaborators at Yale University, we also measured the transcriptional responses to 11β of a panel of oxidative stress inducible genes. The Yale team also continued their work probing the mechanism of toxicity of 11β compounds in animals (biomarkers of oxidative stress) and completed the testing of 11β-dipropyl compound in the adult mouse model of PKD.

15. SUBJECT TERMS
Polycystic kidney disease, ADPKD, PKD1, polycystin1, therapeutic, apoptosis, mitochondria, reactive oxygen species, antioxidant, vitamin E

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1. INTRODUCTION

This project aims to develop synthetic multifunctional compounds as therapeutics for polycystic kidney disease (PKD). In collaboration with the Somlo group at Yale University, we have already shown that two parent compounds, 11β-dichloro and 11β-dipropyl, are effective at preventing and delaying cystic growth in two different mouse models of PKD. To guide the development of new compounds, throughout this entire project, we have been investigating the mechanism by which 11β compounds achieve their efficacy and selectivity against cystic cells. One arm of the project focuses on the synthesis of new molecules from the 11β family, which will inform, through a structure-activity study, the key molecular features required for activity and provide additional hints about the mechanism of action. A second arm of the project focuses on the development of a cell culture model that can be used to screen the new molecules for improved efficacy and selectivity; such molecules will be then validated in the established perinatal and adult-onset ADPKD mouse models and pave the way towards their preclinical and clinical development.

During the last funding period, we continued the work on characterizing cell culture models that recapitulate the efficacy and selectivity of 11β compounds seen in animals. The experiments focused on IMCD3 cells, and their PKD null isogenic counterpart. Together with our collaborators at Yale University, we also focused on investigating the mechanism of toxicity of 11β compounds in cell culture and animals. Several completed mechanistic studies demonstrate the role of oxidative stress as a key effector of 11β-induced toxicity and developed a better understanding of the basis of selectivity of 11β compounds towards cystic cells. We also accumulated more extensive data about the transcriptional responses to 11β compounds in tissues. Finally, together with our collaborators, we completed one of the overarching goals of this project, which is the testing of the 11β-dipropyl compound in the adult-onset mouse model of PKD.

2. KEYWORDS

Polycystic kidney disease, cystic disease, ADPKD, *PKD1*, *PKD2*, therapeutic, polycystin-1, apoptosis, mitochondria, reactive oxygen species, unfolded protein response, mouse model.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Our major goals were:

- Improve cell culture models for PKD to perform mechanistic studies on 11β toxicity and selectivity and examine the therapeutic role of oxidative stress in cell culture; extend findings to mouse models of PKD
- Record transcriptional responses of oxidative stress-inducible genes in both mouse models
- Complete mechanistic studies on the mechanism of action of 11β-dipropyl
- Complete testing of 11β-dipropyl in the adult-onset PKD mouse model

What was accomplished under these goals?

a. Developing a better cell culture model for testing efficacy and selectivity of 11β compounds. (Essigmann, MIT and Somlo, Yale)

Cell culture models for PKD remain a poor substitute for animal models and for the human disease. However, cell culture models are desperately needed to enable more efficient screening of potential therapeutics and understanding of disease mechanism. Our previous report described the use of the LLC-PK1 (ATCC CL-101) cell line, derived from pig kidney. While it showed potential for in vitro screening, being from a different mammalian species than our PKD animal models (which are mouse models) added an additional variable (and experimental challenge) for mechanistic studies. Therefore, during the last funding period, we investigated a mouse cell line for a cell culture model of PKD.

The mIMCD-3 (ATCC CRL-2123) is a murine cell line derived from the inner medullary collecting duct (IMCD) of a SV40 transgenic mouse (1). Despite being an SV40 transformed cell line, and triploid for most chromosomes, IMCD-3 cells retain many characteristics of the kidney epithelia, including polarization and the ability to form tight junctions. They are also capable of withstanding high osmotic stress, which is a physiological occurrence in the kidney collecting ducts (1). The Yale team employed a CRISPR-Cas9 technology to generate a PKD1 null isogenic counterpart to the wild-type IMCD-3 cells procured from ATCC, and both cell lines were subsequently studied in cell culture by the MIT team.

Preliminary studies with the IMCD3 cell lines indicated that they grow well in cell culture and that, unlike LLC-PK1 cells, the viability of the IMCD3 cells can be estimated consistently with several different methods, such as cell counting, viability dyes (e.g., CellTiter-Blue, MTT) and metabolic endpoints (e.g., total ATP concentration via CellTiter-Glo). The IMCD3 cells showed a robust response to both 11β-dichloro and 11β-dipropyl compounds (**Fig. 1**). After continuous exposure to compounds for 24 h, IC50 concentrations were 3-4 μM for both compounds.

However, the compounds' selectivity between WT and PKD1 null cells was less apparent in this isogenic cell line pair (**Fig. 1**).

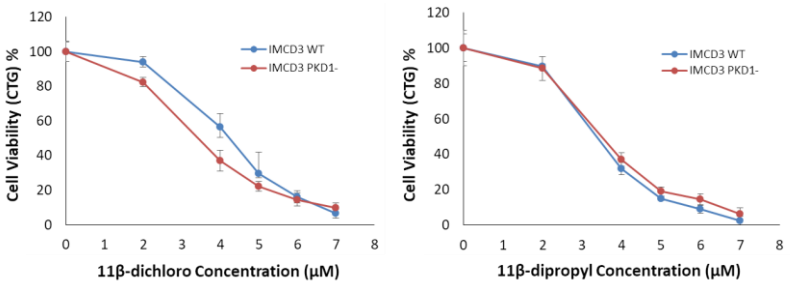

Figure 1. Dose responses of IMCD3 cell lines (WT and PKD1 null) to 11β compounds (11β-dichloro and 11β-dipropyl). Viability estimated using total ATP concentration (CellTiter-Glo).

One possible explanation for the lack of selectivity observed in this model is the fact that, although isogenic, the two IMCD3 cell lines have different growth kinetics. Therefore, it is extremely challenging to insure that the same number of WT and PKD1 null cells is exposed to the drug over the 24 h exposure. One way to address this challenge is to take into account, in our dose response analyses, not only the remaining viable cells, but also the fraction of cells that have died due to drug exposure. An efficient way to perform this analysis is to use the MultiTox-Glo assay, which independently estimates the relative numbers of live and dead cells, by measuring the activities of a live-cell protease and a dead-cell protease, respectively (2). To provide robust estimates, the activities are measured with orthogonal methods; the live-cell protease cleaves off a fluorogenic substrate, whereas the dead-cell protease generates a luminescent signal. Finally, by taking a normalized ratio between the live-cell and dead-cell signals, a much better viability metric is obtained, which is independent of cell number and minimizes the false-positive and false-negative results associated with only one type of measurement.

We repeated the dose-response analyses in the IMCD3 cells using the MultiTox-Glo assay. Owing to the higher sensitivity of the assay, the selectivity of each of the 11β compounds was more apparent (Fig. 2). Both 11β-dichloro and 11β-dipropyl killed IMCD3 PKD1 null cells more readily, with the dose response curves showing IC50 values that were 1-1.5 μM lower than the corresponding IMCD3 WT IC50 values. This is an important breakthrough for these types of assays and the MultiTox approach is easily extendable to other cell lines (e.g., LLC-PK1). Nevertheless, these cell culture models are still far from capturing the exquisite selectivity of the 11β compounds observed in animal models. In the "future directions" section, we outline several new approaches that may help bridge this gap and further improve the usefulness of cell culture models for PKD.

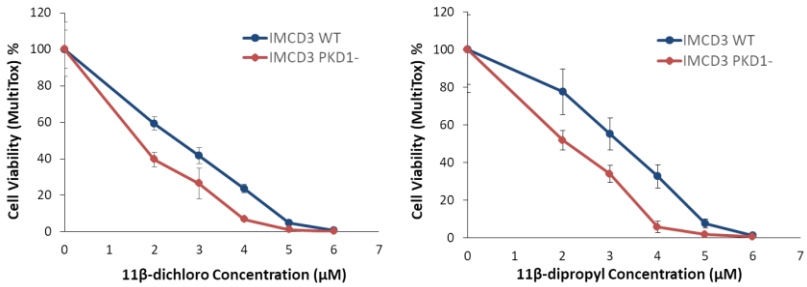

Figure 2. Dose responses of IMCD3 cell lines (WT and PKD1 null) to 11β compounds (11β-dichloro and 11β-dipropyl). Viability estimated using ratiometric assay MultiTox-Glo, which incorporates independent estimates of both the fraction of live cells and the fraction of dead cell.

b. Examining the mechanism of toxicity of 11β compounds in tissues from neonate mouse model of PKD. (Somlo, Yale, and Essigmann, MIT).

Recent studies of ADPKD indicated that cystic cells, akin to certain cancer cells (3,4), harbor fundamental mitochondrial abnormalities (5,6), which manifest through altered organelle morphologies and copy number, decreased respiration and a leaky electron transport chain (ETC) that leads to the excessive formation of reactive oxygen species. It has been argued (7) that the mitochondrial defects are upstream, and perhaps causative, of the metabolic reprogramming that is observed in cystic cells, such as increased glycolysis and decreased oxidative phosphorylation in the mitochondria. We previously showed that 11β compounds induce oxidative stress by localizing in the mitochondria and disrupting the flow of electrons through complex I of the ETC (8), an effect independent, in the case of 11β-dichloro, of the ability to alkylate DNA. Given the dysregulated oxidative metabolism and mitochondrial function of *Pkd1*$^{-/-}$ cells and tissues (5-7,9), the central hypothesis of this project has been that 11β compounds achieve selective killing of cystic cells by inducing oxidative stress (by targeting mitochondria), and the cystic cells are improperly equipped (in terms of antioxidant capacity and enzymes) to handle a burst of ROS. The studies outlined below provide experimental support to this hypothesis.

a.1. Induction of ROS in cystic cells exposed to 11β-dichloro. (Somlo, Yale)

In studies initiated in the last funding cycle, we investigated whether 11β induces ROS in cystic kidneys. Early *Pkhd1-Cre* cystic kidneys were harvested and stained for lipid peroxidation biomarker 4-hydroxynonenal (4-HNE), a common oxidative stress-induced cellular byproduct. Recent evidence suggests that the bulk of 4HNE in a cell is formed from the oxidation of mitochondria-specific phospholipid cardiolipin (10), and thus, it primarily reflects mitochondrial oxidative stress (11). The specificity of the anti-4-HNE antibody was established by treating IMCD3 cells (an established kidney cell line) with antimycin A (a known ROS stressor) and 11β-dichloro (**Fig. 3**); both compounds elicited a positive 4-HNE signal compared with DMSO treated cells.

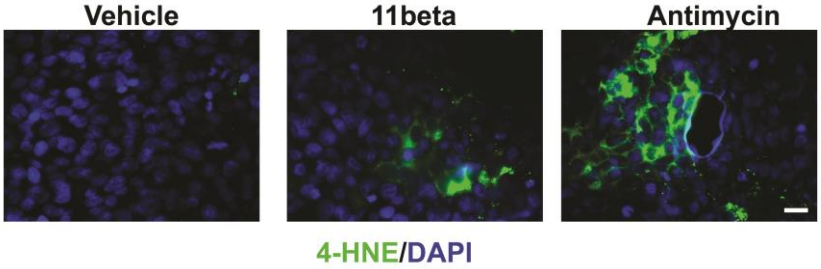

Figure 3. Detection of 4-HNE induced by 11β-dichloro treatment in cells. The green fluorescence denotes 4-HNE staining. The nuclei are stained with DAPI.

Next we investigated the status of 4-HNE in the 11β-treated mice. In the neonate model, the levels of 4-HNE were substantially increased in the cystic kidneys treated with 11β (**Fig. 4a**), but only in the DBA-positive cells (*Pkd1*$^{-/-}$ cells), suggesting a specific induction of oxidative stress in the cystic cells. By contrast, no 4-HNE signal was detected in wild-type (proximal tubules) epithelia in the 11β-treated kidneys, or in any of the vehicle-treated kidneys. To bolster these observations, we also probed for 8-oxoguanine, a DNA oxidative stress biomarker. Uncontrolled production of ROS leads to oxidative damage of all macromolecules in the cell, including nucleic acids. 8-Oxoguanine is the most prevalent oxidation product in DNA and RNA. Corroborating the 4HNE observations, cystic cells treated with 11β were displayed a strong 8-oxoguanine signal, which was absent in all other experimental conditions (**Fig. 4b**).

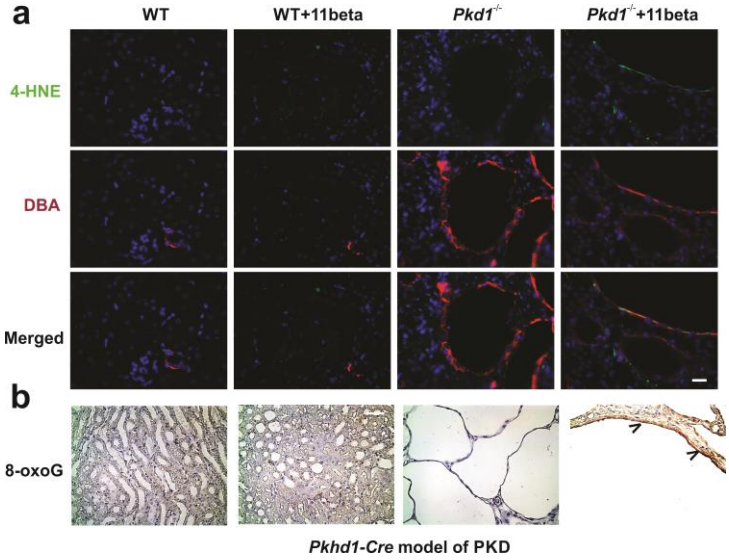

Figure 4. 11β-dichloro induces specific accumulation of the oxidative stress biomarkers 4-HNE (**a**) and 8-oxoguanine (**b**) in cystic cells of the neonate mouse model of PKD. DBA staining is characteristic for the collecting ducts that harbor the mutant cells.

a.2. Transcriptional effect to 11β-induced oxidative stress. (Essigmann, MIT and Somlo, Yale)

The above results are consistent with early observations (described in the proposal for this project) that 11β upregulates transcription of the oxidative stress genes catalase (CAT) and superoxide dismutase (SOD1) in cystic cells and tissues.

Corroborating evidence was recently obtained in our lab, where exposure of LLC-PK1 pig kidney cell lines to increasing concentrations of 11β-dichloro (2, 4, and 6 μM) for 6 hours resulted in a dose-dependent upregulation of transcripts associated with oxidative stress. Specifically, the levels of SOD1 (cytosolic superoxide dismutase), SOD2 (mitochondrial superoxide dismutase), GPX4 (glutathione peroxidase) and TOM20 (mitochondrial translocase) increased

Table 1. Oxidative stress inducible genes of interest.

Gene	Description
CAT	Catalase – breaks down hydrogen peroxide to molecular oxygen and water.
SOD1	Superoxide dismutase 1 (cytosolic) – converts superoxide to water and hydrogen peroxide
GLRX	Glutaredoxin – reduces oxidized glutathione
GPX	Glutathione peroxidase – reduces peroxides to water using glutathione
HMOX	Heme oxygenase – catalyzes breakdown of heme
Hif1a	Hypoxia-inducible factor 1 alpha – key subunit of a master regulator of the cellular response to hypoxia
PRDX	Peroxiredoxin – thioredoxin-dependent peroxide reductase; reduces peroxides

significantly after 6 hour exposure to 11β-dichloro. These cell culture experiments paved the way towards selecting a more comprehensive panel of oxidative stress-induced genes and designing efficient primers for mouse cell lines and tissues.

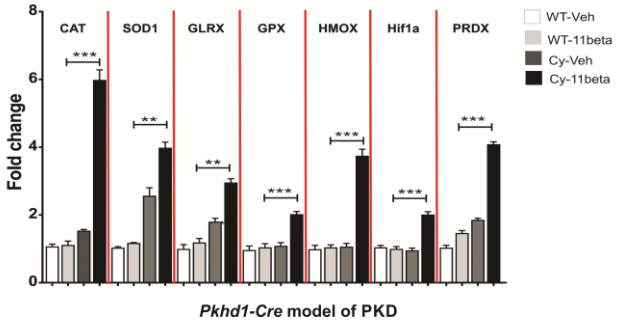

Figure 5. Transcriptional effect of 11β-dichloro on oxidative stress inducible genes described in Table 1.

Once the primer sets were synthesized and validated, the transcriptional responses to 11β-dichloro of a panel of oxidative stress induced genes (**Table 1**) were analyzed in whole kidney extracts from the neonate mouse model of PKD. Only cystic kidneys treated with 11β-dichloro showed statistically significant increases in the mRNA levels of oxidative stress inducible genes CAT, SOD1, GLRX, GPX, HMOX, Hif1a and PRDX; by contrast, 11β-treated wild-type kidneys and vehicle-treated cystic kidneys showed no change in the expression of these genes (**Fig. 5**).

a.3 Co-treatment with the antioxidant α-tocopherol (vitamin E) attenuates the toxicity of 11β-dichloro in cell culture. (Essigmann, MIT)

So far, oxidative stress and production of ROS have been biomarkers of exposure to 11β dichloro with cystic cells, due to their dysregulated metabolism, reacting at lower doses of the drug than wild-type tissues. However, we have long suspected that oxidative stress is more than a side effect of 11β toxicity, but rather a functional biomarker that reflects a key step in the mechanism of action of the drug candidate. To attenuate the oxidative stress induced by 11β, we co-treated IMCD3 cells with increasing concentrations of DL-α-tocopherol (vitamin E), a liposoluble antioxidant that localizes in membranes, including mitochondrial membranes and can efficiently scavenge reactive oxygen radicals. Previous cell culture data from our lab indicated that vitamin E can buffer 11β-induced oxidative stress and ameliorate killing of cancer cell lines (8). Our data now show that vitamin E is also an efficient inhibitor of 11β toxicity in kidney cell lines (**Fig. 6**). Concentrations of 50-150 μM of vitamin E were sufficient to inactivate the cell killing ability of the compound in both WT and PKD1 IMCD3 cells.

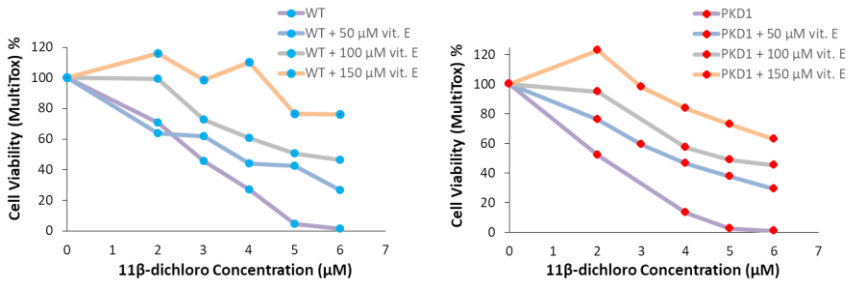

Figure 6. Co-treatment with vitamin E reduces the toxicity of 11β-dichloro in IMCD3 WT and PKD1 cells. The error bars on each data point were omitted for clarity.

a.4 Vitamin E blunts the therapeutic effect of 11β-dichloro in vivo. (Somlo, Yale)

To demonstrate that oxidative stress is an intrinsic component of the mechanism by which 11β-dichloro targets the cyst cells for apoptosis in vivo, we repeated the 11β treatment of *Pkhd1-Cre* mice in the presence of DL-α-tocopherol acetate (vitamin E) administered at 20 IU/ml in the drinking water of nursing mothers. Confirming our expectation, mice that received vitamin E concurrently with 11β (10 mg/kg) showed no therapeutic benefit from 11β (**Fig. 7a**). The therapeutic effect of 11β alone, quantified as kidney/body weight ratio, cystic index and kidney function biomarker BUN disappeared in the mice also receiving vitamin E (**Fig. 7b**). The loss in efficacy was also reflected mechanistically. The increase in the fraction of apoptotic cells (TUNEL assay) induced by 11β alone was abrogated in the presence of vitamin E (**Fig. 7c,d**), along with the characteristic oxidative stress biomarker 4-HNE (**Fig. 7e**).

Taken together, these data argue that the induction of oxidative stress in cystic cells is a key effector step in the mechanism of action of 11β, a step that likely commits the cells to apoptosis. Our findings also suggest that the basis for the selectivity of the compound is the inherent sensitivity to oxidative stress of the *Pkd1*$^{-/-}$ cells, which in turn may be due to their dysfunctional mitochondrial metabolism (6,7).

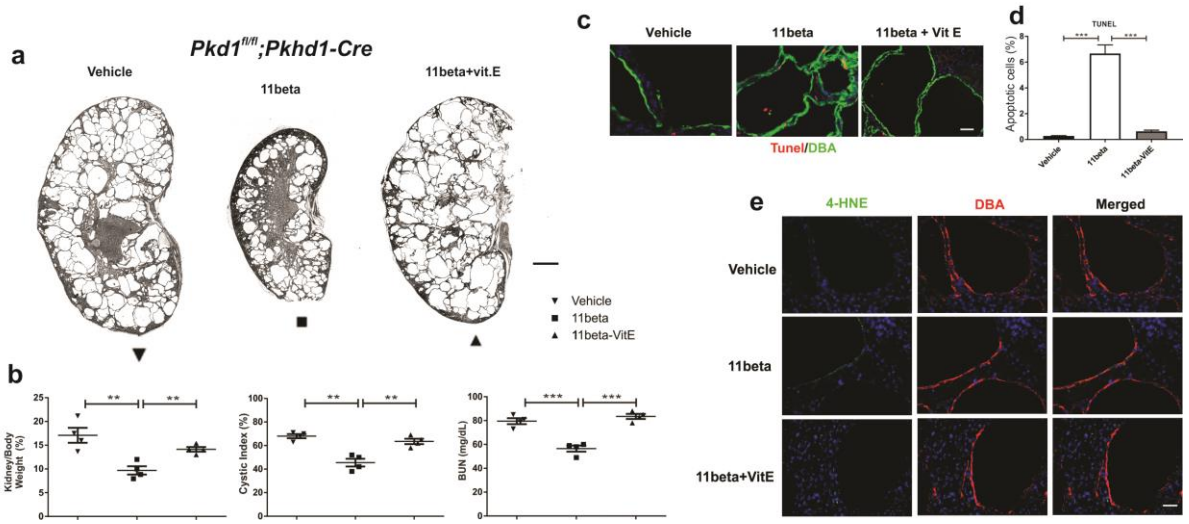

Figure 7. Co-treatment with vitamin E abrogates the therapeutic effect of 11β in the early PKD mouse model. (**a**) Representative whole kidney H&E scans of vehicle-treated (left), 11β-treated (middle) and 11β+vitamin E co-treated (right) kidneys. Scale bar, 1 mm. Vehicle-only or 11β (10mg/kg) was given i.p. daily from P10-P23 and mice were euthanized at P24. In addition to the 11β (10mg/kg) injection, the third group received vitamin E (20 IU/ml) in drinking water. (**b**) Morphological (kidney/body weight ratio, cystic index) and functional parameters (blood urea nitrogen) show that the addition of vitamin E abolishes the PKD amelioration afforded by the 11β treatment alone (n=4 for all groups). (**c**) Apoptosis was evaluated by TUNEL staining in cyst lining epithelia from DBA-positive, Cre-expressing segments. The addition of vitamin E dissipates the increased apoptotic effect of 11β alone. (**d**) Quantitation of the apoptotic index from **c**, 3 kidneys per genotype, >1000 DBA-positive cells were counted. (**e**) 4-HNE staining of tissues from **a** at P24. The oxidative stress induced by 11β alone (middle row) is absent in the 11β+vitamin E co-treated kidneys. Scale bar, 20 μm. **p<0.01; ***p<0.001 determined by ANOVA (**b**) or *t* test (**d**). Data is shown as mean ± s.e.m (**d**).

b. Examining the mechanism of toxicity of 11β compounds in tissues from adult Pax8 mouse model of PKD.

b.1. Induction of ROS in cystic cells exposed to 11β-dichloro. (Somlo, Yale)

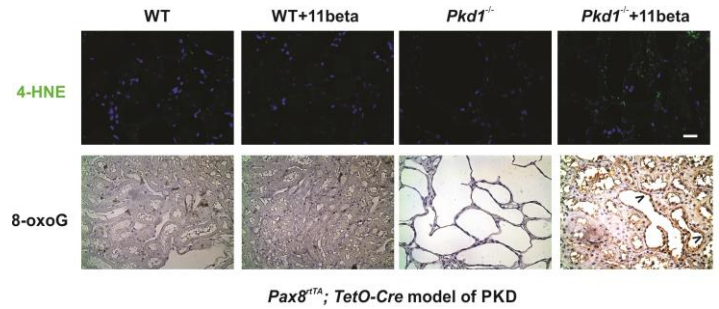

Figure 8. 11β-dichloro induces the accumulation of the oxidative stress biomarkers 4-HNE (**top row**) and 8-oxoguanine (**bottom**) in cystic cells of the Pax8 adult mouse model of PKD. Cell nuclei in top row are stained by DAPI.

The Pax8 adult model recapitulated the mechanistic insights gleaned from the studies in the neonate PKD model. After 12 weeks of treatment with 11β, cystic cells displayed higher level of 4-HNE staining (**Fig. 8**, top panels). By contrast, wild-type cells treated with 11β showed virtually no detectable response. Additionally, we also probed for the 8-oxoguanine oxidative stress biomarker, which was found to be increased, once again, only in the cystic epithelia treated with 11β (**Fig. 8**, bottom panels).

b.2. Transcriptional effects of 11β-induced oxidative stress. (Essigmann, MIT and Somlo, Yale)

During the last funding period, we also analyzed in whole kidney extracts from the adult Pax8 model the transcriptional responses to 11β of a panel of oxidative stress induced genes (**Table 1**). Consistent with the observations in the neonate model, only adult Pax8 cystic kidneys treated with 11β showed statistically significant increases in the mRNA levels of oxidative stress inducible genes CAT, SOD1, GLRX, GPX, HMOX, Hif1a and PRDX; by contrast, 11β-treated WT kidneys and vehicle-treated cystic kidneys showed no change in the expressions of these genes (**Fig. 9**).

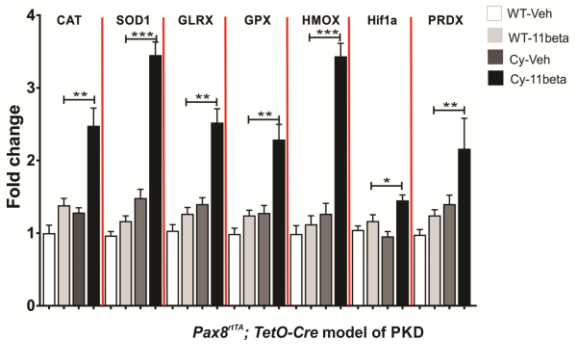

Figure 9. Transcriptional effect of 11β-dichloro on oxidative stress inducible genes in the adult Pax8 mouse model of PKD.

c. Investigating the mechanism of action of 11β-dipropyl in the neonate model of PKD (Somlo, Yale).

An isosteric derivative of 11β-dichloro, 11β-dipropyl (**Fig. 10a**) was initially synthesized to understand the extent to which the ability of the compound to alkylate DNA (and other macromolecules) is necessary for the therapeutic effects in ADPKD mouse models. The nitrogen mustard functionality of 11β-dichloro, which confers ability to alkylate non-specifically

biomolecules, was seen as a liability for the clinical development of the compound. The replacement of the two chlorine atoms of 11β-dichloro with methyl groups inactivates the reactive aniline mustard. While we have previously disclosed preliminary evidence that 11β-dipropyl is effective at ameliorating PKD in the neonate model, the full mechanistic analysis was only completed during the last funding period.

11β-Dipropyl proved effective at slowing PKD development in the neonate model at 5mg/kg daily (half the dose used with 11β-dichloro) (**Fig. 10b**). Efficacy end-points were comparable with those observed for the treatment with 11β-dichloro at 10 mg/kg body weight (**Fig. 10c**). There was a significant decrease in the kidney/body weight ratio, cystic index and a substantial lowering of the BUN (**Fig. 10c**). Similarly to the 11β-dichloro treatment, the mechanism of 11β-dipropyl involved the specific induction of apoptosis in the cystic cells (**Fig. 10d**). The fraction of apoptotic cells (as detected by TUNEL assay) was markedly increased in the treated kidneys, with the apoptotic cells present only in DBA-positive epithelia, where the Cre expression renders them *Pkd1* null (**Fig. 10d,e**). Moreover, the apoptotic markers cleaved PARP and cleaved caspase 3 were also increased in the treated kidneys (**Fig. 10f,g**). Furthermore, the *Pkd1* null cells also experienced increased oxidative stress (reflected by the accumulation of 4-HNE) following treatment with 11β-dipropyl (**Fig. 10h**). Taken together, these findings support the corollary hypothesis that DNA alkylating activity is not necessary for the efficacy and selectivity of 11β compounds in ADPKD, reinforcing the overarching premise that the ability of 11β

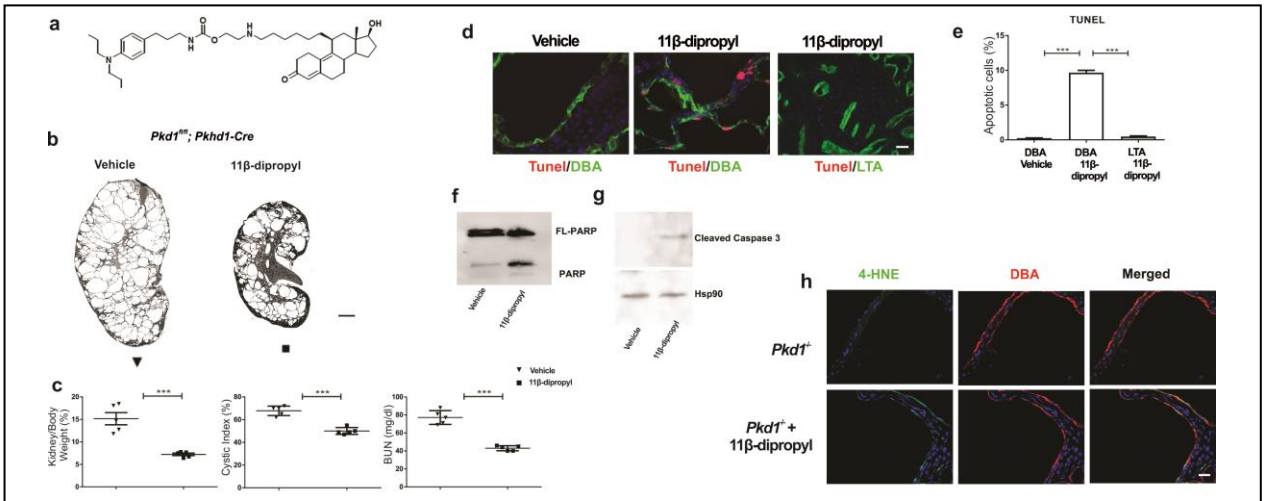

Figure 10. The isostere 11beta-dipropyl ameliorates cystic disease in an early model of PKD by inducing apoptosis in cystic cells. (**a**) Structure of 11β-dipropyl, an isosteric derivative of 11beta. (**b**) Representative whole-kidney H&E scans of vehicle-treated (left), and 11β-dipropyl-treated (right) kidneys. Vehicle-only or 11beta-dipropyl (5 mg/kg) was given i.p. daily from P10-P23 and mice were euthanized at P24. (**c**) Morphological (kidney/body weight ratio, cystic index) and functional parameters (blood urea nitrogen) are all lowered in 11beta-dipropyl-treated kidneys compared to vehicle treated (n=5 for all groups). (**d**) Apoptosis was evaluated in cyst lining epithelia by TUNEL staining. 11beta-dipropyl increased apoptosis levels only in DBA-positive (Cre expressing) regions; vehicle-treated null cells or cells where Cre is not active (LTA positive proximal segments) were not affected. Scale bar, 20 µm. (**e**) Quantitation of the apoptotic index from **d**, 3 kidneys per genotype, >1000 DBA-positive cells were counted. (**f**,**g**) Apoptosis markers cleaved PARP and cleaved caspase 3 were increased in the 11beta-dipropyl-treated vs. vehicle-treated kidneys as seen by immunoblotting. (**h**) 4-HNE staining of tissues from **b**, at P24 indicates that11Beta-dipropyl induces oxidative stress specifically in *Pkd1* null (DBA positive) cells. Statistics: ***p<0.001 determined by ANOVA (**c**) or *t* test (**e**). Data is shown as mean ± s.e.m (**e**).

compounds to target mitochondria and induce oxidative stress are the key mechanisms by which these compounds lead to selective apoptosis of cystic cells.

d. Investigating the efficacy of 11β-dipropyl in the adult onset PKD mouse mode (Somlo, Yale and Essigmann, MIT)

Testing 11β-dipropyl activity in the adult onset PKD mouse model (*Pkd1*$^{fl/fl}$*; Pax8*^{rtTA}*;TetO-Cre*) has been underway since the previous funding period. Given the length of the assay and number of mice required by the power calculations to reach a statistically robust conclusion, our initial batch of 11β-dipropyl was exhausted and we needed to synthesize and purify more compound. The additional synthesis took place during the last funding period.

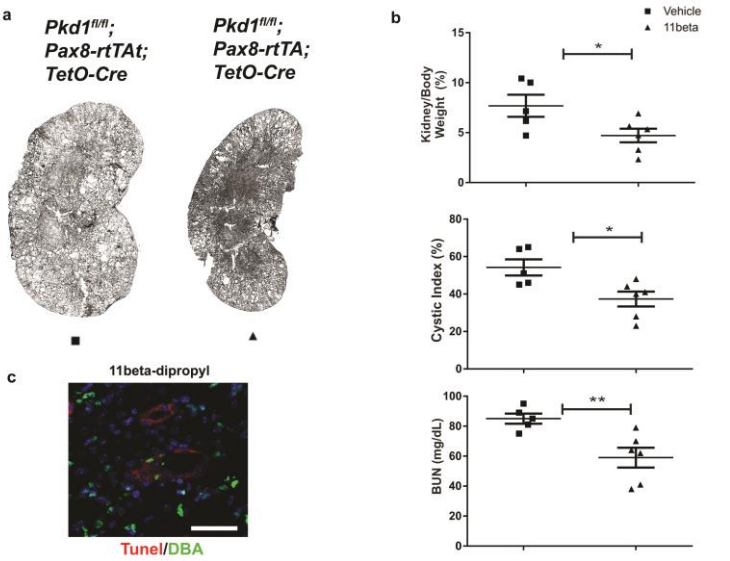

Figure 8. **11β-dipropyl ameliorates cystic disease in the adult Pax8 mouse model of PKD**. (**a**) Representative cross-sections of kidney specimens from vehicle and 11β-dipropyl treated animals. (**b**) Quantification of morphological changes (kidney/body weight ratio, cystic index) and functional changes (blood urea nitrogen). The dots denote individual animals; large bar indicates the mean, and error bars are +/- 1 SD; n=5 (vehicle) and n=6 (treated); *p<0.05, **p<0.01 by ANOVA. (**c**) Representative TUNEL staining of treated kidneys.

We are excited to report that this experiment was completed early last fall. The data indicate that 11β-dipropyl is effective in slowing down cystogenesis in the adult Pax8 model (**Fig. 11a**), as indicated by the decreased kidney/body weight ratio, decreased average cystic index and improved kidney function (i.e., lower levels of blood urea nitrogen) in the treated animals (**Fig. 11b**). From a mechanistic stand point, 11β-dipropyl induces apoptosis of *Pkd1*$^{-/-}$ cells, as evidenced by TUNEL staining (**Fig. 11c**).

What opportunities for training and professional development has the project provided?

Two MIT undergraduate students (Elyse Plachinsky and Sally Liu) and two technical associates (Tania Gonzalez-Robles and Lina Kim) contributed to the project, fully engaged in the scholarly enrichment activities of the MIT Departments of Chemistry and Biological Engineering and the Center for Environmental Health Sciences. A postdoctoral research scientist (Nina Gubina) started working on the project late in the fall of 2018 and is currently supported by this grant.

How were the results disseminated to communities of interest?

Results from this project were disseminated in the form of oral and poster presentations at the 2017 and 2018 annual meetings of the American Society of Nephrology.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, we shall continue our work to investigate the mechanism of action of 11β compounds against PKD cells in both cell culture and animal models, using a multipronged approach. First, we shall continue the development of a better cell culture model for PKD using the IMCD3 cell lines. One major difference between cells in cell culture and the kidney tissues in a live animal is the local oxygen concentration. Traditional cell culture is performed in normoxic atmosphere (~21% oxygen), which is strikingly different than the low oxygen concentration (3-6% oxygen) that exists in tissues. Kidneys are in fact at the lower end of that range, so, under physiological conditions, kidney cells are relatively hypoxic. Given that the mechanism of action of the 11β compounds involves oxidative stress, we suspect that the cellular response would be dependent on the local oxygen concentration. Therefore, in the next reporting period, we propose to study the 11β toxicity in cell culture as a function of oxygen tension. To accomplish this goal, we shall setup a glove box station that enables cellular manipulation under a variety of carefully-controlled oxygen concentrations (including anoxia, and various degrees of hypoxia) and grow cells in gas-tight chambers to maintain the desired (low) oxygen concentration throughout the experiment.

Second, we shall revisit the structure-activity study with all the11β-dipropyl analogs synthesized previously, but this time in the IMCD3 cell line pair, and once the low-oxygen station is setup, we shall also perform the study in hypoxic conditions more reminiscent of the kidney physiology.

Third, we shall investigate the transcriptional responses to 11β-dipropyl, which is now our clinical lead compound, on kidney extracts collected by the Yale team and compare it with: a) the transcriptional responses induced by 11β-dichloro (shown in this report); b) the transcriptional responses of cells grown in hypoxic conditions, when exposed to 11β compounds.

The future directions highlighted here should lead to substantial findings that can enable the formulation of additional, more specific hypotheses regarding the mechanism of action of 11β compounds in PKD mouse models, and even additional insight into the precise molecular target of these agents. These findings, once published, should also accelerate the preclinical and clinical development of the 11β compounds.

4. IMPACT: *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?

This project is likely to make an impact in the area of therapeutics targeted at polycystic kidney disease (PKD). Including experiments recently completed, we have now shown that both 11β compounds (11β-dichloro and 11β-dipropyl) show great efficacy in preventing cystic growth in mouse models, suggesting that they can be developed into clinical candidates. Furthermore, the 11β compounds work by inducing apoptosis in cystic cells, a mechanism of action relatively unique in the field of PKD. Induction of apoptosis is specific to the cystic cells. Because of their mitochondrial dysfunction and reprogrammed metabolism, cystic cells are very sensitive to oxidative stress, which is a major component of the mechanism of action of the 11β compounds. The concept of using drug-induced ROS generation to treat this disease is novel and, aside from our own laboratories, it could inspire others to develop ROS-generating drug candidates.

What was the impact on other disciplines?

The 11β compounds that will be developed in this project for treating PKD are likely to have an impact for the treatment of other diseases, including cystic diseases in other organs (i.e., liver) or proliferative diseases (i.e., cancer). It is noteworthy that 11β-dichloro has already shown efficacy against a number of tumor types in animal xenografts (12,13).

What was the impact on technology transfer?

The new 11β compounds and derivatives synthesized in this project have already been described in a patent application, and the patent was issued earlier this year. Currently, the patent only covers the methods of use of the compounds (primary species is 11β-dipropyl) for treating PKD and related cystic diseases. However, a continuation application is being submitted to secure composition of matter claims. Issuance of intellectual property will be a step toward licensing to a company that can efficiently bring a drug candidate to clinical trials.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS: *The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

As outlined in the future plans section, we propose to perform cell culture experiments on PKD cell lines under hypoxic conditions, which better mimic the physiological state of the kidneys in vivo. Although these experiments were not explicitly proposed in the original proposal, they are a natural extension of our findings regarding the mechanism of action of the 11β compounds, which involves oxidative stress. Moreover, these experiments have the potential to provide a significant breakthrough in developing a robust cell culture model of PKD. The materials required for these experiments (glove box, gas chambers, gaseous mixtures, etc.) are all within the original budget, so we foresee no significant impact on expenditures.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

Publications, conference papers, and presentations

Journal publications.

A manuscript that describes the efficacy of 11β-dichloro and 11β-dipropyl compounds against PKD, as seen in both neonate and adult mouse models, as well as the mechanistic insights underlying the specificity of the compounds is essentially complete and will be submitted within the next few weeks. The manuscript acknowledges the federal support received. The authors and title are as follows:

Fedeles BI, Fedeles SV, Ishikawa Y, Khumsubdee S, Krappitz M, Rodrigues D, Westerling P, Staudner T, Campolo J, Liu S, Dong K, Cai Y, Gallagher AR, Croy RG, Essigmann JM, Somlo S. *"A synthetic anti-tumor agent ameliorates polycystic kidney disease by promoting apoptosis of cystic cells through increased oxidative stress"* (in preparation).

Books or other non-periodical, one-time publications.
Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses

The non-provisional patent application 14/515,441, entitled "Methods for treating polycystic kidney disease and polycystic liver disease" was filed jointly by MIT and Yale on October 15, 2014. While this application was filed prior to the start of the funding for this project, it covers a broad range of compounds that could be used to treat polycystic kidney disease, including the lead compound 11β-dipropyl. In May 2018, the patent US 9,982,009 was issued, based on this application. Recently, through the technology licensing offices at MIT and Yale, we have been discussing potential licensing agreements with several companies, in an effort to accelerate the pre-clinical development of the 11β compounds.

Other Products
Nothing to report.

7. Participants and Other Collaborating Organizations

Name:	*John M. Essigmann*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*Ebrap ID* 237355 ORCID: 0000-0002-2196-5691
Nearest person month worked:	*1*
Contribution to Project:	*John Essigmann has the overall supervisory responsibility for the MIT site of this grant, including organizing deployment of personnel and the preparation of manuscripts and reports. Additionally, he helps interpret the data emerging from the project, especially data that involve the impact of the compounds made on oxidative stress and disruption of metabolic pathways.*
Funding Support:	*See Appendix 2.*

Name:	*Robert Croy*
Project Role:	*Research Scientist*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*6*
Contribution to Project:	*Dr. Croy provided supervision and guidance for the synthesis of 11β-dipropyl and analogs required for structure activity studies. He designed and performed quality control chromatographic analyses of the compounds. He also participated in conference calls to monitor experimental progress and coordinate with our partners at Yale.*
Funding Support:	*See Appendix 3.*

Name:	*Bogdan Fedeles*
Project Role:	*Research Scientist*
Researcher Identifier (e.g. ORCID ID):	ORCID: 0000-0001-5252-826X
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Fedeles has designed, performed and coordinated the cell biology studies aimed at characterizing the efficacy and mechanism of 11β compounds in cell culture models. These included toxicity assays, ROS assays, isolated mitochondria assays, and transcriptional profiling using qPCR. Additionally, he performed quality control analysis for all the materials shared with the Yale collaborators. He also assembled the majority of the project write-ups, including the progress reports and the upcoming manuscript reporting the latest findings.*
Funding Support:	

Name:	*Tania Gonzalez-Robles*
Project Role:	*Technical associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Ms. Gonzalez-Robles has provided the technical support for all the cell culture experiments, including managing the supplies and the cell culture facility, preparation of media and buffers, maintenance of cell lines, and routine testing for viability and contaminants.*
Funding Support:	

Name:	*Lina Kim*
Project Role:	*Technical associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3*
Contribution to Project:	*Ms. Kim has provided the technical and bioinformatics support for the project, including design of qPCR primers, analysis of sequencing data for genotyping, and power calculations and statistical analysis for the transcriptional data.*
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Updated Other Support Pages are included in the Appendices.

What other organizations were involved as partners?

This is a COLLABORATIVE AWARD. Our collaboration partner is Stefan Somlo at Yale University. Details are below:

- **Organization Name:** Yale University.
- **Location of Organization:** New Haven, CT, USA
- **Partner's contribution to the projec**t
    - **Financial support:** none
    - **In-kind support:** development of cell lines for research
    - **Facilities**: none

- **Collaboration:** Development of mouse models for PKD; designing and performing mouse model studies with the 11β compounds; mechanistic studies on mouse tissues and cells.
- **Personnel exchanges**: none
- **Other:** none.

8. SPECIAL REPORTING REQUIREMENTS

This is a COLLABORATIVE AWARD. An independent report from BOTH the initiating PI and Collaborating PI will be provided. The current report is from the Collaborating PI (John Essigmann, MIT). Given the collaborative nature of the work, experiments that involve materials and expertise provided by both institutions are included in this report. The reports are therefore very similar. Throughout the report, the responsible PI and the site where the work was performed are included.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc. Reminder: Pages shall be consecutively numbered throughout the report.* ***DO NOT RENUMBER PAGES IN THE APPENDICES.***

Appendix 1: References

Appendix 2: John Essigmann Updated Active Support Pages

Appendix 3: Robert Croy Updated Active Support Pages

APPENDIX 1

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APPENDIX 2

ESSIGMANN, John M.
ACTIVE SUPPORT

This project, previously reported as Pending, has been funded.

Title:	**Science and Engineering for Sensors, Mechanisms, and Biomarkers of Exposures**
Effort:	2.30 calendar (Entire project)
Supporting Agency:	NIH/NIEHS
Grants Officer:	Lisa Edwards, archer@niehs.nih.gov
Performance Period:	8/1/2017 - 3/31/2022
Funding Amount:	
Project Goals:	This is a sub-project within a larger context of a Superfund Research Project proposal. The major goal of this sub-project is to reveal the mutagenic biomarkers that reflect risk factors of susceptibility to N-nitrosodimenthylamine (NDMA) and benzo(a)pyrene (BP), environmental contaminants found at Superfund sites.
Specific Aims:	The Aims of this sub-project are to determine if a special mouse model of cancer originally developed to study the mutagenic effects of aflatoxin B_1 can distinguish between the mutational spectra of two different environmental toxicants (NDMA and BP), alone or in combination.
Overlap:	No overlap.

Title:	**MIT Center for Environmental Health Sciences**
Effort:	2.40 calendar
Supporting Agency:	NIH/NIEHS
Grants Officer:	James William, williamsjr@niehs.nih.gov
Performance Period:	4/1/2016 - 3/31/2021
Funding Amount:	
Project Goals:	Core Center focused on the impact of the environment on human health and the health of the human ecosystem.
Specific Aims:	This core grant provides support for the administrative structure, community engagement activities and core facilities for the Center for Environmental Health Sciences at MIT. Professor Essigmann is Center Director on this center core grant but does not receive any direct support.
Overlap:	No overlap.

Title:	**Endogenous Nitrate Carcinogenesis in Man - Project 2**
Effort:	1.00 calendar
Supporting Agency:	NIH
Grants Officer:	Joy Kearse, kearsej@mail.nih.gov
Performance Period:	6/1/2014 - 5/31/2019

Funding Amount:
Project Goals: Study of oxidative stress as it contributes to inflammation induced cancer.
Specific AIms: The aim of this project is to identify a population of oxidative stress mediators that in aggregate represent the drivers of genetic changes many researchers believe underpin the conversion of normal cells to cancer cells. Specific attention is given to chemicals that cause oxidative stress associated with inflammation induced by nitric oxide, HOCl acid and related oxidants.
Overlap: No overlap.

Title: **The Therapeutic Effect of the Antitumor Drug 11beta and Related Molecules on Polycystic Kidney Disease**
Effort: 1.00 calendar
Supporting Agency: U.S. Army Medical Research and Material Command
Grants Officer: Elena G. Howell, elena.g.howell.civ@mail.mil
Performance Period: 9/30/2015 - 9/29/2018
Funding Amount:
Project Goals: To develop effective therapeutics of polycystic kidney disease
Specific AIms: Study of the mechanistic basis of activity of drug candidate molecules that have selective activity against polycystic kidney disease in vitro and in vivo. Specific aims are to develop effective therapeutics of polycystic kidney disease.
Overlap: This grant.

Title: **Intra and Extra-Chromosomal Probes for Mutagenesis by Carcinogens**
Effort: 1.00 calendar
Supporting Agency: NIH
Grants Officer: Joy Kearse, kearsej@mail.nih.gov
Performance Period: 7/6/2016 - 6/30/2021
Funding Amount:
Project Goals: Study of mutagenic properties of DNA adducts produced by compounds that cause human cancer.
Specific AIms: The aim of this Project is to investigate the mechanisms by which simple environmental alkylating agents and the potent human liver carcinogen aflatoxin B1 induce mutations. This project involves synthesis of short oligonucleotides containing organic compound-DNA adducts. Typically the adducts are of environmental agents such as vinyl chloride and short-chain alkylating agents. The oligonucleotides are inserted into the genomes of viruses, which are replicated in cells. The type, amount and genetic requirements for mutagenesis of DNA damaging agent-derived adducts are characterized.
Overlap: No overlap.

APPENDIX 3

CROY, Robert C.
ACTIVE SUPPORT

This project, previously reported as pending, has been funded.

Title:	**Science and Engineering for Sensors, Mechanisms, and Biomarkers of Exposures**
Effort:	1.10 calendar
Supporting Agency:	NIH/NIEHS
Grants Officer:	Lisa Edwards, archer@niehs.nih.gov
Performance Period:	8/1/2017 - 3/31/2022
Funding Amount:	
Project Goals:	This is a sub-project within a larger context of a Superfund Research Project proposal. The major goal of this sub-project is to reveal the mutagenic biomarkers that reflect risk factors of susceptibility to N-nitrosodimenthylamine (NDMA) and benzo(a)pyrene (BP), environmental contaminants found at Superfund sites.
Specific Aims:	The Aims of this sub-project are to determine if a special mouse model of cancer originally developed to study the mutagenic effects of aflatoxin B_1 can distinguish between the mutational spectra of two different environmental toxicants (NDMA and BP), alone or in combination.
Overlap:	No overlap.

Title:	**MIT Center for Environmental Health Sciences**
Effort:	1.10 calendar
Supporting Agency:	NIH/NIEHS
Grants Officer:	James William, williamsjr@niehs.nih.gov
Performance Period:	4/1/2016 - 3/31/2021
Funding Amount:	
Project Goals:	Core Center focused on the impact of the environment on human health and the health of the human ecosystem.
Specific Aims:	This core grant provides support for the administrative structure, community engagement activities and core facilities for the Center for Environmental Health Sciences at MIT. Dr. Croy is co-director of the Genomics and Imaging Facilities Core. but does not receive any direct support.
Overlap:	No overlap.

Title:	**The Therapeutic Effect of the Antitumor Drug 11beta and Related Molecules on Polycystic Kidney Disease**

Effort:	8.80 calendar
Supporting Agency:	U.S. Army Medical Research and Material Command
Grants Officer:	Elena G. Howell, elena.g.howell.civ@mail.mil
Performance Period:	
Funding Amount:	
Project Goals:	To develop effective therapeutics of polycystic kidney disease
Specific AIms:	Study of the mechanistic basis of activity of drug candidate molecules that have selective activity against polycystic kidney disease in vitro and in vivo. Specific aims are to develop effective therapeutics of polycystic kidney disease.
Overlap:	This grant.