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TITLE: Intratumoral Steroid Production as a Mechanism of Immune Evasion in Adrenocortical Carcinoma (ACC)

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CONTRACTING ORGANIZATION: University of Michigan, Ann Arbor, MI,

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14. ABSTRACT

Adrenocortical carcinoma (ACC) is a rare, aggressive cancer of the adrenal glands. Up to 75% of ACC patients will develop metastasis, but available therapies are ineffective and toxic. Immunotherapy has emerged as a promising strategy. The FDA accelerated approval of anti-PD-1 therapy for mismatch repair-deficient tumors including ACC, with rationale that genomically unstable tumors accumulate "neoantigens" that can be unmasked for immune clearance. However, therapy success relies on several factors including tumor immune infiltration, characteristically low in anti-PD-1-resistant tumors. In The Cancer Genome Atlas (TCGA) study on ACC, we observed ACC bears low expression of immune genes and is likely anti-PD-1-resistant. Paradoxically, the most aggressive ACC, with highest mutational burden, are the most immune poor. This ACC subset also exhibits pathologic cortisol excess and high expression of genes involved in steroid production. We *hypothesize* that in ACC, high concentrations of locally-produced steroids protect cancer cells from the immune system by impairing an anti-tumoral immune response. Aim 1 – Characterization and quantification of ACC intra-tumoral steroidomics and the molecular phenotype of infiltrating immune cells. Aim 2 – Functional characterization of the impact of ACC-secreted steroids on immune cell function and tumor immune infiltrate.

15. SUBJECT TERMS

None listed.

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1. Introduction

The adrenal glands synthesize and secrete a variety of hormones critical for life. Adrenocortical carcinoma (ACC) is a rare, aggressive cancer of these glands (5-year-survival ~35%). Up to 75% of ACC patients will develop metastasis, but available therapies are ineffective and toxic. ***There is an urgent need to identify novel therapies for advanced ACC that target tumor-specific vulnerabilities while limiting drug toxicity.*** Immunotherapy has emerged as a promising strategy for advanced cancers. The FDA accelerated approval of anti-PD-1 therapy for mismatch repair-deficient tumors including ACC, with rationale that genomically unstable tumors accumulate "neoantigens" that can be unmasked for immune clearance. However, therapy success relies on several factors including tumor immune infiltration, characteristically low in anti-PD-1-resistant tumors. In The Cancer Genome Atlas (TCGA) study on ACC, we observed ACC bears the lowest expression of immune genes of nearly all TCGA cancers, suggesting most ACC is likely anti-PD-1-resistant. Paradoxically, the most aggressive ACC, with highest mutational burden, are the most immune poor. This ACC subset also exhibits pathologic cortisol excess and high expression of genes involved in steroid production. Given the well-known immunosuppressive actions of glucocorticoids, these observations suggest intra-tumoral steroid production may be a novel mechanism of immune evasion in ACC. Our *overall objective* is to characterize a novel mechanism by which cancer cells evade the immune system, which will enable the development of improved therapeutic strategies to target ACC and other malignancies using immunotherapy. We *hypothesize* that in ACC, high concentrations of locally-produced steroids protect cancer cells from the immune system by impairing an anti-tumoral immune response.

2. Keywords

Adrenocortical carcinoma, ACC, immunotherapy, steroids, intra-tumoral steroids, tumor, orthotopic xenograft, transcriptome, steroidomics

3. Accomplishments

The major goals of this project, as stated in two specific aims in the SOW, are:

- Specific Aim 1 – Characterization and quantification of ACC intra-tumoral steroidomics and the molecular phenotype of infiltrating immune cells. We will measure tissue concentrations of steroid hormones in frozen ACC samples from the University of Michigan Endocrine Oncology Repository by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We will characterize the spectrum of infiltrating immune cells (including T cells) present in each sample by immunohistochemistry, and evaluate cytokines, immune signatures, and steroidogenic programs differentially expressed across ACC by RNA-seq. Through integrated analyses, we will characterize the immune cell signature associated with a given steroid profile and transcriptional program.
- Specific Aim 2 – Functional characterization of the impact of ACC-secreted steroids on immune cell function and tumor immune infiltrate. We will characterize secreted steroidomics of ACC-derived cell lines by LC-MS/MS. We will treat CD8+ T cells with conditioned media from ACC-derived cell lines +/- a variety of steroidogenesis inhibitors to evaluate impact of secreted steroids on T cell function in vitro. We will test if disrupting steroid receptor signaling using pharmacological antagonists of steroid receptors on T cells reverse these effects and will extend findings to co-culture experiments with T cells and ACC cell lines. We will treat a steroidogenic, syngeneic xenograft mouse model of metastatic ACC with PD-1 inhibitors +/- inhibitors of steroid production/action, and measure impact on tumor burden and immune infiltrate.

For this reporting period, pertinent tasks as proposed in the SOW and accomplishments are described below:

- Specific Aim 1:
    - Major Task 1: (months 1-10): Collect frozen human ACC samples; collect relevant clinical data.
        - Subtask 1: Protocol review and approval by the University of Michigan IRB. **(approved 06/23/2019)**
        - Subtask 2: Protocol review and approval by the USAMRMC Human Research Protection Office **(initial approval 08/27/2019)**

        - Subtask 3: Select 50 frozen ACC samples from the UM Endocrine Oncology Repository (MEOR); collect relevant clinical and demographic data. **(40/50 samples identified).**
        - Subtask 4: Quality-control checking of samples by histological assessment (will be performed by the Hammer Lab team, UM). We will evaluate sample quality by inspecting for areas of extensive necrosis or acellular material.
    - Major Task 2 (months 10-14): Measure intra-tumoral concentrations of steroid hormones, precursors, and metabolites in human ACC samples by LC-MS/MS. **This task has not begun due to unanticipated technical problems with intra-tissue steroid extraction from OCT-embedded samples (see 5. Changes/Problems).**
        - Subtask 1: Harvest 200 mg of the 50 selected frozen ACC samples using a cryotome (will be performed by the Hammer Lab team, UM). Tissues will be harvested for LC-MS/MS steroid profiling (which will be performed in the next subtask) and for immunohistochemistry/immunofluorescence (will be performed in Major Task 3).
        - Subtask 2: Homogenize tissues and extract steroids for LC-MS/MS steroid profiling (will be performed by the Auchus Lab team, UM).
        - Subtask 3: Perform intra-tumor steroid profiling in collected samples (will be performed by the Auchus Lab team, UM) to measure the concentrations of intra-tumoral steroids in present in each patient sample. LC-MS/MS data will be analyzed as in Rege et al. JCEM 2018
    - Major Task 3 (months 14-18): characterize the spectrum of infiltrating immune cells and PD-1/PD-L1 expression in human ACC samples by immunohistochemistry. **This task has not begun due to the technical problems described in Major Task 2 (see 5. Changes/Problems).**
        - Subtask 1: Perform immunohistochemistry/immunofluorescence staining for selected markers on tissue sections prepared from selected 50 ACC samples.
        - Subtask 2: Quantification of staining for each marker.
    - Major Task 4 (months 10-20): Characterize steroidogenic and immune-related transcriptional programs in human ACC samples by RNA-seq. **This task has not begun due to the technical problems described in Major Task 2 (see 5. Changes/Problems).**
        - Subtask 1: Extract RNA from the 50 ACC frozen samples (will be performed by the Hammer Lab team, UM).
        - Subtask 2: Generation of RNA-seq data.
        - Subtask 3: RNA-seq data analysis.
    - Major Task 5 (months 17-20): Integrate transcriptomics, steroidomics, and immunohistochemistry data obtained from human ACC.
        - Subtask 1: Perform integrative analysis of transcriptome, steroidomics, and IHC data
- Specific Aim 2:
    - Major Task 6 (months 1-6): Characterize spectrum of ACC-secreted steroids in mouse and human models of ACC in vitro (Y1, NCI-H295R, SW13 cell lines). All experiments in this task will be performed in four biologically independent replicates. We performed these experiments in human (NCI-H295R) and mouse (Y1) cell lines. We used ELISA assays to measure cortisol (in the NCI-H295R cell lines) and corticosterone (in the Y1 cell lines) before and after treatment with increasing concentrations of different steroidogenesis inhibitors. We used this ELISA assay to identify the optimal dose (maximum steroidogenesis inhibition with minimal toxicity) for each compound (see Figures 1 and 2 in appendix). We then measured several steroids and metabolites in the Y1 cells by LC-MS/MS before and after treatment with the steroidogenic inhibitors at the determined doses (see Table 1 in appendix). By these measurements we detected that the Y1 cell line is 21-hydroxylase-deficient and therefore does not produce corticosterone. Since our downstream *in vitro* and *in vivo* studies rely on a cell that can produce corticosterone, we had to genetically manipulate the Y1 cells to restore Cyp21 activity (see 5. Changes/problems). We demonstrated that our Cyp21-

positive Y1 (Y1-Cyp21+) cells are able to synthetize corticosterone and express Cyp21 protein (Figure 4 and 5 in the appendix). To fully characterize the Y1-Cyp21+ cells, we are now performing western blot for Cyp21 and measuring *Cyp21a1* transcript levels. The LC-MS/MS determinations also detected an unexpected high concentration of cortisol in the conditioned media of the Y1 cells (Table 1). Since Y1 is a mouse cell line (and therefore not expected to produce cortisol), the likely source of cortisol is the serum (FCS supplemented with horse serum) used in the culture media. This observation is also corroborated by the fact that cortisol concentrations does not change upon treatment with steroidogenic inhibitors (Table 1). Since such high cortisol levels might interfere with the outcomes of our cellular experiments, we are currently developing a protocol to remove cortisol from the culture media based on activated charcoal extraction (see appendix).

- Subtask 1: Measure steroids from conditioned media of ACC cell lines (these cell lines are available at the Hammer Lab; protocols for measuring steroids by mass spectrometry in conditioned media are established in the Auchus Lab).
- Subtask 2: Treat cell lines with the following steroidogenesis inhibitors: metyrapone, trilostane, ketoconazole, etomidate, and abiraterone (this task will be performed in the Hammer Lab).
- Subtask 3: Compare steroids in the conditioned media before and after treatment with steroidogenesis inhibitors. Each experiment will be performed in quadruplicates (each drug versus vehicle-treated cells). Changes in steroid production will be assessed by t-tests.

- Major Task 7 (months 6-14): Measure the impact of ACC-secreted steroids on CD8+ T cell function in vitro. All experiments in this task will be performed in four biologically independent experiments in Dr. Keshamouni's lab, using conditioned media or cells from ACC cell lines maintained in Dr. Hammer's lab. For all experiments, statistical significance for differences in steroid production will be evaluated by Student's t-test for pairwise comparisons or ANOVA with post-hoc tests for greater than pairwise comparisons. **We have developed a protocol to isolate CD8+ T cells from mice. However, we will only proceed with these studies after we optimize the protocol for removing cortisol from the culture media (see major task 6).**
  - Subtask 1: Treat CD8+ T cells with conditioned media from ACC cell lines before and after treatment with steroidogenic inhibitors and steroid receptor inhibitors.
  - Subtask 2: Perform CD8+ T cells activation assay to measure changes in T cell proliferation after exposure to conditioned media.
  - Subtask 3: Perform CD8+ cells chemotaxis assay to measure changes in T cell migration after exposure to conditioned media by flow cytometry.
  - Subtask 4: Perform CD8+ apoptosis assay to measure changes in T cell apoptosis after exposure to conditioned media by TUNEL.
  - Subtask 5: Perform CD8+ cytokine production assay to measure changes in T cell cytokine production by ELISPOT.
  - Subtask 7: Perform co-culture experiments with ACC cell lines and CD8+ T cells and measure changes in ACC cytotoxicity after exposure to different steroidogenesis inhibitors by measuring changes in CD8+, NR5A1+ cells by immunocytochemistry and apoptosis by TUNEL.
- Major Task 8 (months 1-14): Characterize the metastatic potential, tumor dynamics, and steroidogenesis profile of a syngeneic, orthotopic xenograft model of ACC (this is a study utilizing cell lines engrafted in an animal model, which will be performed in the Hammer Lab). **We experienced delays associated with obtaining ACURO approval (see below), COVID-19 restrictions (see 5. Changes/Problems), and cryorecovery of A/HeJ mice with the Jackson Laboratory. After initially expanding the C57L/J and A/HeJ colonies,**

and successfully obtaining the LAF1 mice (F1 colony of the cross between C57L/J and AHe/J), we were not able to maintain our A/HeJ colony due to fertility issues (see 5. Changes/Problems).

- Subtask 1: Obtain ACURO approval for all the studies involving experimental animals. **(approval received 05/28/20)**
- Subtask 2: Cross male A/HeJ mice and female C57L/J mice to generate LAF1 mice.
- Subtask 3: Perform US-guided orthotopic injection of Y1 cells in LAF1 mice – pilot study with 20 mice. 10 will be sacrificed 30 days after injection to measure steroid serum levels and IHC characterization of tumors. The US-guided Y1 cells orthotopic injection will be performed by Dr Erica A. Newman in her Lab, which Dr. Hammer's lab has access to.
- Subtask 4: Perform steroid profiling in tumors originating from Y1 injection in 10 mice (serum steroids will be measured by mass spectrometry in the Auchus Lab) to evaluate if orthotopic xenografts are steroidogenic as predicted.
- Subtask 5: IHC characterization of immune cell infiltration of Y1-derived tumors in 10 mice to determine if immune cells infiltrate orthotopic xenografts.
- Subtask 6: Characterization of the xenografts at the predetermined endpoint or death: evaluation of local tumor growth and metastatic potential. This study will be performed in the 10 remaining mice.

- Major Task 9 (months 14-24): Evaluate if inhibition of steroidogenesis and/or steroid receptor signaling improves efficacy of pembrolizumab.
  - Subtask 1: Perform US-guided orthotopic injection of Y1 cells in LAF1 mice.
  - Subtask 2: Perform drug treatments or placebo/sham according to group assignments.
  - Subtask 3: Perform characterization of immune infiltration and steroidomics as in Major Task 2 and 3. Perform characterization of tumor progression.
  - Subtask 3: Perform statistical and survival analysis.

Training opportunities and professional development:
Nothing to report

Dissemination of results to communities of interest:
Dr. Gary Hammer was invited to participate in a round table discussion focused on the future of ACC treatment/clinical trials at the 2019 Adrenal Cancer Symposium in Clermont-Ferrand, FR. He shared our upcoming and ongoing DOD-supported studies with the adrenal cancer research community. The citation for his contribution to the round table discussion is listed below:

Hammer GD (participant). Round Table on Clinical Trials/ACC Treatment. ACC 2019 (Adrenal Cancer Symposium 2019), Clermont-Ferrand, France, on September 27-28, 2019

4. **Impact:** Nothing major to report at this time. However, this subject is of increasing interest to the adrenal cancer research community and we expect this work when completed will have high impact.

5. **Changes/Problems:** Patient enrollment, sample collecting, and processing got substantially delayed due to lockdowns and access restrictions to facilities related to COVID-19. All human subjects research and the Michigan Endocrine Oncology Repository (MEOR) was shut down from 03/14/2020 to 07/08/2020. All basic science research was shut down beginning 03/20/2020. On 05/26/2020, Wave 1 reactivation began for essential basic science research only. Wave 2 reactivation began on 06/17/2020 and Wave 3 reactivation began subsequently. We have had intermittent occupancy restrictions since Wave 3 began. As a result, the enrollment and processing of human samples (Specific Aim 1) has been substantially delayed.

We also experienced an unanticipated problem with intra-tissue steroid determination from OCT-embedded human samples. Preliminary results from the Auchus Lab team indicated that intra-tissue measurements of sulfated steroids from OCT-embedded samples (obtained from benign adrenal tumors) led to inconsistent results. This could lead to unreliable results especially when associating steroid metabolite measurements with tumor subtypes. While these observations require further troubleshooting, one possible explanation is that the conjugated sulfated steroids are more hydrophilic and are released into the circulation as soon as they are produced. The tissue levels of steroids, thereby, do not reflect their actual concentration at their biosynthetic site. Another possible scenario is the leaching of the steroids (especially sulfated steroids) during sample transporting/processing. Due to these technical issues, this research line (steroid determination by LC-MS/MS on tumor tissues) was discontinued. Without a reliable method to measure intra-tissue steroids, it would not be possible to pursue what we proposed in Aim1, even if we proceeded with the other proposed experiments (RNA-seq and immunophenotyping by IHC). We have recently been in contact with an external collaborator (Dr. Man-Ho Choi, from South Korea Institute of Science and Technology) who has extensive experience with GC-MS/MS and has developed a robust and reliable method for intra-tissue steroid determination. In addition to that, since we proposed the experiments, sequencing technologies have tremendously evolved, and we felt that our original sequencing approach (bulk RNA-seq) had become obsolete. Sequencing technologies based on single-cell or single-nuclei sequencing, like the 10x Genomics multiome platform, have since been extensively used to characterize novel immune cell populations and its distinct cell states from different tissues at single-cell resolution. This technology would fully answer our scientific question (quantitative and qualitative effects of intra-tissue steroids immune cell infiltrates in ACC) and would abrogate the need for performing immunophenotyping by IHC. The UofM Advanced Genomics Core performs the 10x multiome (RNAseq+ATAC) assay as routine. A pilot study from our group using normal adrenal glands demonstrated the presence of a diverse population of immune cells, including distinct subpopulations of macrophages and T-cells. This assay is compatible with frozen tumor tissues from our repository. Therefore, we proposed a modification to our experimental plan (Aim1) as described:

- Single-nuclei multiome (RNAseq+ATAC) in 6 ACC samples – 3 cortisol-producing and 3 hormonally-silent ACC (to be performed at the University of Michigan Advanced Genomics Core)
- Intra-tissue steroid quantification by GC-MS/MS (to be performed by Dr. Man-Ho Choi, South Korea Institute of Science and Technology)

COVID-19 also negatively impacted Specific Aim 2 due to restrictions to lab access/lab resources. We found out that original Y1 cells are CYP21-deficient and therefore do not produce corticosterone (which is essential for our proposed *in vivo* and *in vitro* downstream studies). We were able to overcome this issue with support of our collaborator, Dr. Celso Gomez-Sanchez (University of Mississipi Medical Center), who has recently developed a CYP21-expressing/corticosterone-producing Y1 cell line. Dr. Gomez-Sanchez kindly provided these cells to us as a gift. While we successfully established the breeding colonies to generate the LAF1 mice (F1 generation of the crossing between B6 and A/HeJ), the A/HeJ animals exhibited fertility issues (a known problem of these mice) and we were not able to maintain this colony. Unfortunately, the A/HeJ strain (once available from cryorecovery) is now temporarily unavailable in the JAX Lab inventory. We therefore anticipate we won't be able to conduct our proposed in vivo experiments for Aim2.

6. **Products:** Nothing to report at this time.

7. Participants & Other Collaborating Organizations

Name: Gary D. Hammer
Role: Initiating Principal Investigator
Research Identifier: http://orcid.org/0000-0001-6843-3628
Nearest person month worked: 1
Contribution to Project: Dr. Hammer has supervised the study and lead meetings with all members of the research team.

Name: Richard J. Auchus
Role: Co-Investigator
Research Identifier: https://orcid.org/0000-0001-6815-6181
Nearest person month worked: 1
Contribution to Project: Dr. Auchus has shared his expertise regarding LC-MS/MS analysis of intra-tumoral and secreted steroids in frozen tissue samples and cell lines (respectively) during team meetings.

Name: Venkateshwar Keshamouni
Role: Co-Investigator
Research Identifier: https://orcid.org/0000-0003-1947-791X
Nearest person month worked: 1
Contribution to Project: Dr. Keshamouni has shared his expertise regarding in vitro and in vivo immune/tumor cell studies in team discussions.

Name: Antonio M. Lerario
Role: Co-Investigator
Research Identifier: https://orcid.org/0000-0002-8336-6432
Nearest person month worked: 1
Contribution to Project: Dr. Lerario has worked on optimizing sample acquisition for RNA-seq analysis and has shared bioinformatics expertise to evaluate study design and number and type of samples required for biological/clinical significance during team meetings.

Name: Dipika R. Mohan
Role: Graduate Student
Research Identifier: https://orcid.org/0000-0002-6334-9416
Nearest person month worked: 2
Contribution to Project: Ms. Mohan has worked with Dr. Lerario to optimize sample acquisition for RNA-seq analysis and evaluate study design, and has worked with Ms. Brand to obtain IRB approval. Ms. Mohan has also worked on extraction of ACC cell line secreted steroids with members of Dr. Auchus's team. Finally, Ms. Mohan has completed mouse training and helped with submitting amendments for the University of Michigan IACUC protocol and ACURO.

Name: Lauren Koch
Role: Graduate Student
Research Identifier: https://orcid.org/0000-0003-2276-9251
Nearest person month worked: 2
Contribution to Project: Ms. Koch is conducting the in vitro experiments with the ACC cell lines to assess the effects of different steroidogenesis inhibitors on the steroidomics of conditioned media, which will be used in CD8+ T-cell activation experiments.

Name: Sarah Brand
Role: Coordinator of A5
Research Identifier: N/A
Nearest person month worked: 1
Contribution to Project: Ms. Brand has led submitting and obtaining IRB approval, and will also assist in enrolling and consenting patients at the University of Michigan and managing clinical data.

8. **Special Reporting Requirements:** Not applicable.

9. **Appendices:** Please see subsequent pages in the appendix for the following information –

- Figure 1
- Figure 2
- Table 1
- Figure 3
- Figure 4
- Figure 5

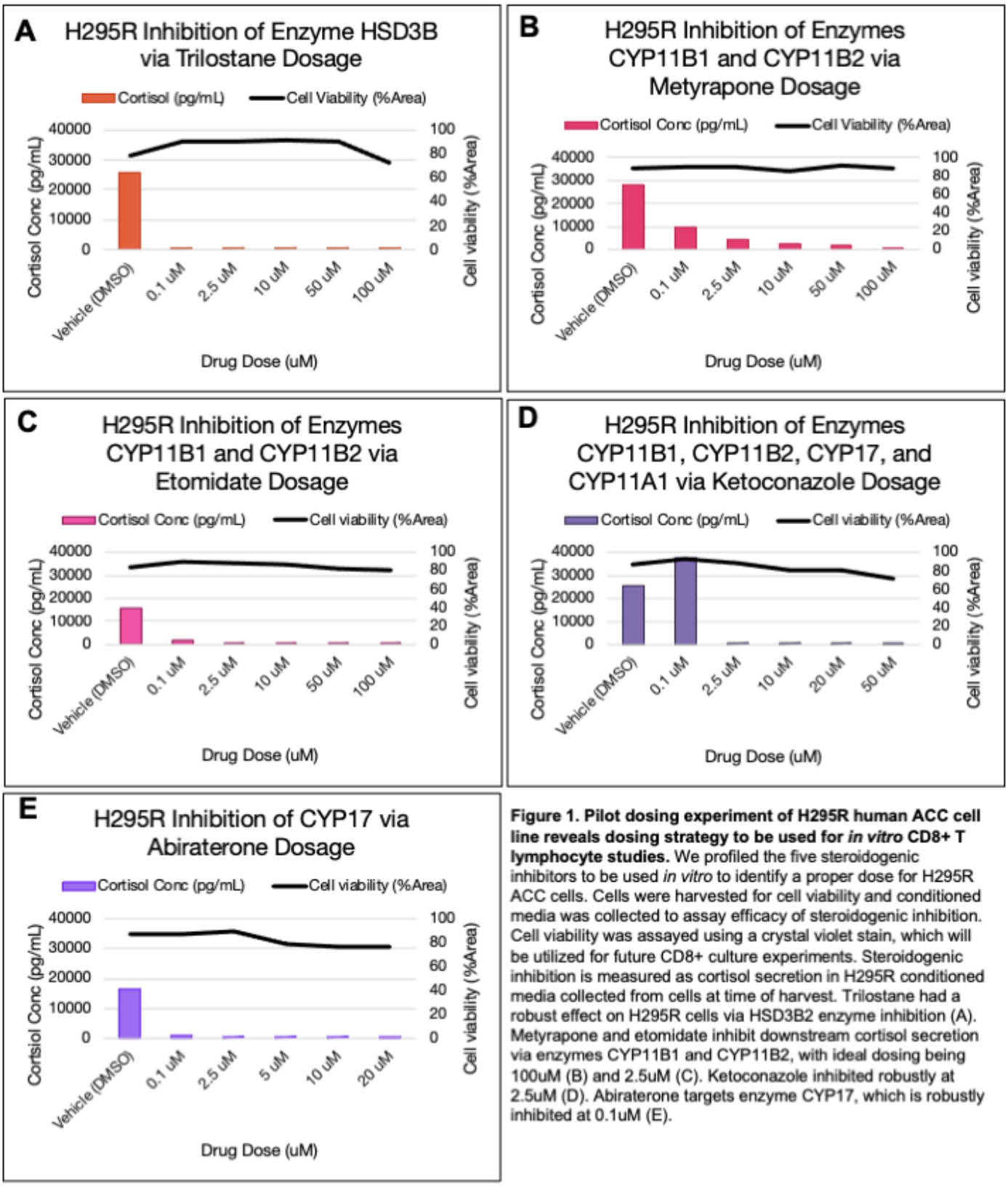

Figure 1. Pilot dosing experiment of H295R human ACC cell line reveals dosing strategy to be used for *in vitro* CD8+ T lymphocyte studies. We profiled the five steroidogenic inhibitors to be used *in vitro* to identify a proper dose for H295R ACC cells. Cells were harvested for cell viability and conditioned media was collected to assay efficacy of steroidogenic inhibition. Cell viability was assayed using a crystal violet stain, which will be utilized for future CD8+ culture experiments. Steroidogenic inhibition is measured as cortisol secretion in H295R conditioned media collected from cells at time of harvest. Trilostane had a robust effect on H295R cells via HSD3B2 enzyme inhibition (A). Metyrapone and etomidate inhibit downstream cortisol secretion via enzymes CYP11B1 and CYP11B2, with ideal dosing being 100uM (B) and 2.5uM (C). Ketoconazole inhibited robustly at 2.5uM (D). Abiraterone targets enzyme CYP17, which is robustly inhibited at 0.1uM (E).

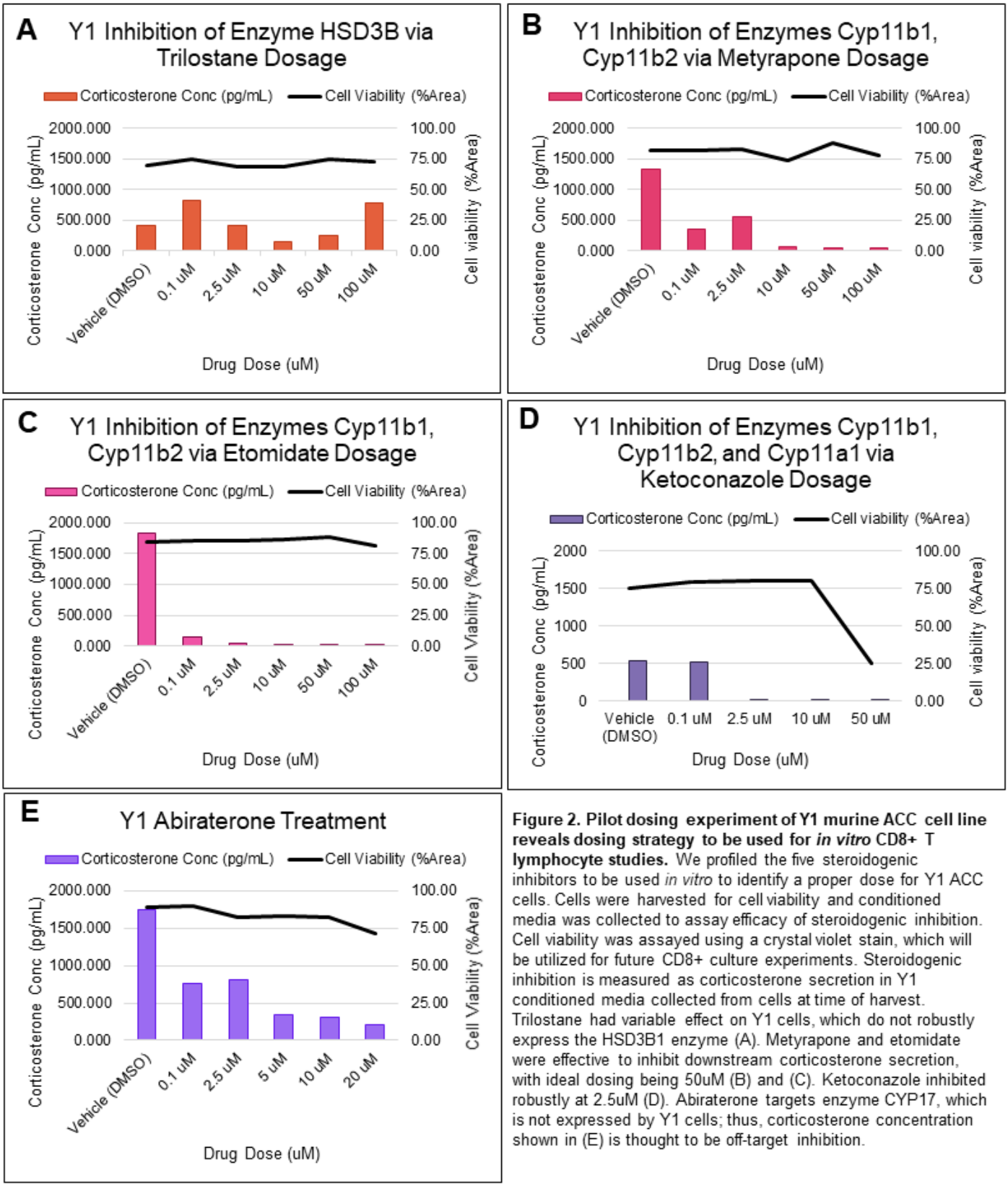

Figure 2. Pilot dosing experiment of Y1 murine ACC cell line reveals dosing strategy to be used for *in vitro* CD8+ T lymphocyte studies. We profiled the five steroidogenic inhibitors to be used *in vitro* to identify a proper dose for Y1 ACC cells. Cells were harvested for cell viability and conditioned media was collected to assay efficacy of steroidogenic inhibition. Cell viability was assayed using a crystal violet stain, which will be utilized for future CD8+ culture experiments. Steroidogenic inhibition is measured as corticosterone secretion in Y1 conditioned media collected from cells at time of harvest. Trilostane had variable effect on Y1 cells, which do not robustly express the HSD3B1 enzyme (A). Metyrapone and etomidate were effective to inhibit downstream corticosterone secretion, with ideal dosing being 50uM (B) and (C). Ketoconazole inhibited robustly at 2.5uM (D). Abiraterone targets enzyme CYP17, which is not expressed by Y1 cells; thus, corticosterone concentration shown in (E) is thought to be off-target inhibition.

	DMSO	DMSO	Abir	Abir	Etom	Etom	Keto	Keto	Metyr	Metyr	Trilo	Trilo
Estriol	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
18-hydroxy-cortisol	40.2	47.7	29.6	30.4	31.7	28.2	33.2	29.3	32.5	30.5	42.1	28.9
18-oxo-cortisol	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Cortisol	4287.8	4641.7	3429.6	3155	3498.7	3525.8	3893.6	3642.4	3302.6	3468.7	4516.3	3499.9
18-hydroxy-corticosterone	64.6	67.9	47.9	45	48.4	48.1	47.1	48.7	53	53.3	66.6	48.5
Cortisone	102.2	103.8	84.2	77.5	88.6	77.5	87.2	75	75.9	76.5	126.9	82.5
21-deoxy-cortisol	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Aldosterone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Estradiol	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
11-hydroxy-progesterone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	330.1	147.6
11-keto-testosterone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
11-deoxy-cortisol	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
11-hydroxy-androstenedione	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
11-keto-androstenedione	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Corticosterone	72	NQ	53.4	NQ	46.9	16.9	53.5	NQ	38.6	NQ	145.9	91.8
16-hydroxy-progesterone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Estrone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Testosterone	42.6	34.1	33.7	28.1	28.9	25.7	34.2	27.3	31.4	31	125.7	86.7
17-hydroxy-progesterone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
11-hydroxy-progesterone	4779.6	14508.5	3386.6	5065.5	32.2	62.4	41.4	106.9	177.9	321.6	1110.9	3738.2
11-keto-progesterone	336.9	968.1	261.4	429.8	NQ	11.4	NQ	22	NQ	33.7	57.1	209.6
Androstenedione	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
11-deoxy-corticosterone	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ	62.1	NQ
Progesterone	38418.9	46643.8	31188.9	18986.5	146.3	119.7	1178	4025.3	30149.4	28921.1	11120.4	13887.7
Prog./11-OH-prog	8.03	3.2	9.21	3.74	4.54	1.91	28.48	37.67	169.47	89.92	10.01	3.72

Table 1: LC-MS/MS quantification of different steroids, precursors, and metabolites in the Y1 cells at baseline (DMSO) and after treatment with different steroidogenesis inhibitors. Low levels of corticosterone and 11-deoxy-corticosterone associated with high levels of progesterone suggests 21-hydroxilase deficiency. Increase in progesterone/11-hydroxy-progesterone ratio upon treatment with ketoconazole and metyrapone suggests normal 11-beta-hydroxylase activity. Abir=abiraterone; Etom=etomidate; Keto=ketoconazole; Metyr=metyrapone, Trilo=trilostane

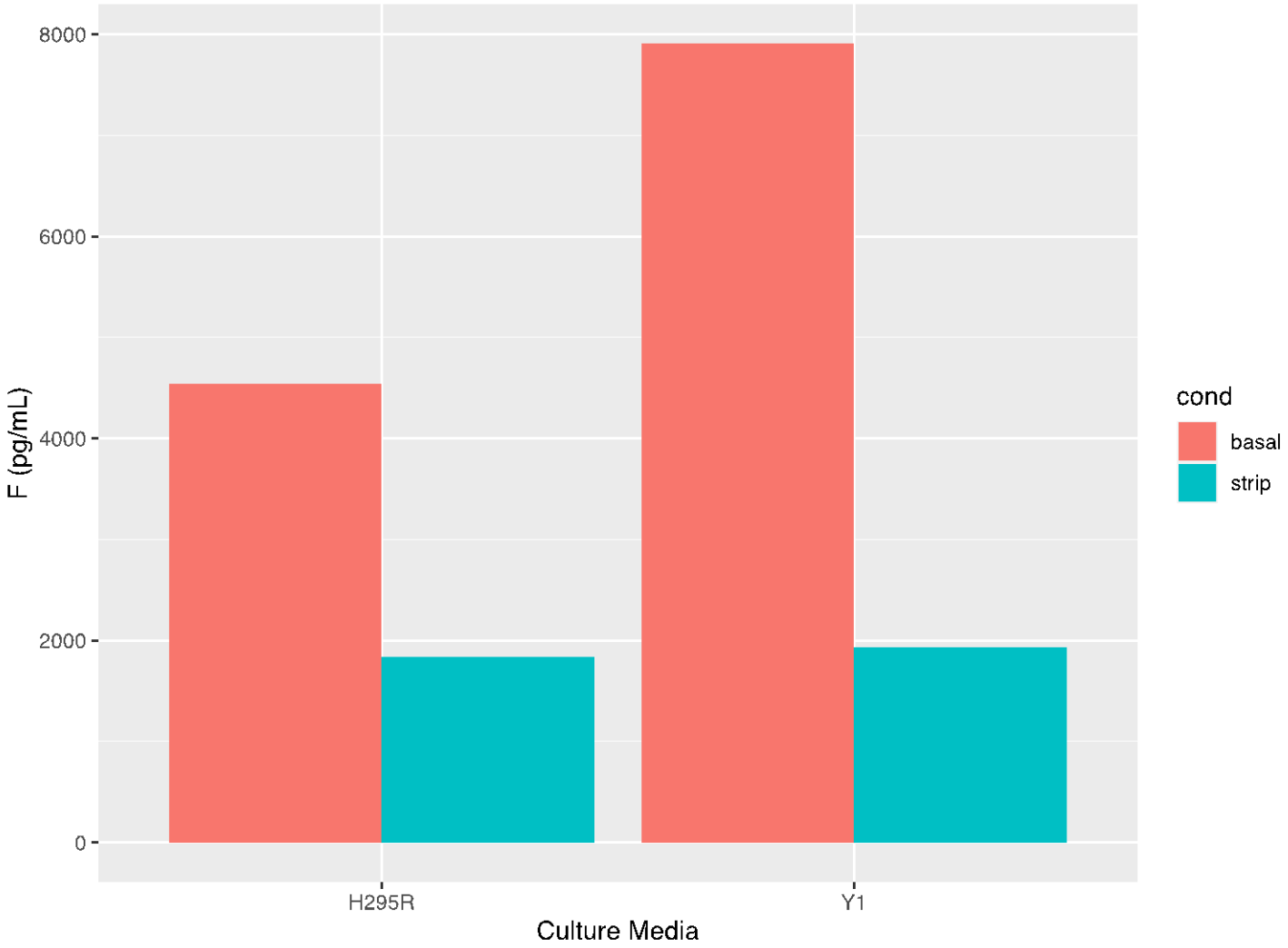

Figure 3: Cortisol (F) levels in the culture medias of human H295R and murine Y1 cell lines at baseline (red bars) and after activated charcoal stripping of the serum (teal bars). H295R media is comprised of DMEM-F12+10% Nu serum+1% ITS. Y1 media is comprised of DMEM+10% fetal calf serum (FCS)+horse serum (3:1). F levels were determined by ELISA.

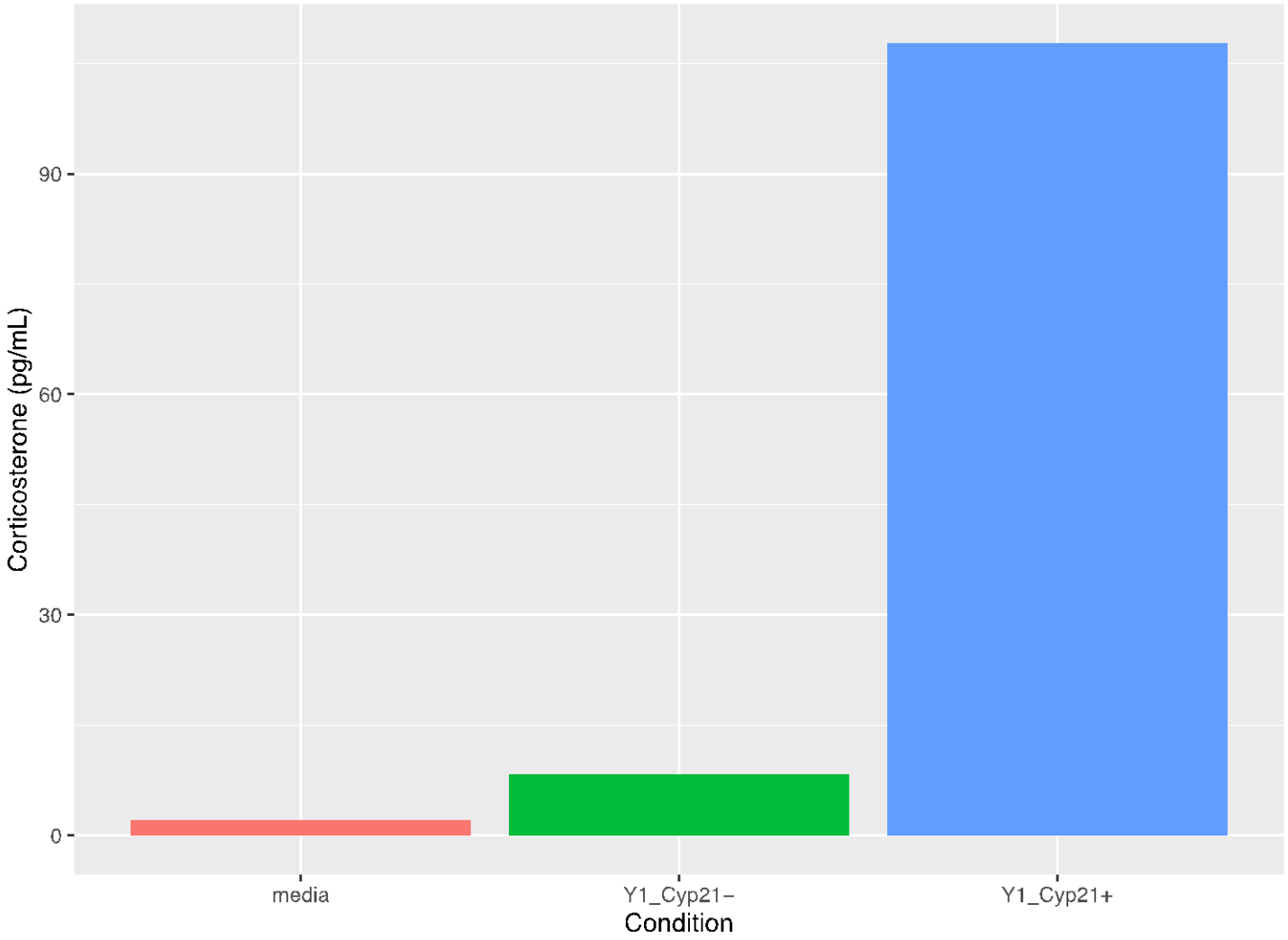

Figure 4: Corticosterone levels in the supernatants of Y1-Cyp21- (green) and Y1-Cyp21+ (blue) cells. Cell-free media is depicted in red. Corticosterone levels were determined by ELISA.

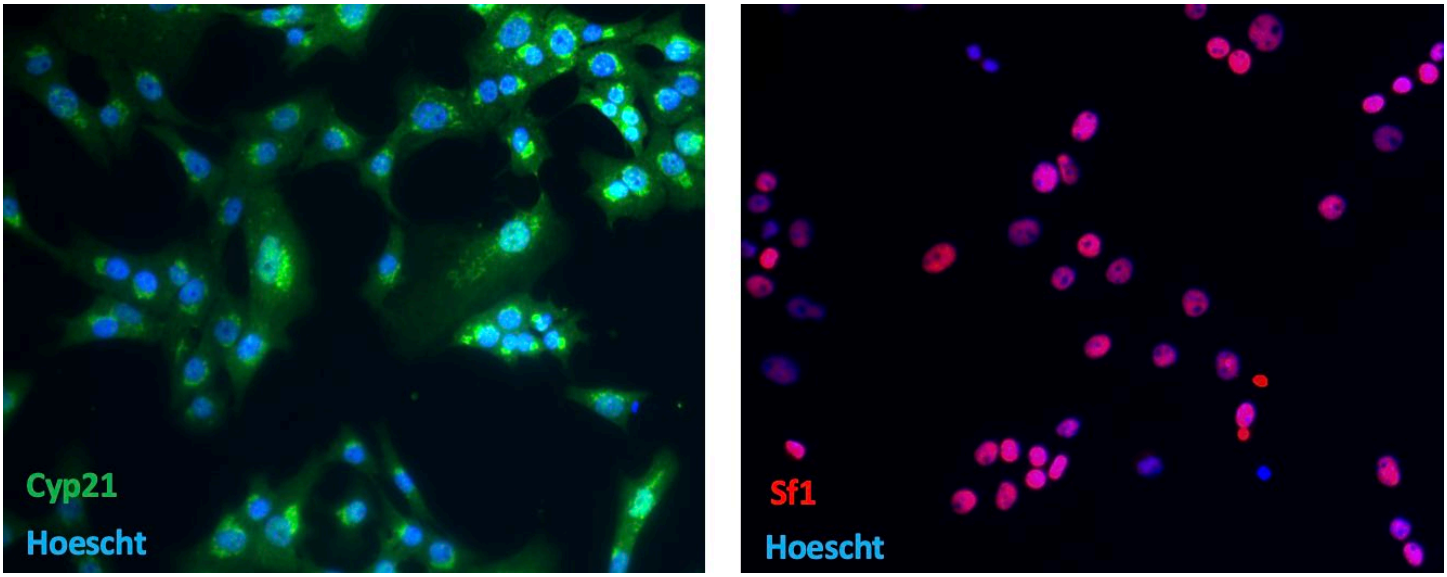

Figure 5: Expression of Cyp21 (green) and the adrenocortical lineage marker Sf1 (red) in the Y1-Cyp21+ cells demonstrated by Immunocytochemistry. Nuclear staining by Hoescht is depicted in blue.