

AWARD NUMBER: W81XWH-20-1-0148

TITLE: TYK2 as a Biomarker and Therapeutic Target for NF1-Associated Malignant Peripheral Nerve Sheath Tumors

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CONTRACTING ORGANIZATION: Washington University, St. Louis, MO

REPORT DATE: March 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE March 2023		2. REPORT TYPE Annual		3. DATES COVERED 01Mar2022-28Feb2023	
4. TITLE AND SUBTITLE TYK2 as a Biomarker and Therapeutic Target for NF1-Associated Malignant Peripheral Nerve Sheath Tumors				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-20-1-0148	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dr. Angela Hirbe E-Mail: hirbea@wustl.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Washington University in St. Louis Couch Building, Room 3304 660 S. Euclid Avenue Campus Box 8076 St. Louis, MO 63110				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT <p>Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas that account for ~5% of all soft tissue sarcomas. These tumors occur at an increased frequency in patients with the Neurofibromatosis Type 1 (NF1) cancer predisposition syndrome, but also occur sporadically or secondary to radiation therapy. In the setting of NF1, MPNST arise from malignant transformation of a benign precursor lesion, a plexiform neurofibroma. Despite aggressive therapy, the recurrence rate is high and the vast majority of people with these cancers will die within 5 years of diagnosis. Given limited treatment options, there is a pressing need to identify novel therapeutic targets. Our laboratory previously identified TYK2 as a gene mutated in a subset of MPNSTs. More recently, we have shown that genetic knockdown of TYK2 in both human and murine MPNST cell lines results in increased cell death <i>in vitro</i> and decreased tumor growth. Here, immunohistochemistry (IHC) shows strong TYK2 staining in 63/112 (56%) MPNST, 13/39 (33%) plexiform neurofibromas and 23/24 (96%) atypical neurofibromas (ANNUBP). In murine MPNST JW23.3 cells, knockout of <i>TYK2</i> and <i>TYK2/STAT3</i> markedly block proliferation, while <i>STAT3</i> knockout only modestly reduces proliferation, as assessed by IncuCyte live cell assays. Similarly, pharmacologic inhibition of TYK2, STAT3 and Bcl2 dose-dependently decreased proliferation and induced apoptosis over time. RNAseq pathway analysis on TYK2 inhibitor treated MPNST demonstrated decreased expression of cell cycle, mitotic, and glycolysis pathways. TYK2 inhibition resulted in upregulation of the MEK/ERK pathway gene expression, by both RNA-seq and qPCR array as well as increased pERK1/2 levels by WES Western system. The compensatory response was tested with dual treatment with TYK2 and MEK inhibitors, which synergistically decreased proliferation and increased apoptosis <i>in vitro</i>. Finally, combination therapy was shown to inhibit growth of MPNST in multiple <i>in vivo</i> models. Finally, combination therapy was shown to inhibit growth of MPNST tumors in multiple mouse models. These findings suggest that the combination of TYK2 and MEK inhibitory drugs serves as a promising therapeutic strategy in NF1-MPNST.</p>					
15. SUBJECT TERMS Malignant peripheral nerve sheath tumor, MPNST, tyrosine kinase-2, TYK2, neurofibromatosis-1, NF1, sarcoma, STAT3					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION:

Neurofibromatosis type 1 (NF1) is one of the most common inherited tumor predisposition syndromes, affecting 1:2500 individuals worldwide. Diagnosis is typically made during childhood and is associated with an increased risk of benign and malignant tumors. Approximately 1/3 of patients with NF1 will develop benign nerve sheath tumors called plexiform neurofibromas (PN), and almost half of these tumors will undergo malignant transformation to malignant peripheral nerve sheath tumors (MPNST), a highly aggressive sarcoma. Currently, there are no predictive biological markers of transformation, few therapeutic options for advanced disease, and dismal survival. Once diagnosed with MPNST, patients with localized disease are treated with curative-intent surgery as well as radiation therapy and chemotherapy. Unfortunately, patients with metastatic disease can rarely be cured, and treatment is limited to palliative chemotherapy. Treatment of PN is usually surgical and is generally limited to symptomatic cases given the high morbidity associated with these surgeries. As such, there is a pressing need to identify biomarkers for transformation to MPNST as well as novel therapeutic targets for both PN and MPNST.

2. KEYWORDS:

Malignant peripheral nerve sheath tumor, MPNST, tyrosine kinase-2, TYK2, neurofibromatosis-1 (NF1), sarcoma, STAT3

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Please see **Appendix A** for detailed major goals and target dates in the SOW:

Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?

Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?

Major Task 3: Determine whether STAT-3 mediated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.

Major Task 4: Explore the impact of TYK2 downregulation on known signaling nodes to be important in MPNST pathogenesis.

Major Task 5: Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.

Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability in vitro?

Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis in vivo in MPNST PDX lines?

Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis in vivo in mice with an intact immune system?

What was accomplished under these goals?

Please see **Appendix B**, **Appendix C** and **Appendix D** for major accomplishments.

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

Nothing to Report.

How were the results disseminated to communities of interest?

N/A

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

Please see **Appendix E** on project plans.

4. IMPACT:

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

The most common malignancy affecting adults with neurofibromatosis type 1 (NF1) is the malignant peripheral nerve sheath tumor (MPNST), a highly aggressive sarcoma that commonly develops from benign plexiform neurofibromas. In this regard, 8-13% of individuals with NF1 will develop these tumors during young adulthood. Currently there are few therapeutic options, and the vast majority of people with these cancers will die within 5 years of diagnosis. Moreover, there is a pressing need to identify accurate biological markers of plexiform neurofibroma malignant transformation. Leveraging next generation sequencing of NF1-MPNSTs, we recently identified Tyrosine Kinase 2 (TYK2) as a frequently mutated gene, whose function is critical for mouse MPNST survival *in vitro* and *in vivo*. Based on these exciting findings, we hypothesize that TYK2 promotes MPNST growth through inhibition of cell death and that TYK2 is a biomarker and therapeutic target for MPNSTs. In this proposal, we will assess the utility of TYK2 expression as a potential prognostic marker for MPNST progression. Additionally, we aim to define the mechanism by which TYK2 promotes MPNST development and growth using engineered primary MPNST cell lines *in vitro* and complementary patient derived xenograft models *in vivo*. Finally, we will assess the utility of therapeutically targeting TYK2. Given the dismal overall survival for patients with MPNSTs and the frequency of these cancers in NF1, there is a pressing need to develop more accurate markers of MPNST development and better targeted therapies for these malignancies. This work has the potential to lead to a biomarker driven therapeutic option and clinical trial for patients in desperate need of an effective therapy.

What was the impact on other disciplines?

The development of new therapeutics and identification of biomarkers in our studies have the potential to be extended to other types of sarcomas to potentially develop more effective therapies.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

As a result of variability in the short-hairpin system in genetic knockdown, we are now moving to the CRISPR/Cas9 system for Aim 2 as it provides a better genetic knockout and has become a more widely accepted technique than shRNA.

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

With the Covid-19 pandemic, in-person laboratory activities at Washington University were restricted starting in March 2020. While the grant official start date was March 1, 2020, we were unable to begin work on this grant until July 1, 2020. Covid-19 restrictions limited our lab to 50% capacity between July 1-September 1, 2020, and to 80% capacity between September 1, 2020, to March 1, 2021.

For Aim 1, the commercial antibody against TYK2 (ab39550, Abcam) used in our previous immunohistochemistry (IHC) studies became unavailable during the pandemic. Thus, other antibodies for TYK2 were evaluated and one antibody (ab223733, Abcam) was subsequently optimized for concentration and other IHC conditions before being used on MPNST patient samples.

As a result of variability in the short-hairpin system in genetic knockdown, we moved to the CRISPR/Cas9 system for Aim 2 as it provides a better genetic knockout and has become a more widely accepted technique than shRNA. Thus, we proceeded to Aim 3.1 earlier than planned, while switching to CRISPR/Cas9 from shRNA (Aim 2) and due to Covid-19 limitations on in-person staff.

Changes that had a significant impact on expenditures

Due to Covid-19 limitations on in-person staff, work did not begin on this grant until July 1, 2020. Thus, we will request a 6 month no-cost extension at the end of the 3-year grant period.

The new anti-TYK2 antibody for IHC (Aim 1) needs to be used at a higher concentration (1:100 instead of 1:500), which has increased costs for IHC. We are also transitioning from short hairpin RNA (shRNA) to the CRISPR/Cas9 system, which is more expensive, but also provides more consistent genetic knockout. The difference in cost for these experimental changes will be covered by the lab's start-up funding.

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

Significant changes in use or care of human subjects

For the studies in Aim 1, we obtained subject protocol approval by the Washington University (WU) Institutional Review Board (IRB) on February 28, 2020, and by the Human Research Protection Office (HRPO) in June, 2020, prior to this grant award. Annual renewals occur each year. No changes have been made since protocol approval.

Significant changes in use or care of vertebrate animals

For the mouse studies in Aim 3, we obtained protocol approval from IACUC (protocol #20-0117) in April, 2020 and from ACURO (NF190033.e001) in May, 2020. An amendment to our IACUC/ACURO animal protocol that added two drugs (TYK2 inhibitor, deucravacitinib, and MEK inhibitor, mirdametinib) was approved in March, 2022.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

• Publications, conference papers, and presentations

Journal publications.

Borcherding DC, Amin NV, He K, Zhang X, Lyu Y, Dehner C, Bhatia H, Gothra A, Daud L, Ruminski P, Pratilas CA, Pollard K, Sundby T, Widemann BC, Hirbe AC. MEK inhibition synergizes with TYK2 inhibitors in NF1-associated Malignant Peripheral Nerve Sheath Tumors. *Clinical Cancer Research*. 2023 Feb 17;CCR-22-3722. doi: 10.1158/1078-0432.CCR-22-3722. Epub ahead of print. PMID: 36799629.

Borcherding DC, He K, Amin NV, Hirbe AC. TYK2 in Cancer Metastases: Genomic and Proteomic Discovery. *Cancers*. 2021; 13(16):4171. <https://doi.org/10.3390/cancers13164171>

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

D.C. Borcherding, N. Amin, K. He, C. Dehner, P. Ruminski, S. Dahiya, J. Chrisinger, T. Sundby, K. Pollard, B.C. Widemann, C.A. Pratilas, J.F. Shern, and A.C. Hirbe. TYK2 is a Biomarker and Therapeutic Target in Malignant Peripheral Nerve Sheath Tumors. Poster presentation. Connective Tissue Oncology Society (CTOS) 2021 Annual Meeting. Vancouver, Canada (moved online).

• Website(s) or other Internet site(s)

1. Synapse NF-OSI project space (Synapse Project) for the Hirbe Lab for data related to this grant: <https://www.synapse.org/#!Synapse:syn23639889/wiki/607570>
2. Hirbe lab website at Washington University: <https://hirbelab.wustl.edu/>

• Technologies or techniques

Nothing to report.

• Inventions, patent applications, and/or licenses

Patent application in place through the Office of Technology Management at Washington University: U.S. Provisional Patent Application Serial No. 63/277,331, filed 11/09/2021; and U.S. Patent Application Serial No. 18/053,935, filed 11/09/2022 and entitled "Methods of Treating Cancer and Inhibiting TYK2 and MEK."

• Other Products

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Angela Hirbe, M.D./Ph.D.
Project Role: PI
Researcher Identifier (e.g. ORCID ID): 0000-0003-1719-0771
Nearest person month worked: 6
Contribution to Project: Dr. Hirbe coordinated and supervised all the research activities of this grant project.

Name: Dana Borcharding, Ph.D.
Project Role: Senior Scientist
Researcher Identifier (e.g. ORCID ID): 0000-0002-8849-2139
Nearest person month worked: 8
Contribution to Project: Dr. Borcharding conducted and supervised research activities and analyzed data in this grant project, including IncuCyte assays, IHC, western blotting, qPCR, transfections and mouse experiments.

Name: Sonika Dahiya, M.D.
Project Role: Professor, Pathology & Immunology
Researcher Identifier (e.g. ORCID ID): 0000-0002-5843-6646
Nearest person month worked: 1
Contribution to Project: Dr. Dahiya provided pathology expertise for IHC scoring.

Name: John Chrisinger, M.D.
Project Role: Assistant Professor, Pathology & Immunology
Researcher Identifier (e.g. ORCID ID): 0000-0002-7138-0923
Nearest person month worked: 1
Contribution to Project: Dr. Chrisinger provided pathology expertise for IHC scoring.

Name: Carina Dehner, M.D./Ph.D.
Project Role: Pathology Resident (Co-Chief)
Researcher Identifier (e.g. ORCID ID): 0000-0001-5214-4813
Nearest person month worked: 1
Contribution to Project: Dr. Dehner provided pathology expertise for IHC experiments and scoring.

Name: Kevin He
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1.5
Contribution to Project: Mr. He conducted IHC experiments and imaging.

Name: Neha Amin
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 1
Contribution to Project: Ms. Amin conducted IncuCyte experiments, western blotting and qPCR.

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

Nothing to Report.

What other organizations were involved as partners?

Dr. Christine Pratilas
John Hopkins University (JHU)
Baltimore, MD
Partner's contribution: Provided MPNST and plexiform neurofibroma specimens for IHC and human MPNST cell-lines.

Dr. Brigitte Widemann
National Cancer Institute (NCI)
Bethesda, MD
Partner's contribution: Provided MPNST and plexiform neurofibroma specimens for IHC.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: See Appendices A-E.

Appendix A: STATEMENT OF WORK

Grant Official Start Date: March 1, 2020

Note: No work started until July 1, 2020, and then only at 50% capacity until September 1st, followed by 80% capacity for September 1, 2020, to March 1, 2021, due to pandemic restrictions. New timeline assumes a no cost extension of 6 months due to the pandemic (extending to month 42).

Washington University
660 S. Euclid Ave, St. Louis, MO 63110
PI: Angela C Hirbe, MD, PhD

Specific Aim 1: We will determine whether TYK2 is a prognostic biomarker for MPNST.	Timeline (Original)	Washington University	Percent Complete	Timeline (New)
Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?	Months			Month
Subtask 1: ACURO/HRPO Approval	0	Dr. Hirbe (obtained prior to funding)	100%	0
Subtask 1: Re-review of all 60 MPNST cases with Dr. Dahiya and Chrisinger	0-3	Dr. Hirbe	100%	0-9
Subtask 2: Generation of unstained slides and clinical chart review	3-6	Dr. Hirbe	100%	3-9
Subtask 3: TYK2 staining	6-8	Dr. Hirbe	100%	6-18
Subtask 4: Blinded TYK2 scoring by Dr. Dahiya, Chrisinger	8-10	Dr. Hirbe	100%	10-21
Subtask 5: Statistical analysis	10-12	Dr. Hirbe	100%	12-36
<i>Milestone(s) Achieved: Determine whether TYK2 expression is correlated with clinical prognostic parameters.</i>	12	Dr. Hirbe	100%	36
Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?				
Subtask 1: Re-review of all 45 cases of atypical neurofibroma and low grade MPNST cases by Dr. Dahiya and Chrisinger	0-3	Dr. Hirbe	100%	0
Subtask 2: Generation of unstained slides and clinical chart review	3-6	Dr. Hirbe	100%	3-9
Subtask 3: TYK2 staining	6-8		100%	6-18
Subtask 4: Blinded TYK2 scoring by Dr. Dahiya, Chrisinger	8-10	Dr. Hirbe	100%	10-21
Subtask 5: Statistical analysis	10-12	Dr. Hirbe	100%	12-36
<i>Milestone(s) Achieved: Determine whether TYK2 can be used to distinguish atypical neurofibromas from MPNSTs.</i>	12	Dr. Hirbe	100%	36

Specific Aim 2: We will delineate the mechanism of action of TYK2 in MPNST cells.				
Major Task 3: Determine whether STAT-3 mediated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.				
Subtask 1: Generate and test <i>shSTAT3</i> , <i>shBcl2</i> , and <i>shLacZ</i> control lentivirus	0-4	Dr. Hirbe	75%	4-42
Subtask 2: Incubate cell growth and death assays using lentivirus generated in subtask 1. Cell lines: JW23.3 murine MPNST cells (initially obtained from Dr. Karlyne Reilly), human JH 2-002 cells (obtained from Dr. Christine Pratilas at Johns Hopkins University)	4-8	Dr. Hirbe	50%	16-42
Subtask 3: Incubate cell growth and death assays using napabucasin (STAT3 inhibitor) and venetoclax (Bcl2 inhibitor) compared to vehicle Cell lines: JW23.3 murine MPNST cells, human JH 2-002 cells	8-12	Dr. Hirbe	100%	10-14
Subtask 4: Evaluation of type of cell death Cell lines: JW23.3 murine MPNST cells, human JH 2-002 cells	12-16	Dr. Hirbe	0%	36-42
<i>Milestone(s) Achieved: Understand the mechanism of TYK2 in MPNST growth and survival.</i>	16	Dr. Hirbe	70%	42
Major Task 4. Explore the impact of TYK2 downregulation on known signaling nodes to be important in MPNST pathogenesis.				
Subtask 1: Western blot analysis to evaluate activation of pathways involved in neurofibromin signaling Cell lines: JW23.3 murine MPNST cells, human JH 2-002 cells	16-20	Dr. Hirbe	75%	6-42
<i>Milestone(s) Achieved: Determine whether TYK2 signaling intersects with neurofibromin signaling.</i>	20	Dr. Hirbe	75%	42
Major Task 5. Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.				
Subtask 1: RNA extraction <i>shTYK2</i> and <i>shLacZ</i> control cells was the initial plan. We have since generated CRISPR knockout to obtain complete knockdown. Cell lines: JW23.3 murine MPNST cells, human JH 2-002 cells	20-21	Dr. Hirbe	100%	4-28
Subtask 2: RNA sequencing and analysis.	21-24	Dr. Hirbe	100%	6-36
<i>Milestone(s) Achieved: Determine the global expression changes upon knockdown of TYK2.</i>	24	Dr. Hirbe	100%	36

Specific Aim 3: Evaluate pharmacologic inhibition of TYK2 with in MPNSTs <i>in vivo</i>.				
Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability <i>in vitro</i> ?				
Subtask 1: Incucyte cell growth and death assays using the TYK2 inhibitors TC JL37, WU12, and WU76. Cell lines: JW23.3 murine MPNST cells, human MPNST 724 cells, JH 2-002 cells	16-20	Dr. Hirbe	100%	2-8
Subtask 2: Statistical analysis	20-21	Dr. Hirbe	100%	6-8
<i>Milestone(s) Achieved: Determine IC₅₀ for TYK2 inhibitors in MPNST cell lines</i>	21	Dr. Hirbe	100%	8
Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis <i>in vivo</i> in MPNST PDX lines?				
Subtask 1: Sciatic nerve injections Patient Derived Xenograft lines: MPNST PDX1, MPNST PDX2, MPNST PDX3, MPNST PDX4, MPNST PDX5 (described in background data, Dehner et al. JCI Insight)	21-25	Dr. Hirbe	100%	28-30
Subtask 2: Randomization for drug treatment	22-26	Dr. Hirbe	100%	30-32
Subtask 3: Monitoring tumor burden and response to treatment	22-32	Dr. Hirbe	100%	32-36
Subtask 4: Statistical analysis and histopathological correlations (Drs. Dahiya and Chrisinger)	32-36	Dr. Hirbe	100%	34-36
<i>Milestone(s) Achieved: Determine whether TYK2 inhibition reduces MPNST tumor growth in a genomically heterogenous set of patient derived lines.</i>	36	Dr. Hirbe	100%	36
Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis <i>in vivo</i> in mice with an intact immune system?				
Subtask 1: Maintenance of <i>Nfl</i> heterozygous and wildtype mice	1-36	Dr. Hirbe	100%	1-36
Subtask 2: Injection of <i>Nfl</i> and wildtype mice	21-24	Dr. Hirbe	100%	25-28
Subtask 2: Monitoring tumor burden and response to treatment	24-33	Dr. Hirbe	100%	26-30
Subtask 3: Histopathological correlations (Drs. Dahiya and Chrisinger)	33-36	Dr. Hirbe	100%	30-33
<i>Milestone(s) Achieved: Determine whether TYK2 inhibition reduces MPNST growth in the context of an intact immune system and within the <i>Nfl</i> heterozygous context</i>	36	Dr. Hirbe	100%	33

Appendix B: What was accomplished under these goals?

Major Accomplishments:

Notes: While the grant official start date was March 1, 2020, no work started until July 1, 2020 given the shutdown necessitated by the pandemic. Due to pandemic restrictions, our lab was only at 50% capacity for July 1-September 1, 2020, and then at 80% capacity for September 1, 2020, to March 1, 2021.

Most data presented in this report was recently published in *Clinical Cancer Research* (Borcherding *et. al.* 2023, **Appendix D**).

Specific Aim 1: We will determine whether TYK2 is a prognostic biomarker for MPNST.

Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?

Accomplishments: We obtained ACURO/HRPO approval for these studies and completed a clinical re-review of all 60 MPNST cases. We also generated unstained slides and H&E stained slides of MPNST tumors from these patients at Washington University and from our collaborators at John Hopkins University (JHU), the National Cancer Institute (NCI), and the University of California at San Francisco (UCSF) (**Table 1**).

The commercial antibody against TYK2 (ab39550, Abcam) used in our previous immunohistochemistry (IHC) studies became unavailable during the pandemic. Thus, other antibodies for TYK2 were evaluated and the lead antibody (ab223733, Abcam) was optimized for concentration and other IHC conditions.

IHC for TYK2 has been completed on 112 MPNST samples from Washington University, JHU, NCI and UCSF patients (**Table 1**). Three independent observers scored these slides in a blinded fashion (**Fig. 1**). Moderate to strong TYK2 staining (score ≥ 2) was observed in 56% of high-grade MPNST samples, with 44% having weak or negative TYK2 staining (**Table 1**). Sporadic MPNST had a higher rate of strong TYK2 staining than NF1 MPNST samples, at 62% vs. 53% strong TYK2 (**Table 1**). We then compared overall survival for MPNST patients vs. TYK2 levels by Kaplan Meier analysis (**Fig. 1B-D**). For NF1 MPNST samples, weak/negative TYK2 (<2) was associated with poorer overall survival (**Fig. 1C**), with a median overall survival of 28 months vs. 100 months for strong TYK2 (≥ 2). In contrast, for sporadic MPNST, weak/negative TYK2 (<2) was associated with better overall survival (**Fig. 1D**), with a median overall survival of 94 months vs. 27 months for strong TYK2 (≥ 2).

Tumor Type	Weak TYK2 (<2)	Strong TYK2 (≥ 2)
MPNST (all)	49 (44%)	63 (56%)
MPNST: NF1	33 (47%)	37 (53%)
MPNST: Sporadic	16 (38%)	26 (62%)
Plexiform Neurofibroma	26 (67%)	13 (33%)
ANNUBP	1 (4%)	23 (96%)

Table 1. Immunohistochemistry (IHC) for TYK2 protein levels in patient tumor samples. MPNST, plexiform neurofibroma, and ANNUBP samples were stained for TYK2 and the proportion of positive staining was scored on a 0-3 scale. Modified from Borcherdig *et. al.* Clinical Cancer Research. 2023.

Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?

Accomplishments: Our inventory list quoted in the grant included cases we identified in our pathology clinical database. However, upon requesting cases, a significant number of cases did not have tissue available. As such, we obtained cases from our collaborators to reach the goals set by our power calculations. Slides were obtained from Johns Hopkins University given that an MTA was already in place. An MTA was put in place with NCI and they also provided slides. All cases of plexiform neurofibroma, atypical neurofibroma and low grade MPNST have been re-reviewed with pathology. We have conducted IHC for TYK2 on 112 MPNST, 24 Atypical neurofibromatous neoplasm of uncertain biologic potential (ANNUBP), and 39 Plexiform Neurofibroma samples from Washington University, JHU, NCI and UCSF patients (**Fig. 1**). These samples have been scored by three independent observers in a blinded fashion, and we are now completing statistical analyses. Plexiform neurofibromas were largely weak/negative for TYK2 (67%), while most of the ANNUBP tumors (96%) were strongly positive for TYK2 (**Table 1**). This suggests that TYK2 may be a transformative factor from benign precursor tumor to MPNST.

Specific Aim 2: We will delineate the mechanism of action of TYK2 in MPNST cells.

Major Task 3: Determine whether STAT-3 mediated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.

Accomplishments: We initially generated lentivirus for mouse and human sh*STAT3*, sh*Bcl2* and sh*LacZ* control to transduce MPNST cells to produce stable shRNA lines, and conducted initial knockdown experiments in human MPNST-724 cells and mouse JW23.3 cells. However, due to variability in the short-hairpin system in genetic knockdown, we moved to the CRISPR/Cas9 system as it provides a better genetic knockout and has become a more widely accepted technique than shRNA.

We have generated colonies for two MPNST cell-lines (murine JW23.3 *Nf1/Trp53*-mutant and human JH-2-002) with CRISPR/Cas9 knockout of *TYK2*, *STAT3*, or double knockout (DKO) of both genes. The JH-2-002 cell line was added to ensure the translatability of our results. Knockout of *TYK2* and/or *STAT3* were verified by next generation sequencing (NGS), by qPCR for mRNA gene expression and/or by WES western blotting system (Protein Simple) for protein levels (**Fig. 2A-B**).

The effect of genetic depletion of *TYK2*, *STAT3* or the combination over time was evaluated by IncuCyte Zoom Live Cell proliferation assays (Sartorius). In JW23.3 cells, *TYK2* knockout (T15-4) dramatically inhibited proliferation to less than 25% of control cells, with DKO of *TYK2* and *STAT3* (TS14-10) having a similar inhibitory effect (**Fig. 2B**). However, *STAT3* knockout (S13-3) only modestly decreased proliferation to about 50-75% of control over time, suggesting that TYK2

proliferative effects may only be partially mediated through STAT3 (**Fig. 2B**). *TYK2* knockout modestly increased the number of apoptotic cells, but *STAT3* knockout or DKO did not affect apoptosis (**Fig. 2B**). We plan to evaluate proliferation and apoptosis for CRISPR knockout lines (*TYK2*, *STAT3* and DKO) in JH 2-002 cells by IncuCyte Live Cell assays. Additionally, we will evaluate which other STAT proteins may be mediating the downstream effects of *TYK2*.

MPNST Cell-line	Drug	IC50
JW23.3	WU-12	27.7 μ M
JW23.3	WU-76	18.7 μ M
JW23.3	Napabucasin (NP)	0.34 μ M
JW23.3	Venetoclax (ABT-199)	10.3 μ M
JW23.3	Mirdametininib (PD0325901)	1.22 μ M
MPNST-724	WU-12	32.1 μ M
MPNST-724	WU-76	13.9 μ M
MPNST-724	Napabucasin (NP)	0.04 μ M
MPNST-724	Venetoclax (ABT-199)	1.10 M
MPNST-724	Mirdametininib (PD0325901)	0.28 μ M
JH 2-002	WU-12	26.5 μ M
JH 2-002	WU-76	30.8 μ M
JH 2-002	Napabucasin (NP)	0.30 μ M
JH 2-002	Venetoclax (ABT-199)	*undetermined
JH 2-002	Mirdametininib (PD0325901)	0.22 μ M

Table 2. IC50 concentrations for drugs inhibiting *TYK2*, *STAT3* and *Bcl-2* in MPNST cell-lines. *undetermined value above dose curve upper threshold. Modified from Borcharding *et. al.* Clinical Cancer Research. 2023.

To further examine the role of *STAT3* and *Bcl-2* in MPNST cell proliferation and apoptosis, JW23.3 cells were incubated for 72 hours with the *STAT3* inhibitor, napabucasin (NP), or *Bcl2* inhibitor, venetoclax (ABT-199). Napabucasin and venetoclax both dose-dependently decreased cell confluence over time, which corresponded with increased cell death, as determined by incorporation of the green fluorescent dye YOYO-1 iodide and IncuCyte Zoom (Sartorius) analysis (**Fig. 3**). Thus, pharmacologic inhibition of *STAT3* and *Bcl2* mimics the anti-proliferative and pro-apoptotic effects that we previously reported with *TYK2* genetic deficiency. These initial studies are needed to identify the IC50 for each drug in each cell line. Similarly, the *STAT3* and *Bcl2* inhibitors decreased proliferation in two other MPNST cell-lines: JH-2-002 and MPNST-724 (**Table 2**). IC50s were calculated for inhibition of cell confluence by the drugs in each cell-line using GraphPad Prism software (**Table 2**).

Our initial data indicated that genetic depletion of *TYK2* resulted in both decreased proliferation and increased apoptosis. However, expanded studies in a larger panel of cell-lines and with *TYK2* inhibitory drugs revealed a greater effect on proliferation than on apoptosis. Thus, we are focusing on proliferation and survival currently. We may turn back to evaluating cell death mechanisms, i.e. via *Bcl-2* knockout and apoptotic assays at a later time as the true effect of *TYK2* is likely multifactorial.

Major Task 4: Explore the impact of TYK2 downregulation on signaling nodes known to be important in MPNST pathogenesis.

Accomplishments: In MPNST, the loss of neurofibromin leads to overactivation of Ras and downstream activation of MEK and mTOR. To investigate the interaction of TYK2 with these signaling pathways, we evaluated the activation of STAT3, ERK, and S6K by western blot (WES system, Protein Simple) in MPNST cells using TYK2 inhibitor drugs. In JW23.3 and JH-2-002 cells, the TYK2 inhibitors WU-12 and WU-76 (both developed at Washington University) decreased pSTAT3 protein levels at 1, 24 and 48 hours, as evaluated by the WES western blotting system (**Fig. 4A-D**). Interestingly, pERK2 levels were strongly stimulated by 2-4 fold over time by treatment with TYK2 inhibitors (WU-12 and WU-76) in both cell-lines, suggesting that the cells are compensating for TYK2 suppression by upregulating the MEK/ERK pathway.

Major Task 5: Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.

Accomplishments: While generating CRISPR/Cas9 knockout cell-lines, we conducted a subset of qPCR pathway array and RNAseq experiments using specific TYK2 inhibitors, WU-12 and WU-76. We examined the effect of the TYK2 inhibitor WU-12 on gene expression in human JH-2-002 cells with a qPCR array targeted for JAK-STAT pathway-related genes (**Fig. 5A**). JH-2-002 cells were incubated with WU-12 or vehicle control (DMSO) for 48 hours, and RNA was isolated. A qPCR array was utilized to analyze changes in mRNA for 88 genes of interest (GOIs) related to the JAK-STAT pathway. Gene expression changes revealed a general upregulation of the MEK/ERK pathway (**Fig. 5A**). TYK2 inhibition significantly increased ERK pathway transcriptional output, including *Sprouty 4 (SPRY4)*, *SPRED1* and *Cyclin D1 (CCDN1)*, while decreasing inhibitors of the ERK pathway, including *CBL* and *CBLB*. Members of the P13K/AKT/mTOR pathway that were decreased with TYK2 blockade included *PIK3R3* and *SOS1/2* (**Fig. 2A**). WU-12 incubation also had variable effects on interleukins (IL) and related genes, including increasing *IL7R*, and *IL6R*, while decreasing *IL7*, *IL10RB*, and *oncostatin M receptor (OSMR)*. We validated these results for select significantly changed genes by qPCR using different primer sets (**Fig. 6A-B**). Thus, TYK2 inhibition induced a rapid and sustained increase in pERK1/2 protein levels and MEK/MAP-kinase pathway target gene expression, which we believe is a compensatory mechanism (**Fig. 5C**).

In addition, RNA was isolated from JW23.3 cells treated with vehicle control (DMSO), WU-12 or WU-76 for 48 hours and the impact of TYK2 inhibition on the global expression profile was determined by RNAseq. Pathway analysis revealed that inhibition of TYK2 stimulated KRAS signaling, GPCR pathways, and Oxidative Phosphorylation, while decreasing Cell Cycle, Mitotic, and Glycolysis pathways (**Fig. 5B**). Down-regulated genes involved in proliferation and the G1 to S phase transition include *Ccnd2*, *Cdkn1a/c*, *E2f3/4*, *Pole2* and *Mcm7* (**Fig. 6A**). Incubation with TYK2 inhibitors decreased STAT3 target gene expression (**Fig. 5C-D and 6C-D**), while increasing expression of genes in the MEK/ERK pathway, including *Hras*, *Nras*, *Map3k2/3*, *MAP2k2/4*, *Map2*, and *Dusp4* (**Fig. 5E-G and 6E-G**). These experiments provide important insight on the mechanism of TYK2 inhibitor action in MPNST and potential pathways that could be targeted in combination treatments in order to improve therapeutic efficacy.

Specific Aim 3: Evaluate pharmacologic inhibition of TYK2 with in MPNSTs *in vivo*.

Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability *in vitro*?

Accomplishments: Three MPNST cell-lines, JW23.3, JH-2-002 and MPNST-724, were incubated for 72 hours with potent TYK2 inhibitors developed at Washington University (WU-12, WU-76) or a control compound (WU-18). All three MPNST cell-lines expressed moderate to high levels of TYK2 protein (**Fig. 7A-B**). WU-12 and WU-76 dose-dependently decreased the percent cell confluence, while WU-18 had no effect, in all three MPNST cell-lines, as determined by an IncuCyte cell proliferation assay (**Fig. 7C-D and 8C**). Conversely, apoptosis was increased by treatment with high doses of WU-12 and WU-76 in MPNST cells, as assessed by an IncuCyte cell death assay with YOYO-1 dye (**Fig. 8A-B, D**). IC50s were calculated for inhibition of cell confluence by the TYK2 inhibitors in each cell-line (**Table 2**).

In order to expedite translation to the clinic, we subsequently tested a specific TYK2 inhibitor, deucravacitinib (BMS-986165), and a pan-JAK/TYK2 inhibitor, baricitinib. Both deucravacitinib and baricitinib are used clinically in patients. Deucravacitinib has completed Phase 3 clinical trials and the FDA has accepted the application for approval for treatment of plaque psoriasis. The TYK2 inhibitor, deucravacitinib, dose-dependently reduced JW23.3 and JH-2-002 cell proliferation with IC50 values of 6.76 μM and 26.15 μM , respectively (**Fig. 9A and C**). In contrast, baricitinib decreased cell proliferation with IC50 values of 27.3 μM and 39.91 μM , respectively (**Fig. 9B and D**). Baricitinib is specific for JAK1/2 as well as TYK2 at higher doses. These results indicate that the inhibition of MPNST cell proliferation is predominantly mediated through TYK2, and not the other JAKs. Interestingly, the specific TYK2 inhibitor, deucravacitinib, was more potent in NF1-MPNST cells (JW23.3 and JH-2-002) than in sporadic MPNST cells (MPNST-724), while similar potency was observed for deucravacitinib and baricitinib in sporadic MPNST-724 cells (**Fig. 9E-F**).

Given that inhibition of TYK2 stimulates the MEK/MAP-kinase pathway, we investigated whether adding a MEK inhibitor improved the efficacy of TYK2 inhibitor drugs. Treatment of JW23.3 cells with a MEK inhibitor, mirdametinib, dose-dependently increased the percent inhibition of cell confluence after 72 hours (**Table 2 and Fig. 10A**). The combination of the TYK2 inhibitor, deucravacitinib, and the MEK inhibitor, mirdametinib, synergistically inhibited cell proliferation and increased apoptosis in JW23.3 cells (**Fig. 10**). Similarly, the combination of deucravacitinib and mirdametinib synergistically decreased proliferation and stimulated apoptosis in JH-2-002 and MPNST-724 cells (**Fig. 11 and 12**).

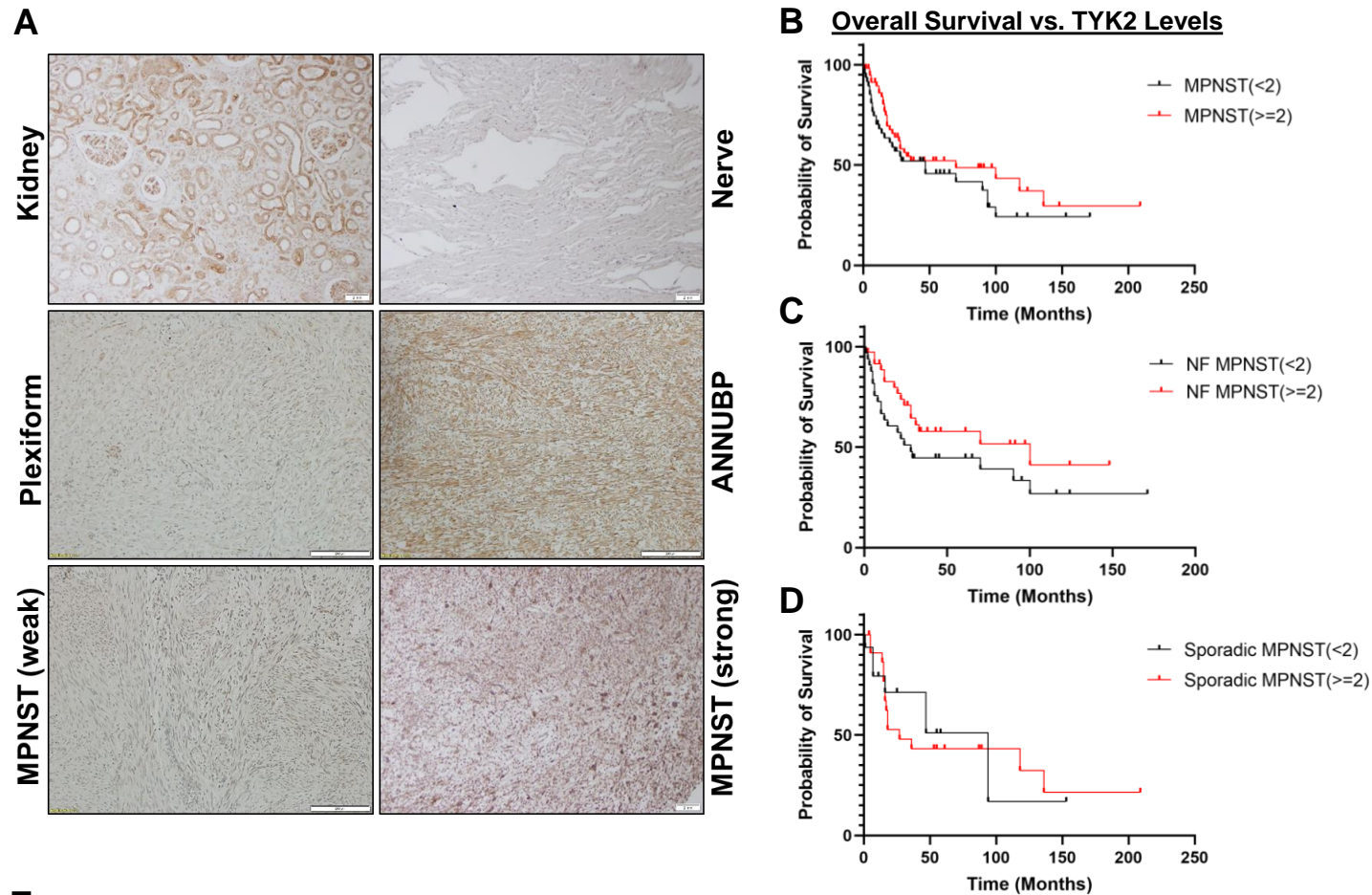
Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in MPNST PDX lines?

Accomplishments: Patient Derived Xenograft (PDX) lines were maintained in mice and *in vitro* cultures. We examined the combination of TYK2 and MEK inhibitors in mice with MPNST patient derived xenograft (PDX) tumors (**Fig. 13A**). Mice were randomized into 4 groups: (1) vehicle control, (2) mirdametinib, (3) deucravacitinib (BMS-986165), and (4) the combination of mirdametinib and deucravacitinib. Consistent with *in vitro* synergy studies, the drug combination

of mirdametinib, a MEK inhibitor, or deucravacitinib, a TYK2 inhibitor, inhibited human WU-386 MPNST PDX tumor growth significantly to about 25% of control, and was more effective than either of the single agents (**Fig. 13C**). For treatment with mirdametinib or deucravacitinib on their own, there was a non-significant trend toward decreased tumor size (**Fig. 13C**). In a tumor model using human JH-2-002 MPNST cell line xenografts, combination TYK2 and MEK inhibition significantly reduced tumor growth compared to control or either drug alone (**Fig. 13D**).

Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in mice with an intact immune system?

Accomplishments: In immunocompetent mice implanted with murine JW23.3 (*Nf1/Trp53*-mutant) MPNST cells, treatment with mirdametinib or deucravacitinib alone significantly reduced tumors to nearly half the volume of vehicle control (**Fig. 13B**). The combination of mirdametinib and deucravacitinib decreased tumor growth to less than one-third of control, and was significantly more effective than either drug alone (**Fig. 13B**). Thus, the combination of drugs inhibiting TYK2 and MEK acted synergistically to improve the therapeutic efficacy in both murine and human MPNST models. Mice given the mirdametinib/deucravacitinib drug combination did not significantly lose weight (**Fig. 14**) or show adverse health effects in any of the mouse models.



E

Characteristics	MPNST TYK2 Strong (Proportion ≥ 2)	MPNST TYK2 Weak (Proportion < 2)	MPNST	Plexiform Neurofibromas
Sex—no.				
Male	23	26	49	21
Female	40	23	63	18
Age Category—no.				
<18 yo	6	7	13	16
18-65 yo	55	38	93	23
>65 yo	1	3	4	0
Unknown	1	1	2	0
NF Status—no.				
NF1	37	33	70	39
Sporadic	26	16	42	0

Fig. 1. Tyrosine kinase 2 (TYK2) protein expression by immunohistochemistry (IHC). (A) Representative strong positive TYK2 staining in human kidney and negative staining for TYK2 in mouse nerve. Representative TYK2 staining in plexiform neurofibroma, ANNUBP, and high-grade Malignant Peripheral Nerve Sheath Tumor (MPNST). Kaplan Meier plots of overall survival time for patients by TYK2 staining in (A) all MPNST samples, (B) NF1 MPNST samples and (C) sporadic MPNST samples. The proportion of positive TYK2 staining was scored on a 0-3 scale (0.5 increments). (E) Table of patient characteristics.

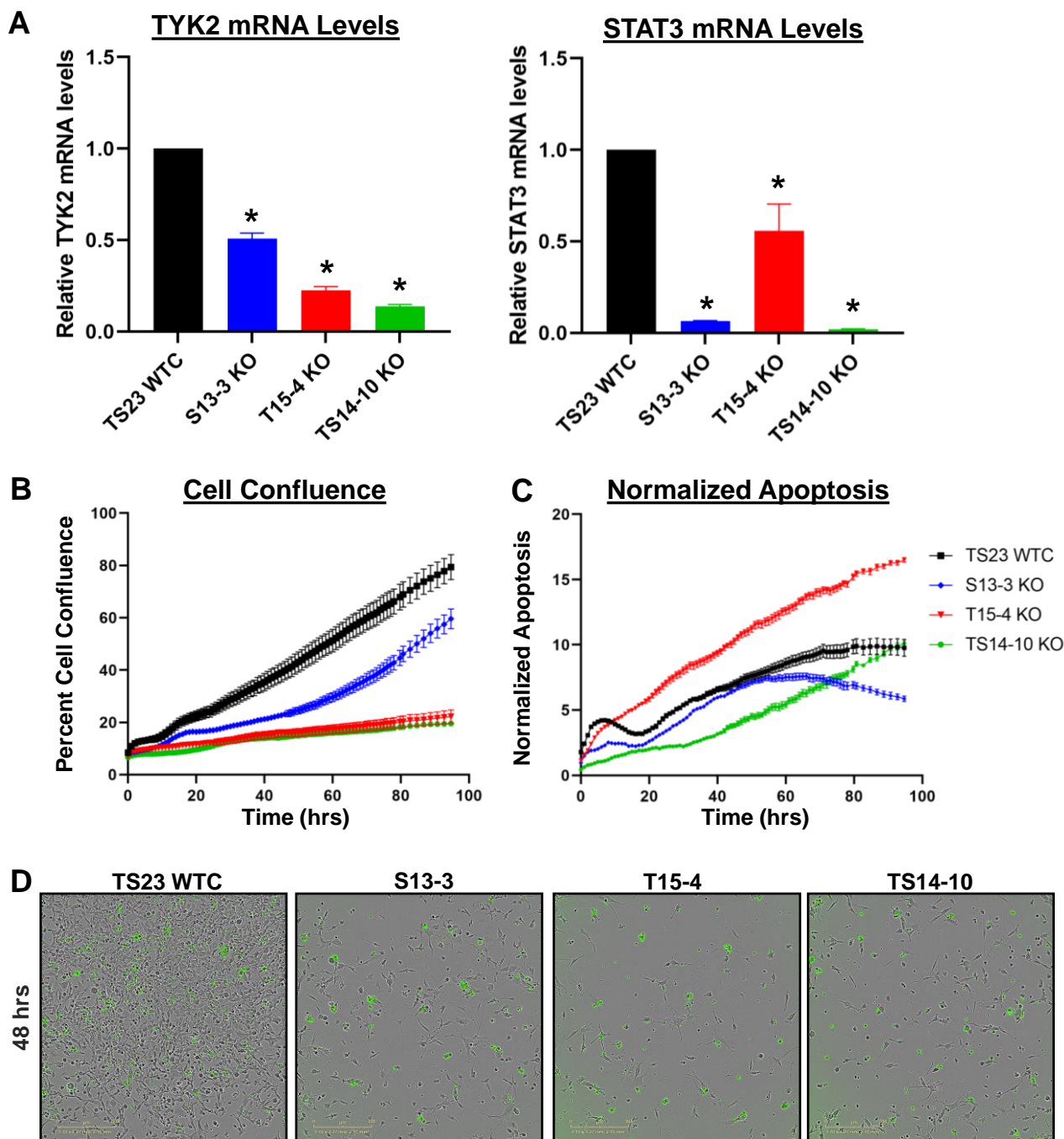


Fig. 2. TYK2 inhibitors reduce proliferation and induce apoptosis in MPNST cells. (A) Relative *TYK2* and *STAT3* mRNA expression in JW23.3 cells for CRISPR/Cas9 knockouts of *TYK2* (T15-4), *STAT3* (S13-3), *TYK2/STAT3* (TS14-10) or wild-type control (TS23 WTC), normalized to housekeeping gene. (B) Percent cell confluence and (C) normalized apoptosis were determined by the IncuCyte live cell imaging assay over 4 days in lines of JW23.3 cells with CRISPR/Cas9 knockout of *TYK2* and/or *STAT3*, in media with 2.5% FBS. Apoptotic cell count (YOYO-1 green fluorescence count), was normalized to percent cell confluence at the same time-point. (D) Representative images of IncuCyte assay at 48 hours, with YOYO-1 green fluorescence as an indicator of apoptosis.

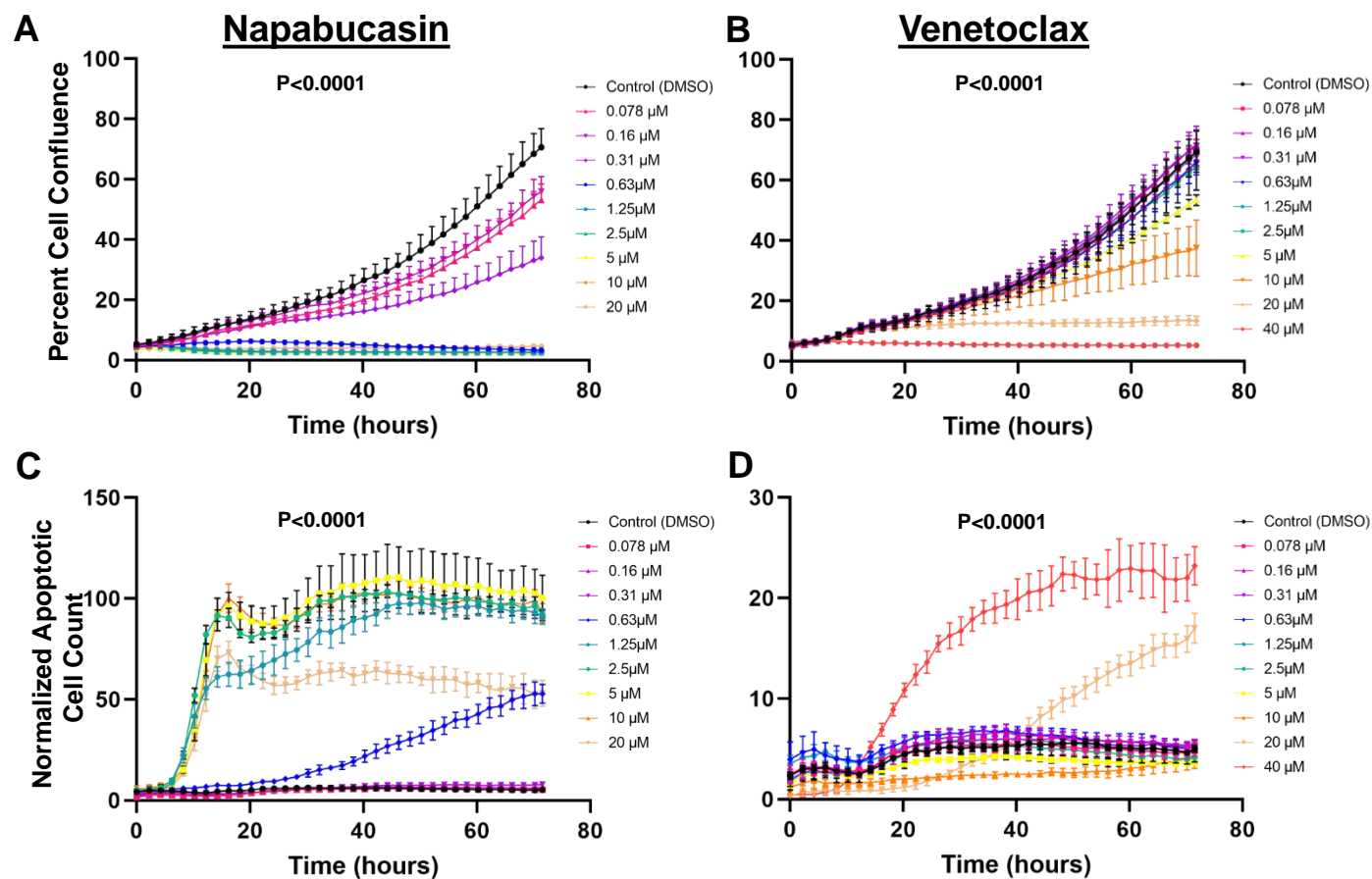
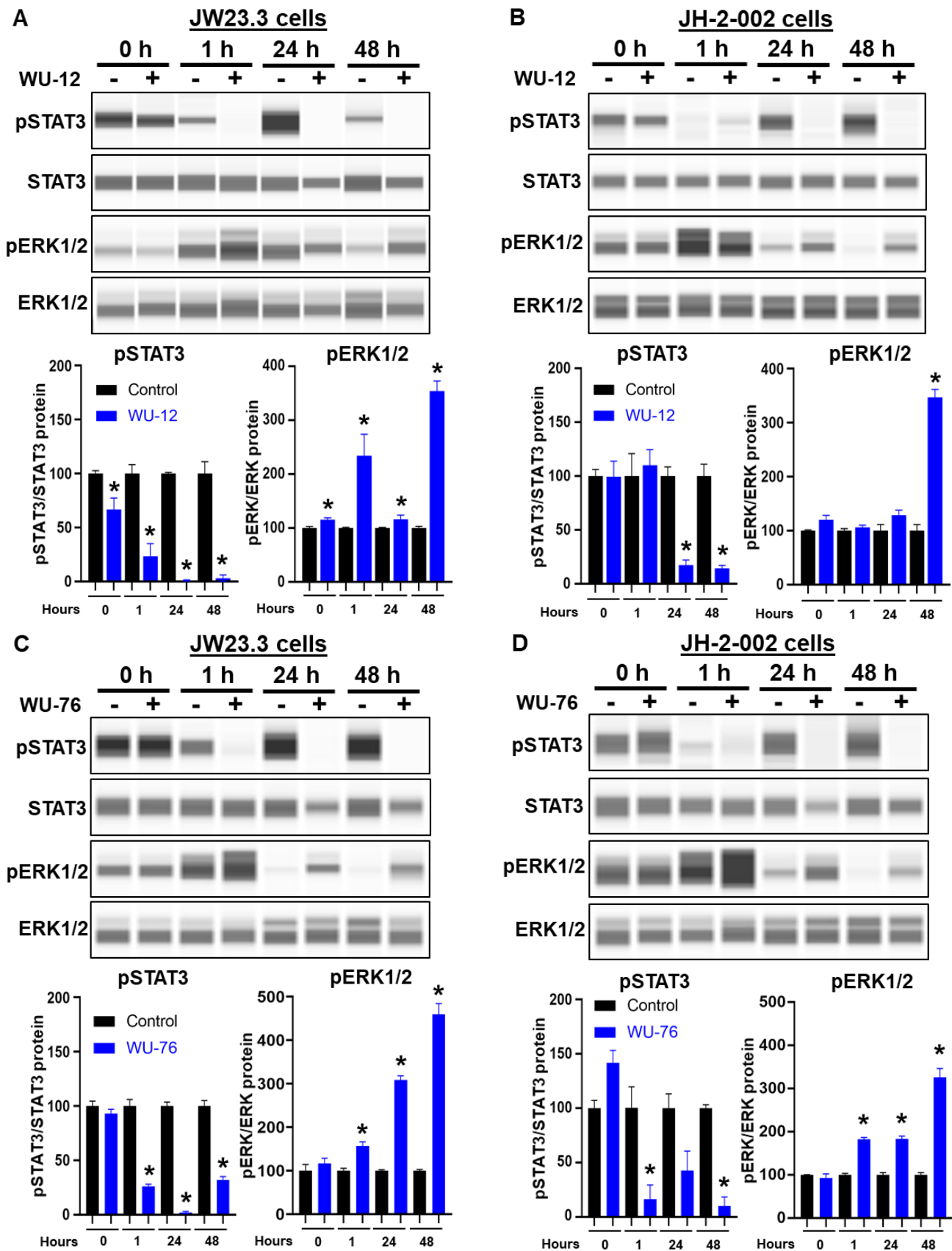


Fig. 3. Pharmacological inhibition of STAT3 or Bcl2 decreases proliferation and increases apoptosis in MPNST cells. JW23.3 cells were treated with inhibitors of (A, C) STAT3 (napabucasin, NP) and (B, D) Bcl2 (venetoclax, ABT-199) for 72 hours. Proliferation was determined by an IncuCyte proliferation assay (A, B) and apoptosis by an IncuCyte cell death assay (C, D), as assessed by YOYO-1 iodide green fluorescence.



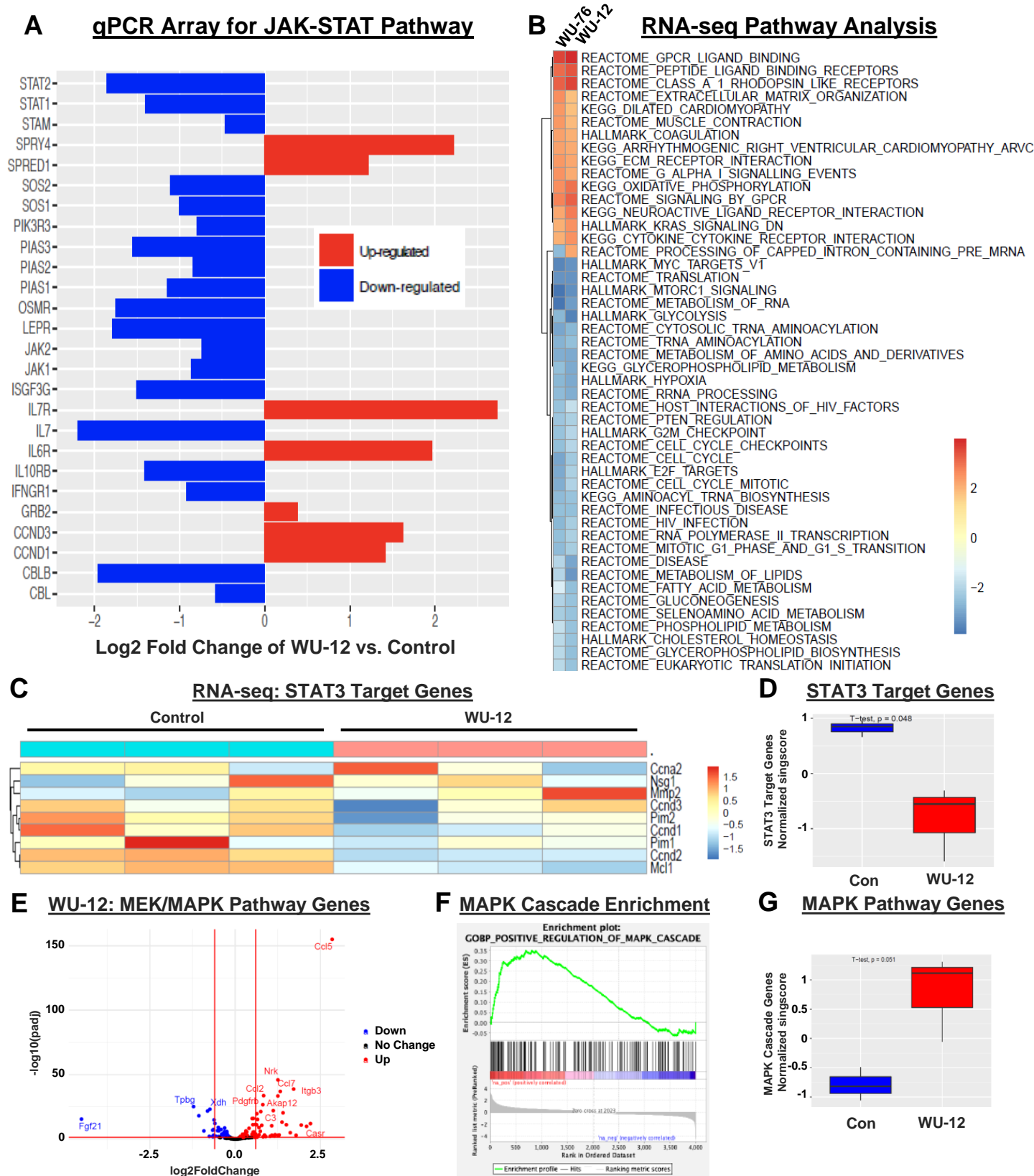


Fig. 5. TYK2 inhibition leads to compensatory stimulation of the MEK/MAP-kinase (MAPK) pathway in MPNST cells. (A) JH-2-002 cells were treated with 40 μ M WU-12 or control for 48 hours, and gene expression was analyzed by qPCR array for JAK/STAT pathway related genes. Log₂ fold-change of significantly changed genes are graphed. (B) JW23.3 cells were treated with control or 40 μ M WU-12 or WU-76 for 48 hours. Global gene expression was determined by RNA-seq pathway analysis. Changes in gene expression for WU-12 vs. control were examined by RNA-seq for genes downstream of STAT3 in a (C) heatmap and (D) boxplot, as well as MEK/MAPK pathway genes in a (E) volcano plot, (F) enrichment plot, and (G) boxplot. $P < 0.05$ vs. vehicle control.

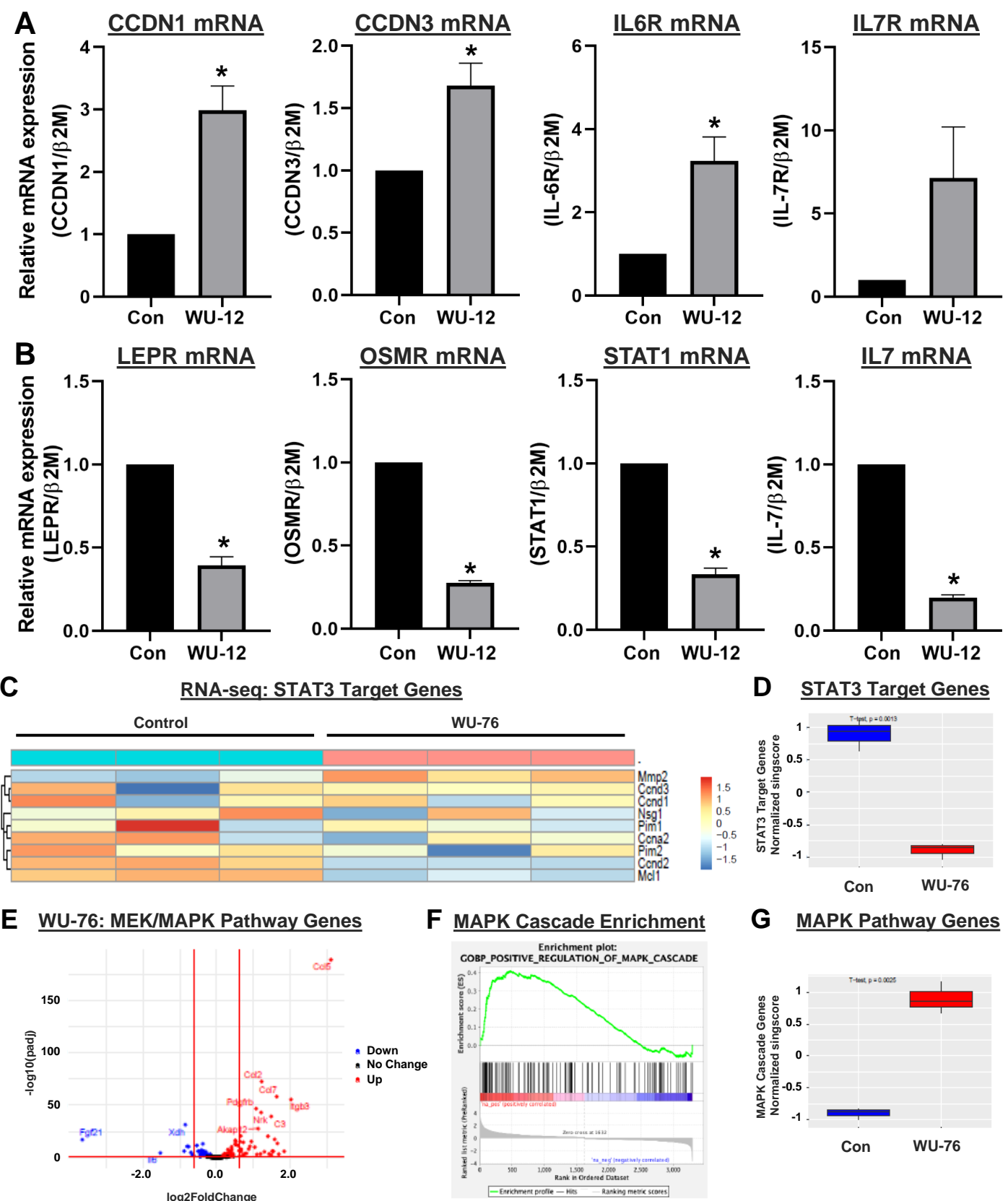


Fig. 6. TYK2 inhibition modifies gene expression in MPNST cells. JH-2-002 cells were incubated with 40 μ M WU-12 or control for 48 hours, and gene expression was analyzed by qPCR for select genes that were (A) up-regulated or (B) down-regulated in a qPCR array. Gene expression normalized to β 2M expression. $P < 0.05$ vs. vehicle control. (C-G) JW23.3 cells were incubated with control or 40 μ M WU-76 for 2 days. Changes in expression of genes were analyzed by RNA-seq for genes downstream of STAT3 in a (A) heatmap and (B) boxplot, as well as genes in the MEK/MAPK pathway in a (C) volcano plot, (D) enrichment plot, and (E) boxplot.

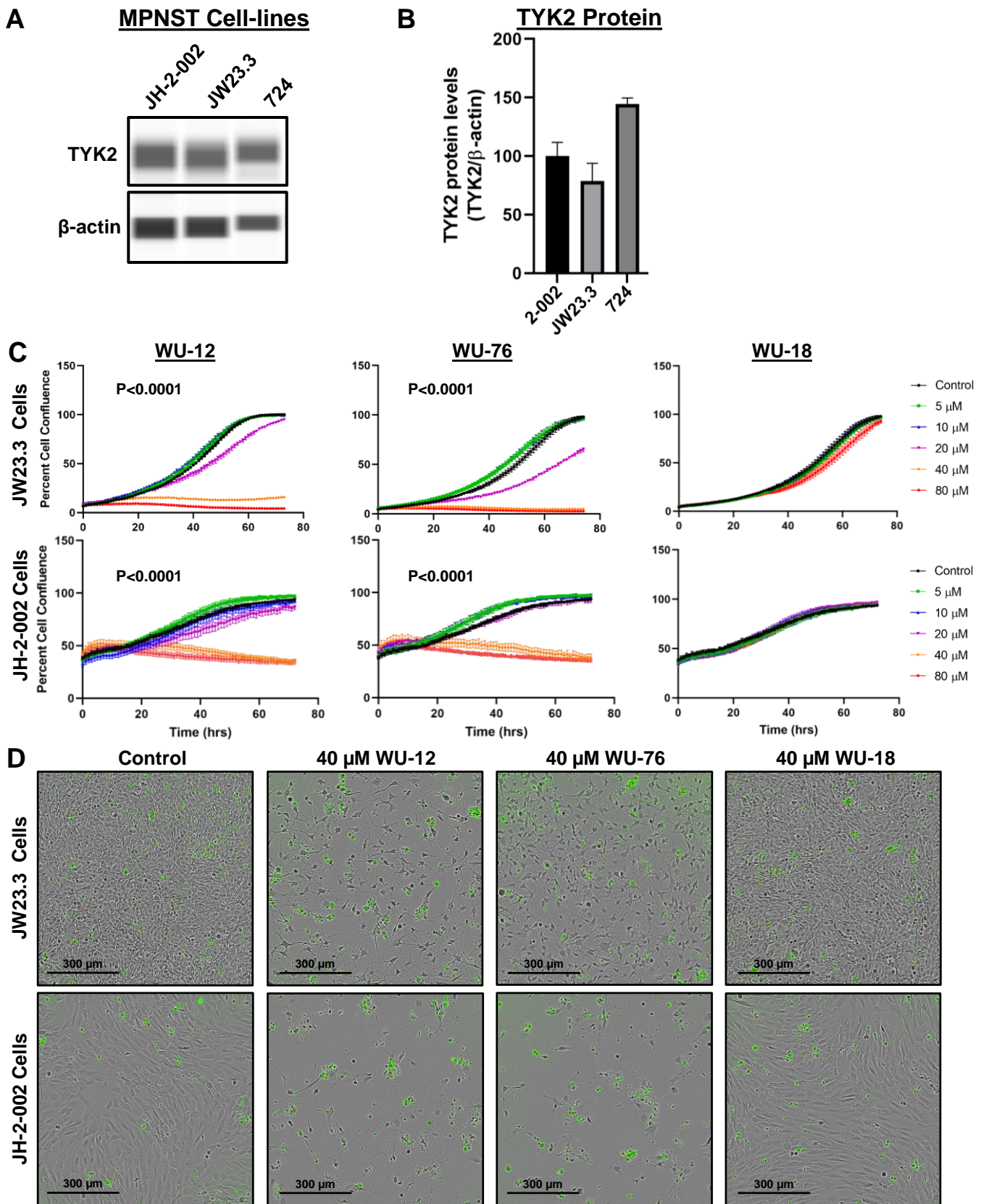


Fig. 7. TYK2 inhibitors reduce proliferation in MPNST cells. (A) TYK2 protein levels were visualized by immunohistochemistry (IHC) in MPNST and plexiform neurofibroma, with positive staining scored on a 0-3 scale. (B) Relative TYK2 protein levels in MPNST cell lines by WES western analysis. Protein bands analyzed by densitometry, with TYK2 normalized to β -actin. (C) JW23.3 and JH-2-002 cells were treated with TYK2 inhibitors (WU-12, WU-76) or inactive control (WU-18) for 3 days, and cell confluence was determined by IncuCyte assay. (D) Representative images of IncuCyte assay at 72 hours, with YOYO-1 green fluorescence as indicator of apoptosis.

Normalized Apoptosis in MPNST Cell-lines

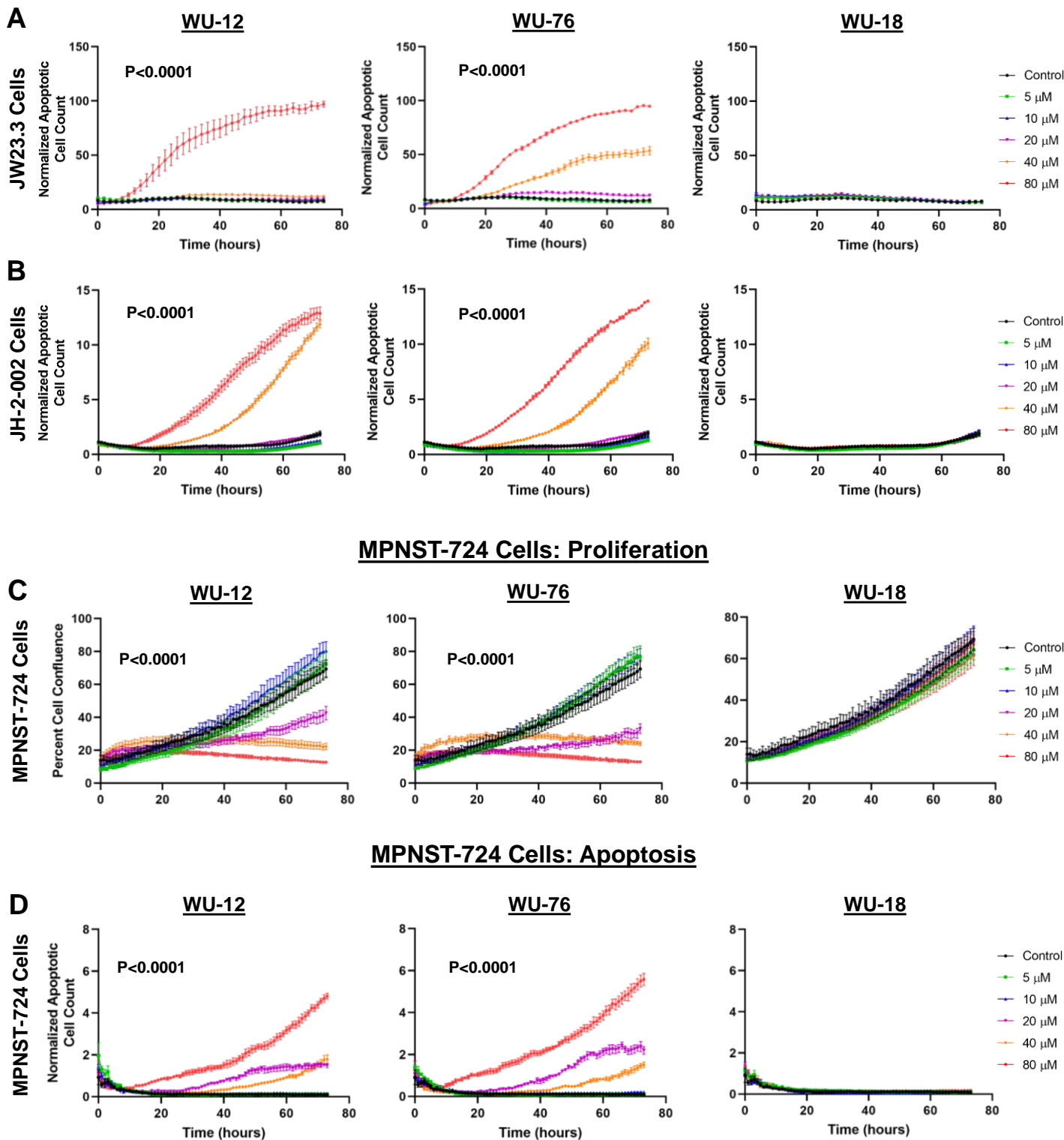


Fig. 8. TYK2 inhibitors reduce proliferation and induce apoptosis in MPNST cells. (A) JW23.3 and (B) JH-2-002 cells were incubated for 72 hours with TYK2 inhibitors, WU-12 or WU-76, or with an inactive control compound, WU-18. The IncuCyte cell death assay with YOYO-1 green fluorescent dye was used to measure apoptosis in cells over time. Apoptotic cell count was normalized to percent cell confluence over time. (C-D) MPNST-724 cells were treated with the indicated doses of TYK2 inhibitors, WU-12 or WU-76, or control compound, WU-18, for 3 days. (C) Percent cell confluence or (D) normalized apoptotic cell count over time were determined by the IncuCyte assay. * $P < 0.0001$ vs. control.

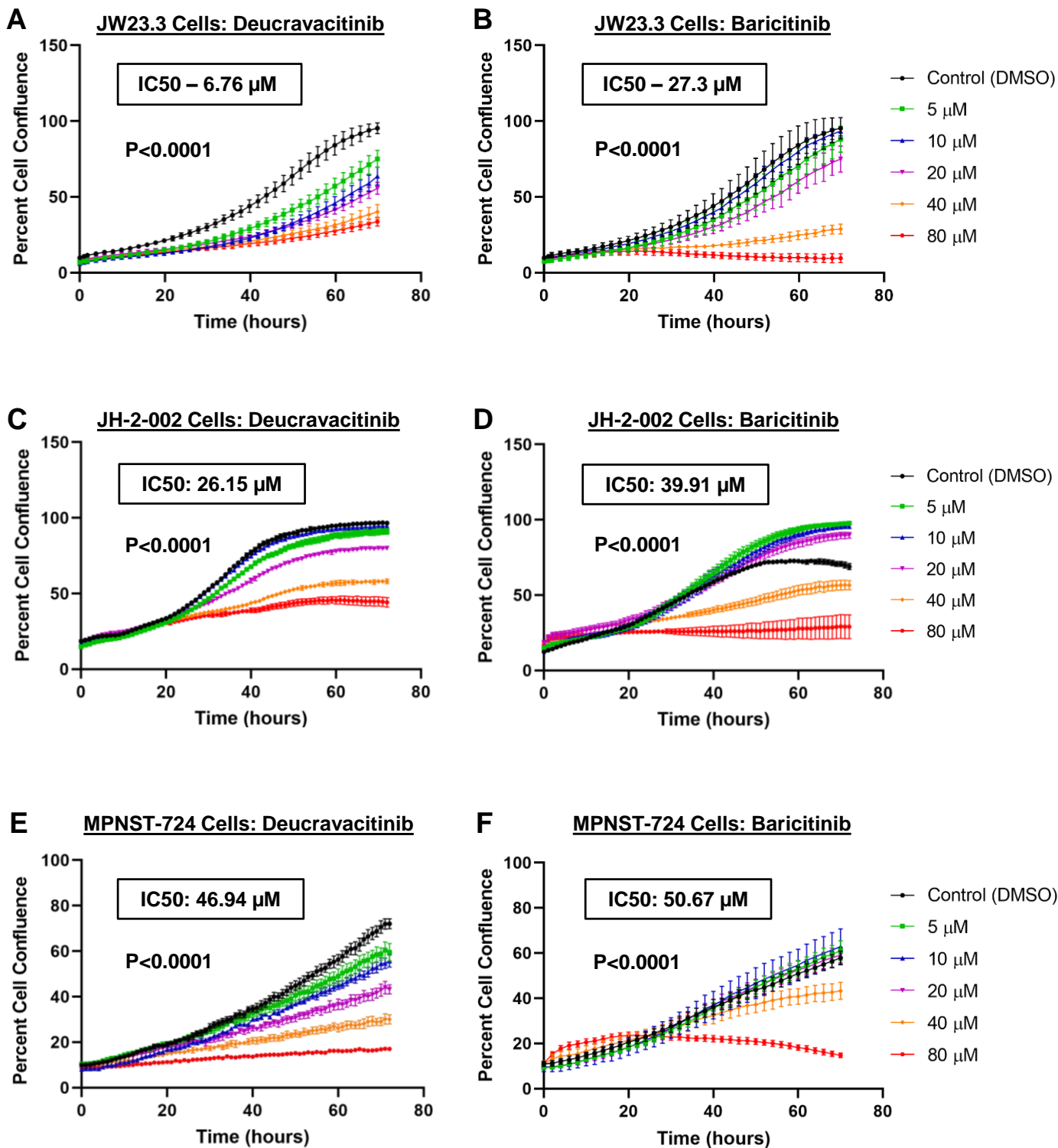
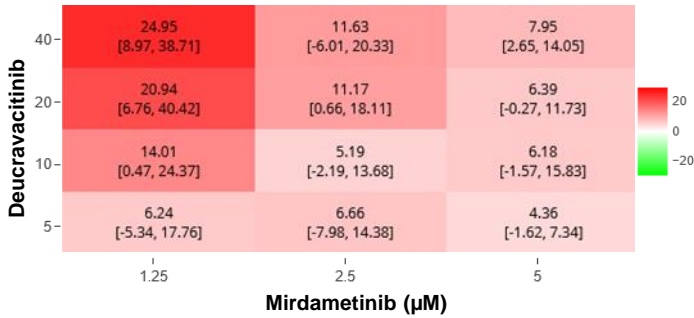


Fig. 9. The specific TYK2 inhibitor, deucravacitinib (BMS-986165), decreases MPNST cell proliferation at lower doses. The MPNST cell lines JW23.3 (A-B), JH-2-002 (C-D), and MPNST-724 (E-F) were treated with the indicated doses of the specific TYK2 inhibitor, deucravacitinib, or the pan-JAK inhibitor, baricitinib, for 3 days in InCuCyte live cell proliferation assays.

A Proliferation HSA Synergy Scores:
Mean 10.47



B Apoptosis HSA Synergy Scores:
Mean 9.84

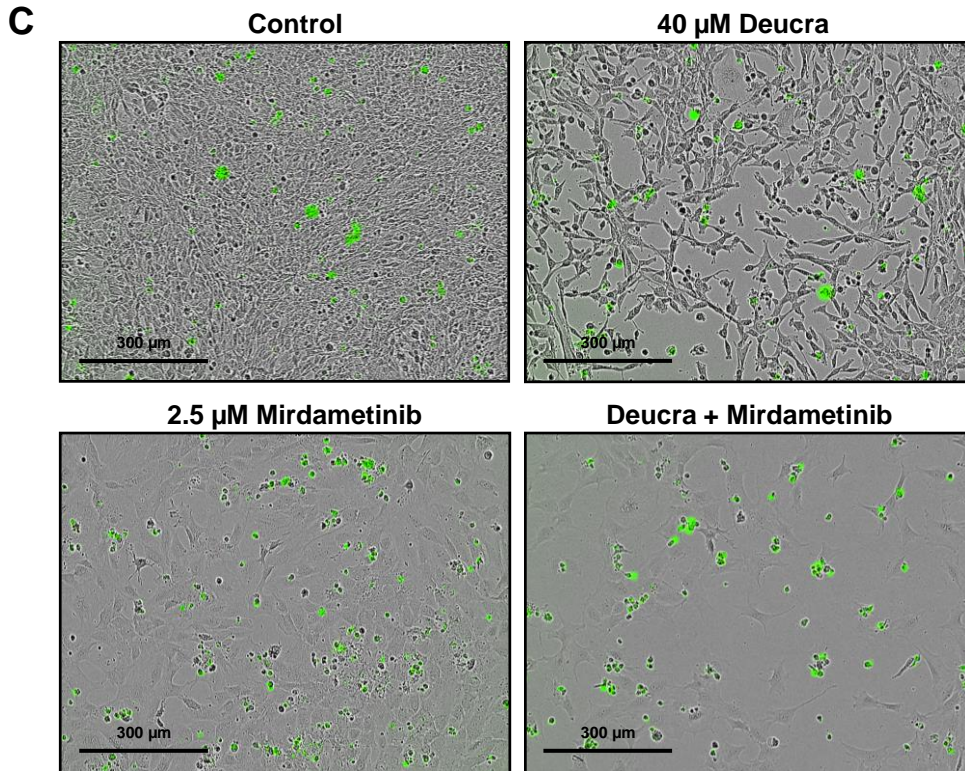
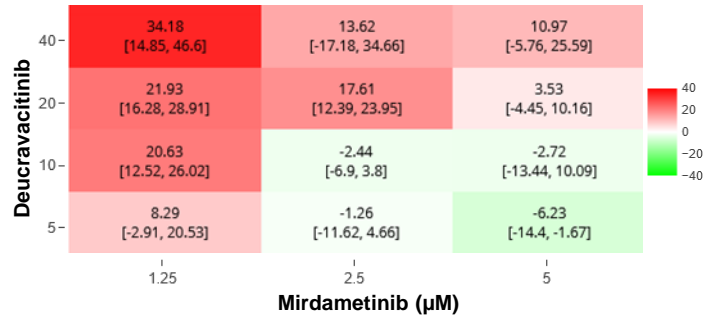


Fig. 10. Inhibitors of TYK2 (deucravacitinib) and MEK (mirdametininib) act synergistically to reduce proliferation and increase apoptosis in JW23.3 MPNST cells. Cell confluence and apoptosis was analyzed by the IncuCyte assay after 48 hour incubation with drugs. Synergy was analyzed using Synergy Finder software by the HSA method for (A) inhibition of cell proliferation or (B) apoptosis, and mean synergy score is reported ($P < 0.05$ vs. drug alone). Intensity of red color indicates synergy score for each dose combination, while green indicates antagonism. (C) Representative images of JW23.3 cells treated for 48 hours. YOYO-1 green fluorescence indicates apoptotic cells.

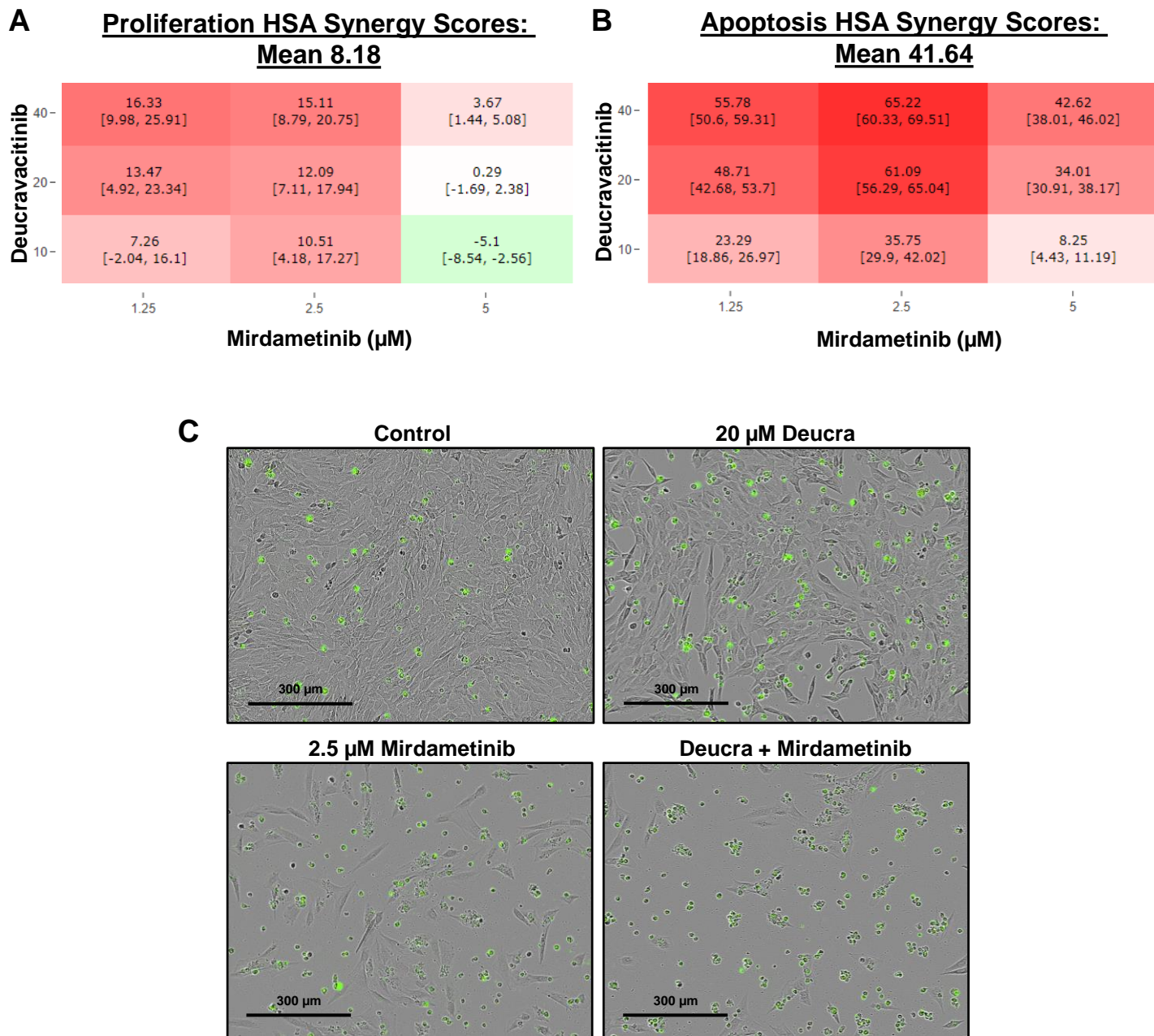


Fig. 11. Inhibitors of TYK2 (deucravacitinib) and MEK (mirdametininib) act synergistically to reduce proliferation and increase apoptosis in JH-2-002 MPNST cells. Cell proliferation and cell death were determined by the IncuCyte assay after 72 hour treatment. Synergy was analyzed using Synergy Finder software by the HSA method for (A) inhibition of cell proliferation or (B) apoptosis. Mean synergy score was calculated ($P < 0.05$ vs. drug alone). Intensity of red color indicates synergy, while green indicates antagonism, for each dose combination. (C) Representative images of JH-2-002 cells treated with the indicated doses for 72 hours. Apoptotic cells are stained with YOYO-1 green fluorescence dye.

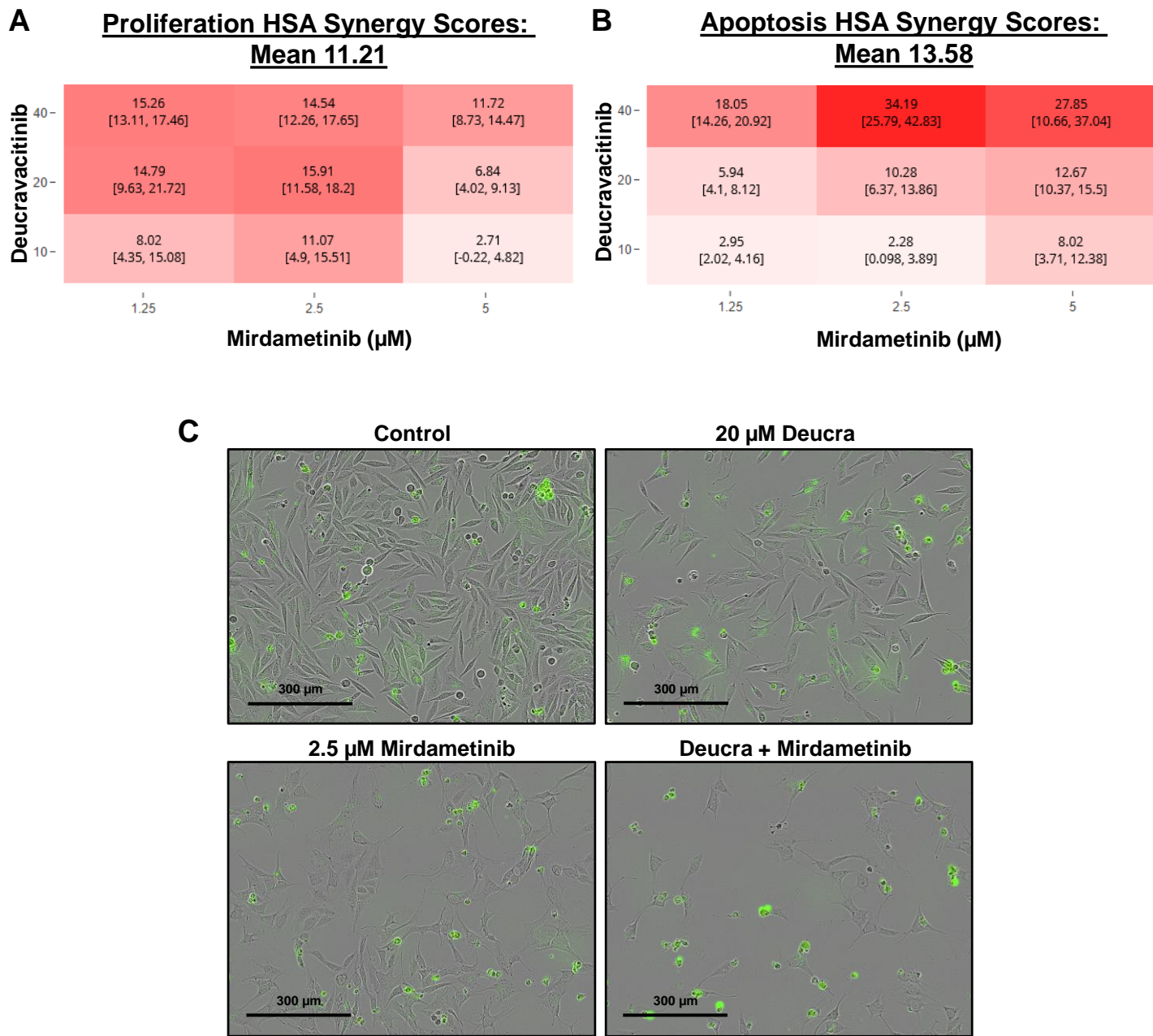


Fig. 12. Synergy of TYK2 and MEK inhibitors in MPNST-724 cells. Cells were treated with deucravacitinib (Deucra) and/or mirdametininib (Mirda) for 4 days in IncuCyte live cell proliferation and apoptosis assays. Synergy was determined by the HSA method (Synergy Finder) for (A) inhibition of cell proliferation or (B) normalized apoptosis. Mean synergy score was calculated ($P < 0.05$ vs. drug alone). (C) Representative images of MPNST-724 cells incubated with the indicated doses of drugs for 96 hours, with apoptotic cells stained with green fluorescent YOYO-1 dye.

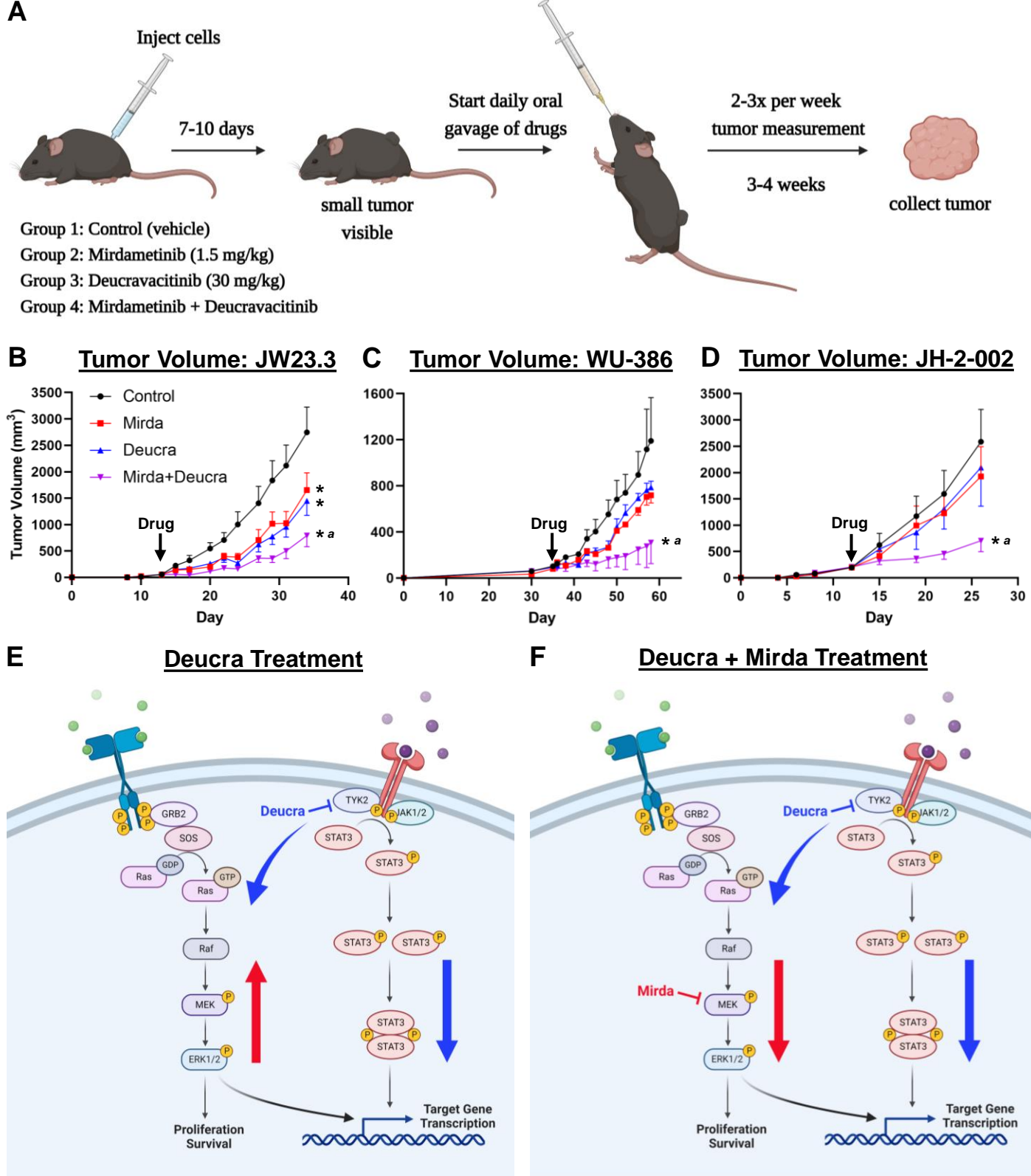
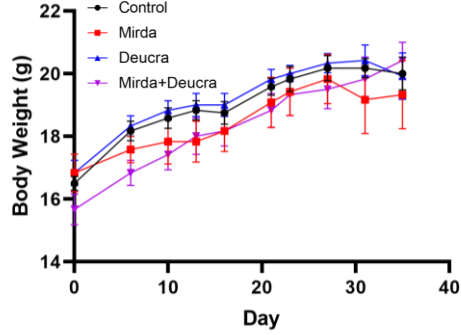
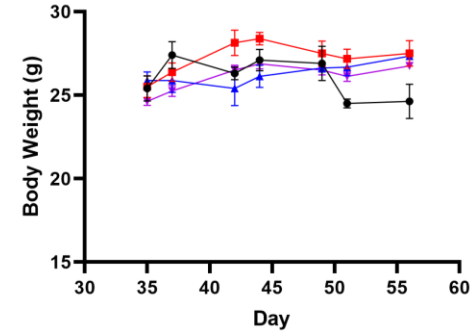


Fig. 13. The combination of drugs inhibiting TYK2 and MEK block MPNST tumor growth in mice. (A) Schematic diagram of treatment paradigm. Mice with JW23.3 MPNST xenograft tumors (n=6 per group) (B), WU-386 MPNST PDX tumors (n=3 per group) (C), or JH-2-002 MPNST xenograft tumors (n=5 per group) were treated daily with 1.5 mg/kg mirdametinib (Mirda), 30 mg/kg deucravacitinib (Deucra, BMS-986165), the combination of drugs, or vehicle control for three weeks or until tumors reached the maximum allowed volume. * P < 0.05 vs. vehicle control. ^a P < 0.05 for drug combination vs. drugs alone. (E-F) Diagram of TYK2/STAT3 and MEK/ERK pathways after treatment with Deucra and/or Mirda in MPNST cells. Illustrations were created with BioRender.com.

A Mouse Weight: JW23.3



B Mouse Weight: WU-386



C Mouse Weight: JH-2-002

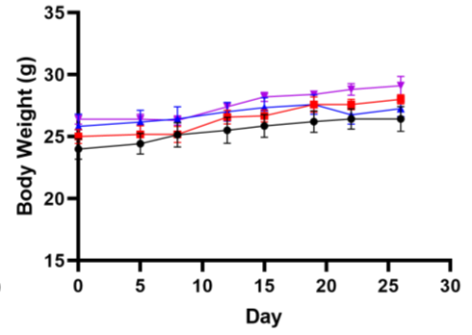


Fig. 14. Treatment with deucravacitinib and/or mirdametininb did not decrease mouse body weight over time. Mice were implanted with (A) JW23.3, (B) WU-386, or (C) JH-2-002 cells. After tumors reached 50-200 mm³, mice were treated daily with 1.5 mg/kg mirdametininb (Mirda), 30 mg/kg deucravacitinib (Deucra, BMS-986165), the combination of drugs, or vehicle control for three weeks or until tumors reached the maximum allowed volume. Mouse body weight, an indicator of health, was measured 2-3 times per week.



MEK Inhibition Synergizes with TYK2 Inhibitors in NF1-Associated Malignant Peripheral Nerve Sheath Tumors

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ABSTRACT

Purpose: Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas with limited treatment options and poor survival rates. About half of MPNST cases are associated with the neurofibromatosis type 1 (NF1) cancer predisposition syndrome. Overexpression of TYK2 occurs in the majority of MPNST, implicating TYK2 as a therapeutic target.

Experimental Design: The effects of pharmacologic TYK2 inhibition on MPNST cell proliferation and survival were examined using IncuCyte live cell assays *in vitro*, and downstream actions were analyzed using RNA-sequencing (RNA-seq), qPCR arrays, and validation of protein changes with the WES automated Western system. Inhibition of TYK2 alone and in combination with MEK inhibition was evaluated *in vivo* using both murine and human MPNST cell lines, as well as MPNST PDX.

Results: Pharmacologic inhibition of TYK2 dose-dependently decreased proliferation and induced apoptosis over time. RNA-seq pathway analysis on TYK2 inhibitor-treated MPNST demonstrated decreased expression of cell cycle, mitotic, and glycolysis pathways. TYK2 inhibition resulted in upregulation of the MEK/ERK pathway gene expression, by both RNA-seq and qPCR array, as well as increased pERK1/2 levels by the WES Western system. The compensatory response was tested with dual treatment with TYK2 and MEK inhibitors, which synergistically decreased proliferation and increased apoptosis *in vitro*. Finally, combination therapy was shown to inhibit growth of MPNST in multiple *in vivo* models.

Conclusions: These data provide the preclinical rationale for the development of a phase I clinical trial of deucravacitinib and mirdametinib in NF1-associated MPNST.

Introduction

Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas, which often arise in people with neurofibromatosis type 1 (NF1), the most common cancer predisposition syndrome (1). Individuals with NF1 have one mutated copy of the *NF1* gene, increasing the risk of developing benign plexiform neurofibromas (PN), which can later undergo malignant transformation to MPNST (2). The *NF1* gene codes for neurofibromin, a tumor suppressor that is a negative regulator of RAS (1). Approximately half of MPNST cases occur in patients with NF1, whereas the other half occur sporadically or as a secondary complication of radiotherapy (3). In addition to *NF1*, the genes encoding *CDKN2A*, *TP53*, *EED*, and *SUZ12* are frequently altered in MPNST (1). Despite aggressive treatments including surgery, chemotherapy, and radiation, these cancers recur in about 50% of

individuals and the majority of patients die within 5 years of their diagnosis (2). Targeted treatment approaches have not demonstrated activity to date, and currently, there are no effective treatments for patients with metastatic disease, thus, necessitating development of more efficacious targeted therapies for MPNST (3).

Our laboratory previously conducted genomic screening using next-generation sequencing (NGS) and identified activating mutations in tyrosine kinase 2 (*TYK2*) in a small subset of NF1-associated MPNST. However, by IHC, *TYK2* was found to be highly expressed in the majority of MPNST (4). A member of the JAK family of proteins, *TYK2* is a receptor-associated kinase that mediates cytokine signaling through phosphorylation of STAT proteins (5). STATs subsequently translocate to the nucleus to regulate the transcription of over 300 target genes, including those involved in cancer cell proliferation, apoptosis, survival, and invasion (6). In subsequent studies, we reported that knockdown of the *TYK2* gene reduced MPNST cell proliferation and increased cell death (7). *TYK2* deficiency led to the decreased activation of downstream targets, including STAT1 and STAT3 (7). Furthermore, *TYK2* genetic knockdown also decreased MPNST tumor growth and metastasis in mice (7).

As an intermediary of immune system and inflammatory cytokines, *TYK2* overexpression and hyperactivation promotes development and metastasis of multiple types of cancer, including leukemia, lymphoma, colorectal, breast, cervical, and prostate cancers (8–13). In line with this, genomic profiling of over 100 advanced sarcoma samples reveals mutations in *TYK2*, *JAK1*, *JAK2*, and *JAK3* (14). Pharmacologic inhibitors of JAKs, including *TYK2*, have been developed clinically for autoimmune diseases, such as inflammatory bowel disease, psoriasis, and rheumatoid arthritis (15, 16). Ruxolitinib and other pan-JAK inhibitory drugs (JAKinibs) are also FDA-approved or in clinical trials for treatment of various hematologic cancers (17, 18). In addition, several specific *TYK2* inhibitors (“*TYK2*inibs”) block growth

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Clin Cancer Res 2023;XX:XX-XX

doi: 10.1158/1078-0432.CCR-22-3722

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Translational Relevance

Malignant peripheral nerve sheath tumors (MPNST) are aggressive sarcomas with dismal prognosis. Thus, there is an urgent need for more effective treatment strategies. Our lab previously found that tyrosine kinase 2 (TYK2) is overexpressed in the majority of MPNST. TYK2 and other JAKs mediate cytokine signaling, thereby influencing inflammation, immune function, and cancer progression. Herein, we demonstrate that drugs targeting TYK2 decrease proliferation and induce apoptosis in MPNST, while inhibiting STAT3 activation. TYK2 inhibitors also stimulate the MEK/ERK pathway, which may be a compensatory survival mechanism for MPNST. Addition of the MEK inhibitor, mirdametinib, synergizes with the TYK2 inhibitor deucravacitinib to block proliferation and promote apoptosis of MPNST both *in vitro* and in three different *in vivo* models. These data provide the preclinical rationale for the development of a phase I clinical trial of deucravacitinib and mirdametinib in patients with NF1-associated MPNSTs.

of leukemia and solid cancer tumors in mice (19, 20). However, intrinsic or acquired resistance to JAK or TYK2 inhibitors can lead to treatment failure in cancer (21–23). Combination strategies with JAKinibs or TYKinibs have been evaluated preclinically to overcome resistance and improve efficacy, including with the addition of chemotherapy agents or inhibitors targeting Hsp90, histone deacetylase (HDAC), mTOR, or MEK (20–22, 24, 25). Many of these pathways are also active in MPNST.

In MPNST, RAS hyperactivation due to loss of neurofibromin can lead to downstream activation of multiple RAS effector pathways including RAF/MEK/ERK, which can stimulate cancer cell proliferation, migration, and invasion (26). In addition to phosphorylated ERK1/2 protein levels, expression of a gene signature, including *DUSP6*, *SPRED1*, *SPRY2/4*, and *CCND1*, are representative of ERK-dependent transcriptional output and therefore activation of the Raf/MEK/MAPK pathway (27–29). In addition, MEK inhibitors, such as mirdametinib and selumetinib, show therapeutic promise in clinical trials for precursor benign PN (3), and the FDA recently approved selumetinib for NF1 patients with inoperable PN tumors (30).

In this study, we sought to evaluate the efficacy of small molecule TYKinibs and examine signaling pathways downstream of TYK2 in MPNST to identify potential targets for combination drug therapy. Herein, we found that pharmacologic inhibition of TYK2 dose-dependently decreased cell proliferation and increased apoptosis in MPNST cells. In addition, TYK2 inhibitors reduced STAT3 activation, while increasing ERK activation and expression of MEK/ERK pathway target genes. Combination treatment with a TYK2 inhibitor and a MEK inhibitor synergistically blocked proliferation in MPNST, thus improving therapeutic efficacy and allowing for lower doses of each drug. Finally, we demonstrated that combination therapy with clinically available TYK2 and MEK inhibitors markedly reduced MPNST tumor growth in mice.

Materials and Methods

Cell culture

Human-derived MPNST cell lines were generated by the Pratilas lab at Johns Hopkins University (JH-2-002 cells), and obtained through the Johns Hopkins NF1 biospecimen repository (31), or obtained from

the Fletcher lab at Dana Farber Cancer Institute (MPNST-724 cells, RRID:CVCL_AU20; ref. 32). Murine MPNST JW23.3 cells were established previously from C57BL6/J *Nf1*[±];*Trp53*[±]*cis* (*NPcis*) mice (33). Cells were cultured in growth media consisting of DMEM high glucose with 10% FBS (Gibco Life Technologies, Thermo Fisher Scientific) and penicillin–streptomycin (200 µg/mL, Thermo Fisher Scientific). Cell cultures were maintained at 37°C and 5% CO₂. Cell lines were verified as mycoplasma negative by regular testing with the MycoAlert Kit (Lonza). For cell line authentication, short tandem repeat (STR) profiling was performed for human MPNST cell lines by the Genome Engineering and Stem Cell Center (GESC) at Washington University.

Patient-derived xenograft (PDX) lines

The WU-386 PDX line was generated previously (32) and maintained through passage in mice.

IHC

Formalin-fixed paraffin-embedded (FFPE) slides of MPNST and PN were obtained from Washington University, Johns Hopkins University (JHU), the NCI, and the University of California at San Francisco (UCSF). The institutional review board (IRB) at Washington University in St. Louis approved the use of de-identified patient samples (Protocol No. 201203042). Immunostaining for TYK2 was performed on 112 MPNST and 39 PN. Sections were first deparaffinized and rehydrated, followed by antigen retrieval in sodium citrate buffer for 15 minutes and blocking in 3% H₂O₂ and avidin/biotin. After blocking, the sections were incubated with TYK2 primary antibody (ab223733, 1:100; Abcam; RRID:AB_2928057) overnight, followed by anti-rabbit HRP-conjugated secondary antibody (1:200). Diaminobenzidine (DAB) was the chromogen with hematoxylin counterstaining. IHC staining was scored independently by three investigators (including board certified pathologist, CD) blinded to patient data with the proportion score (0: 0% TYK2 staining, 1: 1%–33% TYK2 staining, 2: 34%–66%, 3: ≥66% TYK2 staining) in 0.5 intervals.

Cell proliferation and apoptosis assays

MPNST cell lines were plated at 2,500 cells/well in 96-well plates in growth media and incubated overnight. Cells were then treated for 72 to 96 hours in phenol-red free media containing 5% FBS, with various doses of WU-12, WU-76, WU-18 (all gifted by Peter Ruminski, Washington University), deucravacitinib (MedChemExpress), and/or mirdametinib (SpringWorks Therapeutics). The WU-12 and WU-76 compounds are small molecule kinase inhibitors that are derivatives of a 7H-pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol core molecule. The IC₅₀s for WU-12, WU-76, and WU-18 against a panel of kinases, including JAK1–3 and TYK2, were determined by Thermo Fisher's SelectScreen Biochemical Kinase Profiling Service using a Z'-LYTE Screening Assay following manufacturer instructions. ATP concentrations were either 1 mmol/L or set at ATP *K_m* apparent for each kinase, as previously determined using a Z'-LYTE assay. The negative control, WU-18, had an IC₅₀ >1,000 nmol/L for all four of these kinases. The IC₅₀ values of WU-12 and WU-76 for JAK1–3 and TYK2 are presented in Supplementary Table S1. For proliferation and apoptosis assays, cells were imaged every 1 to 2 hours by IncuCyte Zoom Live-Cell Analysis System (Essen Bioscience). Cell proliferation was determined as percent confluence from phase images and was analyzed by IncuCyte image analysis software (Sartorius). For cell death assays, 50 nmol/L YOYO-1 green fluorescent dye (Thermo Fisher Scientific) was added to treatment medium, and apoptosis was calculated as green objects normalized to the confluency factor and the

initial timepoint. IC₅₀ values were calculated in GraphPad Prism version 9 (GraphPad Software).

Generation of *TYK2* knockout cells

The CRISPR-Cas9 system was used to generate *TYK2* knockout (KO) and control in JW23.3 cells using two gRNAs provided by the Genome Engineering & Stem Cell Center (GESC), against murine *TYK2* (GAATCCCCACGGCAGCCGGTNGG; CTTCCCCGACCG-GCTGCCGTNGG). JW23.3 cells were cotransfected with *TYK2* gRNAs and FUW-FLG GFP plasmid (RRID:CVCL_AU20; ref. 33) using TransIT-LT1 transfection reagent (Mirus), sorted by FACS using a MoFlo Cell Sorter (Beckman Coulter), and clones were grown from single cell colonies. Gene KO was confirmed by NGS and qPCR for *TYK2* expression. CRISPR-Cas9 *TYK2* KO and control clones for MPNST-724 cells were described previously (7).

Synergy analysis for drug combination studies

For synergy calculations of drug combinations, IncuCyte dose response data for cell proliferation or apoptosis, normalized to maximum inhibition values, were analyzed using SynergyFinder 2.0 software (RRID:SCR_019318) and the HSA model. The mean synergy score and a *P* value were calculated for the entire matrix by SynergyFinder software (34). Synergy values >10 were considered synergistic, whereas −10 to 10 was additive, and <−10 was antagonistic.

RNA isolation and qPCR

Total RNA was isolated using an RNeasy Mini Kit (Qiagen), and genomic DNA was removed by adding DNase I for 15 minutes to the RNA samples. Total RNA concentrations were determined using a Nanodrop 2000 (Thermo Fisher Scientific). cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using the Power SYBR Green Master Mix Kit (Thermo Fisher Scientific) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Relative gene expression was calculated using CFX Manager Software version 3.1 (Bio-Rad), and values were normalized to housekeeping gene. Fold-changes in gene expression compared with control were calculated using the $2^{-\Delta\Delta C_t}$ method.

Western analysis

MPNST cell lines were plated in 6-well plates at 200,000 cells/well in growth media, and the next day treated for the indicated times with WU-12a, WU-76, deucravacitinib (Deucra), or vehicle control (DMSO). Cells were washed with 1× PBS and lysed in 1× Cell Lysis Buffer (Cell Signaling Technology). Equal amounts of protein were run on the WES capillary electrophoresis western system (ProteinSimple, Bio-Techne), following manufacturer protocol and standard instrument settings. Protein levels were analyzed using Compass Simple-Western software (ProteinSimple). Primary antibodies included total STAT3 (4904S; RRID:AB_331269), pSTAT3 (Tyr705; 9145S; RRID:AB_2491009), total p44/42 ERK1/2 (4695S; RRID:AB_390779), and pERK1/2 (Thr202/Tyr204; 4370S; RRID:AB_2315112), all from Cell Signaling Technologies, and TYK2 (A2128; Abclonal; RRID:AB_2764147). Phosphorylated protein levels were normalized to the respective total protein levels and expressed as a percent of control for each time point.

qPCR pathway array

The effect of WU-12 on gene expression of potential downstream targets known to be in the JAK–STAT pathway was assessed by a PrimerArray JAK–STAT Signaling Pathway (Human) qPCR array in

96-well plate format (Takara Bio). JH-2-002 cells were plated in growth media in 6-well plates at a density of 200,000 cells per well. The next day, the cells were treated in triplicate with vehicle control (DMSO) or 40 μmol/L WU-12a, a TYK2 inhibitor (Washington University), for 48 hours. RNA was isolated and cDNA synthesized as above. qPCR was performed with TB SYBR Green Premix Ex Taq II (Takara Bio) and the JAK–STAT primer arrays on the CFX96 PCR instrument, with gene expression assessed by CFX Manager Software. The PrimerArray Analysis Tool Version 2.2 software (Takara) was utilized for pathway analysis of three replicate plates per condition. Briefly, gene expression was normalized to 8 housekeeping genes and fold-change was expressed versus control using the Ct method. The PrimerArray includes primers for 88 JAK–STAT biological pathway related genes and 8 housekeeping genes.

Bulk RNA-seq analysis

JW23.3 cells were treated with 40 μmol/L WU-12a, 40 μmol/L WU-76, or vehicle control (DMSO) for 48 hours. RNA was isolated as described above, and samples contained at least 5 μg of purified total RNA with RIN >9.0. At least three biological replicates were performed. Samples were aligned against Mouse Ensembl GRCm38.76. Total RNA integrity was determined using Agilent Bioanalyzer or 4200 TapeStation. Library preparation was performed with 5 to 10 μg of total RNA with a Bioanalyzer RIN score greater than 8.0. Ribosomal RNA was removed by poly-A selection using Oligo-dT beads (mRNA Direct Kit, Life Technologies). mRNA was then fragmented in reverse transcriptase buffer and heating to 94° for 8 minutes. mRNA was reverse transcribed to yield cDNA using SuperScript III RT enzyme (Life Technologies, per manufacturer's instructions) and random hexamers. A second strand reaction was performed to yield ds-cDNA. cDNA was blunt ended, had an A base added to the 3' ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 12 to 15 cycles using primers incorporating unique dual index tags. Fragments were sequenced on an Illumina NovaSeq-6000 using paired end reads extending 150 bases. Differential expression analysis was performed with the DESeq2 package (R Bioconductor software; RRID:SCR_015687).

Animals

Mice were housed and treated in compliance with our approved protocol for the Institutional Animal Care and Use Committee (IACUC) of Washington University (Protocol No. 20-0117). For experiments with murine MPNST cells, 5- to 6-week-old C57BL6 mice (Taconic; RRID:IMSR_TAC:b6) were implanted with 400,000 JW23.3 cells subcutaneously on the dorsal surface (*n* = 6 mice per group). For experiments with human MPNST cells, 6- to 10-week-old immunodeficient NOD-*Rag1*^{null} *IL2rg*^{null} (NRG) mice (strain #007799, Jackson Laboratories; RRID:IMSR_JAX:007799) were implanted on the subcutaneous dorsal surface with a PDX single cell suspension of WU-386 cells (3×10^6 cells per mouse; *n* = 3 mice per group) or JH-2-002 cells (1×10^6 cells per mouse; *n* = 5 mice per group). A sample size of three has been previously reported to be sufficient for drug studies with PDX tumors (35). Tumors were monitored by calipers two to three times per week, and tumor volume was calculated as volume = $0.52 \times \text{length} \times \text{width} \times \text{width}$. Drug treatments were initiated when tumors reached ~50 to 200 mm³. Mice were randomized into four groups and given 30 mg/kg deucravacitinib (MedChemExpress, in 5% ethanol/5% TPGS/90% PEG300) and/or 1.5 mg/kg mirdametininib (SpringWorks Therapeutics, in 0.5% HPMC/0.2% Tween-80/water), or vehicle control daily via oral gavage for 3 to

4 weeks. Tumors were extracted, photographed, and weighed at the end of experiments.

Statistical analysis

Data were analyzed and graphed in GraphPad Prism version 9 (RRID:SCR_002798). Data were expressed as mean \pm SEM. Two-way ANOVA or Student *t* test were used to calculate statistical significance where appropriate. *P* < 0.05 was considered significantly significant.

Data availability

Reported data are stored in the SYNAPSE repository (Project SynID: syn23639889; DOI: <https://doi.org/10.7303/syn23639889>) and will also be made available to researchers upon request.

Results

TYK2 is expressed in the majority of MPNST

We conducted IHC for TYK2 protein levels in 112 primary patient MPNST and 39 PN tumor samples. Strong TYK2 staining (score ≥ 2) was observed in 56% of high-grade MPNST samples, with 44% having weak or negative TYK2 staining (Fig. 1A). In contrast, benign precursor PN were largely weak or negative for TYK2 (67%; Fig. 1A). Patient characteristics are detailed in Supplementary Table S2. TYK2 protein expression was also verified in three MPNST cell lines (JW23.3, JH-2-002, and MPNST-724) by the WES Western system. All cell lines had moderate to high levels of TYK2 protein (Fig. 1B).

Pan-JAK/TYK2 inhibitors decrease MPNST cell proliferation

To gain further insight into the role of TYK2 in MPNST, we examined the effect of pharmacologic inhibition of TYK2 using IncuCyte Live Cell Imaging experiments for proliferation and cell death assay with YOYO-1 green fluorescent dye. Murine JW23.3 and human JH-2-002 MPNST cell lines were incubated for 72 hours with novel in-house pan-JAK inhibitor compounds (WU-12 or WU-76) that exhibit potent inhibition against TYK2, or an inactive control compound (WU-18; Fig. 1C and D). WU-12 and WU-76 dose-dependently decreased the percent cell confluence and increased apoptosis, whereas WU-18 had no effect, in either MPNST cell line (Fig. 1C and D; Supplementary Fig. S1). Representative pictures of both cell lines after incubation with the TYK2 inhibitors for 3 days are shown in Fig. 1D. Similarly, in a human sporadic MPNST cell line, MPNST-724, treatment with WU-12 and WU-76 reduced proliferation, suggesting inhibition of TYK2 is generally effective in MPNST and is not specific to a single cell line (Supplementary Fig. S2; Supplementary Table S3). IC_{50} values were calculated for inhibition of cell confluence by the pan-JAK/TYK2 inhibitors in each cell line, and ranged from 18.7 to 32.1 μ mol/L for JW23.3, JH-2-002, and MPNST-724 cells (Supplementary Table S3). Consistent with our previous reports, KO of TYK2 in JW23.3 and MPNST-724 cells decreased proliferation compared with control cells (Supplementary Figs. S3A and S3B and S4A and S4B). In both cell-lines, treatment with TYK2 inhibitors, WU-12 and WU-76, reduced proliferation of control cells, but not of TYK2 KO cells (Supplementary Figs. S3C–S3E and S4C–S4E), indicating that the anti-proliferative effects of WU-12 and WU-76 are primarily mediated through TYK2.

TYK2/JAK inhibitors stimulate activation of the MEK/MAPK pathway

Next, we explored the impact of TYK2/JAK downregulation on gene expression changes using a high throughput qPCR array targeted for JAK–STAT pathway-related genes. Human JH-2-002 cells were

incubated with WU-12 or vehicle control (DMSO) for 48 hours (Fig. 2A). A qPCR array was utilized to analyze changes in mRNA for 88 genes of interest (GOI) known to be downstream to the JAK–STAT pathway. Gene expression changes revealed a general upregulation of the MEK/ERK pathway (Fig. 2A). TYK2 inhibition significantly increased ERK pathway transcriptional output, including *Sprouty 4* (*SPRY4*), *SPRED1*, and *Cyclin D1* (*CCDN1*), while decreasing inhibitors of the ERK pathway, including *CBL* and *CBLB*. Members of the P13K/AKT/mTOR pathway that were decreased with TYK2 blockade included *PIK3R3* and *SOS1/2* (Fig. 2A). WU-12 incubation also had variable effects on ILs and related genes, including increasing *IL7R*, and *IL6R*, while decreasing *IL7*, *IL10RB*, and *oncostatin M receptor* (*OSMR*; Fig. 2A). Changes in expression of select genes identified by the qPCR array were then validated by qPCR using unique PCR primers pairs (Supplementary Fig. S5A and S5B).

Global gene expression changes with TYK2 inhibition

To determine the impact of TYK2 downregulation on the global expression profile of MPNST, we conducted RNA-seq experiments using potent TYK2 inhibitors, WU-12 and WU-76. RNA was isolated from JW23.3 cells treated with vehicle control (DMSO), WU-12 or WU-76 for 48 hours and the impact of TYK2 inhibition on the global expression profile was determined by RNA-seq. Pathway analysis revealed that inhibition of TYK2 stimulated GPCR pathways, MEK/ERK signaling, and oxidative phosphorylation, while decreasing cell cycle, mitotic, and glycolysis pathways (Fig. 2B). Downregulated genes involved in proliferation and the G₁ to S phase transition include *Ccnd2*, *Cdkn1a/c*, *E2f3/4*, *Pole2*, and *Mcm7*. Overall expression of genes targeted by the transcription factor STAT3 were also reduced by treatment with WU-12 (Fig. 2C and D) or WU-76 (Supplementary Fig. S6A and S6B). In line with the qPCR array experiment (Fig. 2A), incubation with TYK2 inhibitors, WU-12 and WU-76, increased expression of genes in the MEK/ERK pathway, for example, *Map3k2/3*, *MAP2k2/4*, *Map2*, and *Dusp4* (Fig. 2E–G; Supplementary Fig. S6C–S6E, respectively). These experiments provide important insight on the mechanism of TYK2 inhibitor action in MPNST and potential pathways that could be targeted in combination treatments to improve therapeutic efficacy.

TYK2 inhibition lowers pSTAT3 while raising pERK1/2 levels

Next, we sought to validate the effects of TYK2 inhibition on STAT and MEK activation by WES. In MPNST, the loss of neurofibromin leads to overactivation of Ras and downstream activation of MEK/ERK. To investigate the interaction of TYK2 on these signaling pathways, we evaluated the activation of STAT3 and ERK in MPNST cells using the ProteinSimple WES system. In JW23.3 and JH-2-002 cells, the pan-JAK/TYK2 inhibitor WU-12 decreased pSTAT3 protein levels at 1, 24, and 48 hours, as evaluated by the WES Western blotting system (Fig. 3A and B). Interestingly, pERK1/2 levels were strongly stimulated by 2- to 4-fold over time by treatment with WU-12 at 1 to 48 hours in the MPNST cell lines, suggesting that the cells are compensating for TYK2 suppression by rapidly upregulating the MEK/ERK pathway, and this increase is sustained over several days. Similarly, another pan-JAK/TYK2 inhibitor, WU-76, increased pERK1/2 while decreasing pSTAT3 levels in both MPNST cell lines (Supplementary Fig. S7A and S7B). Thus, TYK2 inhibition induced a rapid and sustained increase in pERK1/2 protein levels and MEK/ERK pathway target gene expression, which we believe is a compensatory mechanism (Fig. 3).

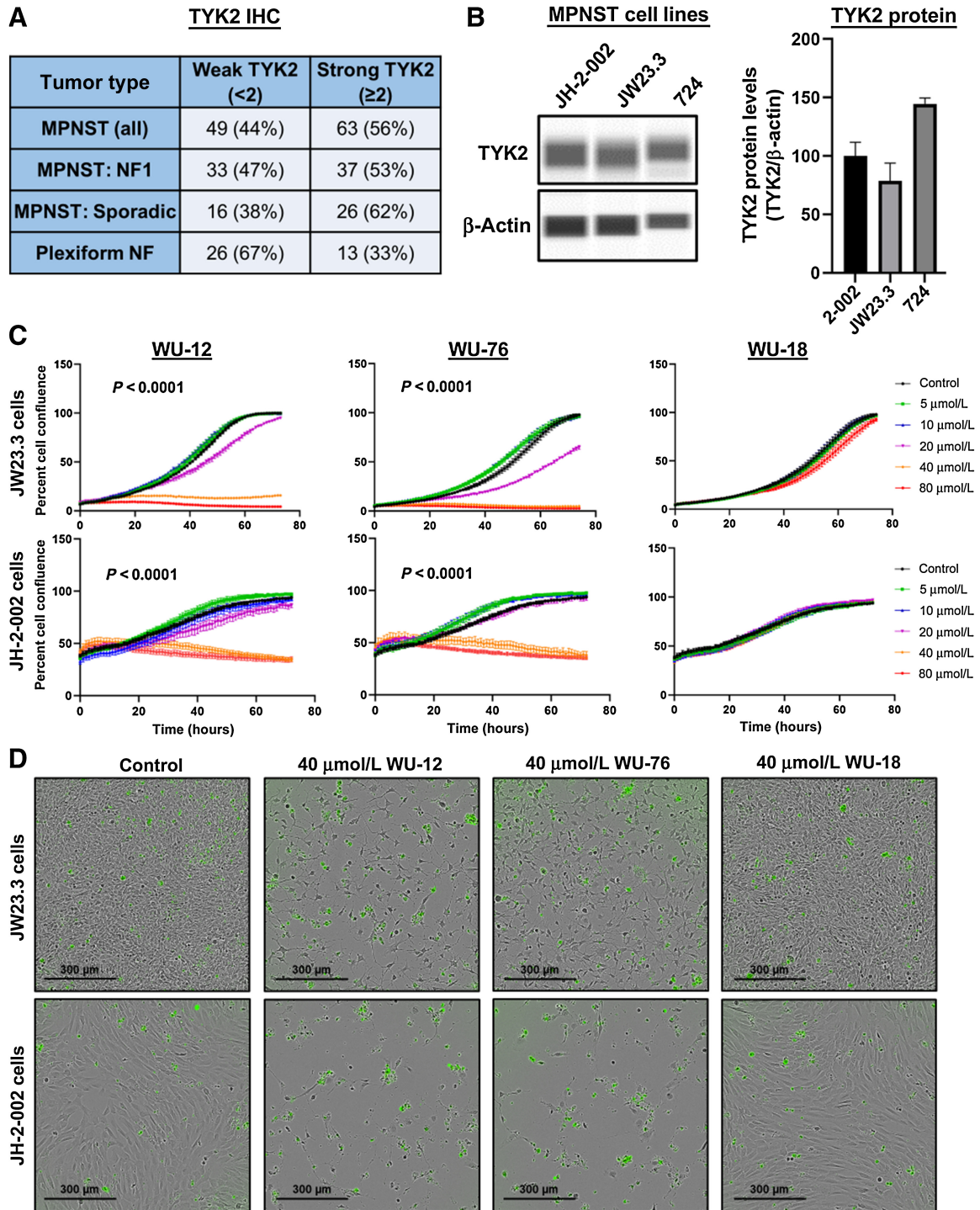


Figure 1.

TYK2 inhibitors reduce proliferation in MPNST cells. **A**, TYK2 protein levels were visualized by IHC in MPNST and plexiform neurofibroma, with positive staining scored on a 0 to 3 scale. **B**, Relative TYK2 protein levels in MPNST cell lines by WES Western blot analysis. Protein bands analyzed by densitometry, with TYK2 normalized to β -actin. **C**, JW23.3 and JH-2-002 cells were treated with TYK2 inhibitors (WU-12, WU-76) or inactive control (WU-18) for 3 days, and cell confluence was determined by IncuCyte assay. **D**, Representative images of IncuCyte assay at 72 hours, with YOYO-1 green fluorescence as indicator of apoptosis.

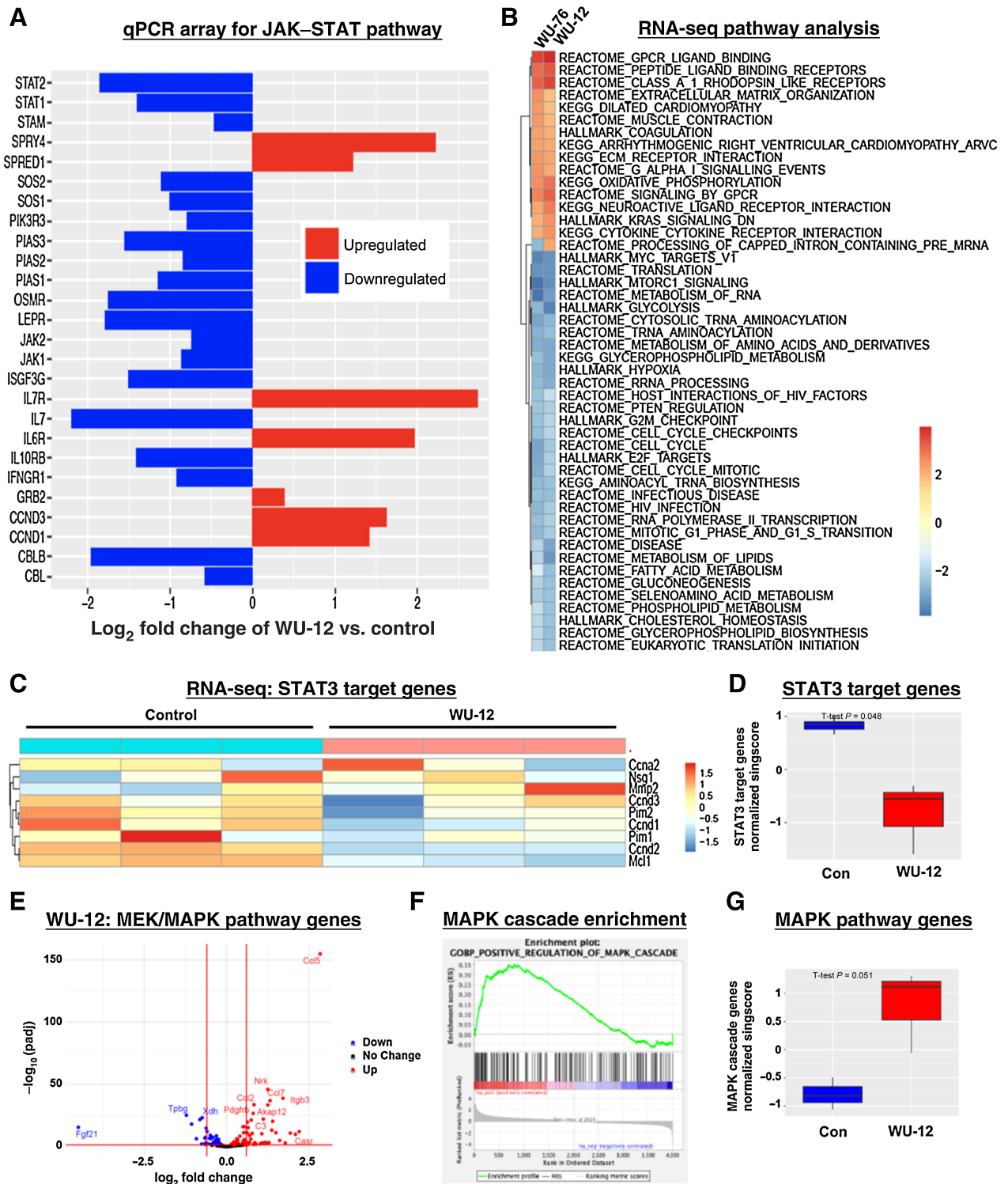
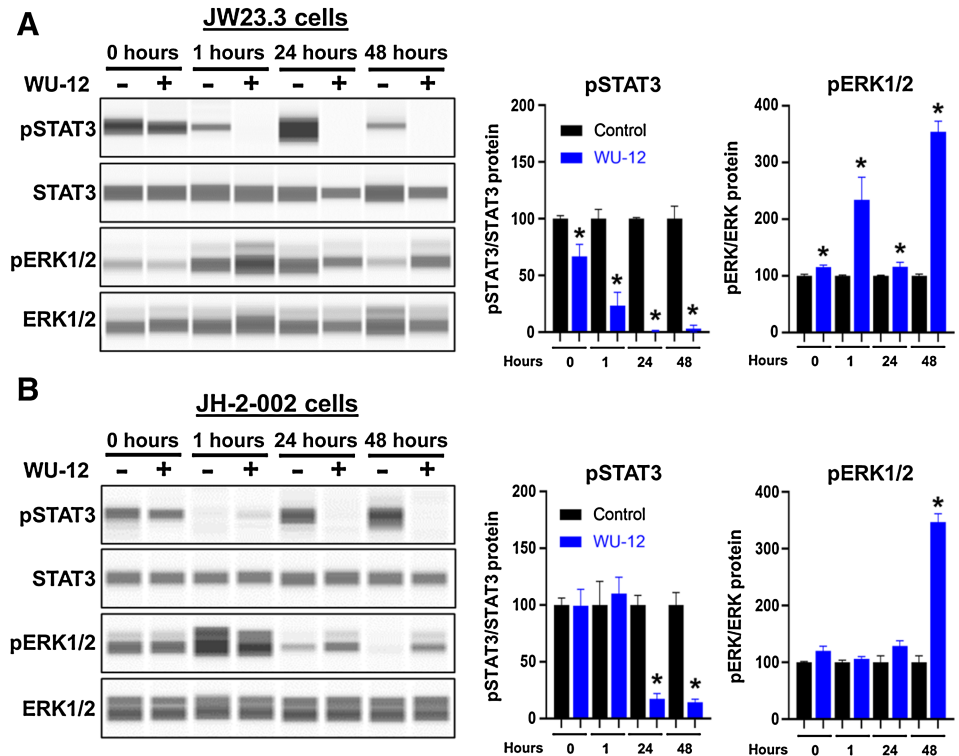


Figure 2.

TYK2 inhibition leads to compensatory stimulation of the MAPK pathway in MPNST cells. **A**, JH-2-002 cells were treated with 40 μmol/L WU-12 or control for 48 hours, and gene expression was analyzed by qPCR array for JAK/STAT pathway-related genes. Log₂ fold changes of significantly changed genes are graphed. **B**, JW23.3 cells were treated with control or 40 μmol/L WU-12 or WU-76 for 48 hours. Global gene expression was determined by RNA-seq pathway analysis. Changes in gene expression for WU-12 versus control were examined by RNA-seq for genes downstream of STAT3 in a heatmap (**C**) and boxplot (**D**), as well as MEK/MAPK pathway genes in a volcano plot (**E**), enrichment plot (**F**), and boxplot (**G**). *P* < 0.05 vs. vehicle control.

Figure 3.

TYK2 inhibitory drugs decrease activation of STAT3 while increasing activation of ERK1/2 in MPNST cells. **A**, JW23.3 cells and **(B)** JH-2-002 cells were incubated with a TYK2 inhibitor (40 $\mu\text{mol/L}$ WU-12) or vehicle control for the indicated times. Phosphorylated and total protein levels for STAT3 and ERK1/2 were analyzed by the WES Western system. Bands were analyzed by densitometry in the WES software, with phosphorylated protein normalized to the matching total protein and expressed as percent of control at the same time point. *, $P < 0.05$ vs. vehicle control at the same time point.



A clinically relevant TYK2 inhibitor, deucravacitinib, decreases MPNST cell proliferation

To prove these effects are driven by TYK2, which is overexpressed in MPNST, as well as to expedite translation to the clinic, we subsequently tested a specific, potent TYK2 inhibitor, deucravacitinib (BMS-986165). Deucravacitinib, an allosteric inhibitor that selectively binds to the TYK2 pseudokinase (JH2) domain, is used clinically in patients with autoimmune conditions, and the FDA recently approved deucravacitinib for treatment of plaque psoriasis. The TYK2 inhibitor, deucravacitinib, dose-dependently reduced murine JW23.3 MPNST cell proliferation (Fig. 4A) as well as that of human JH-2-002 (Fig. 4C) and MPNST-724 cells (Fig. 4E). In contrast, a clinically used pan-JAK/TYK2 inhibitor, baricitinib, decreased cell proliferation at higher doses in JW23.3 and JH-2-002 cells (Fig. 4B and D). These results suggest that the inhibition of NF1-MPNST cell proliferation is predominantly mediated through TYK2, and not the other JAKs (Fig. 4). Interestingly, the specific TYK2 inhibitor, deucravacitinib, was more potent in NF1-MPNST cells (JW23.3 and JH-2-002) than in sporadic MPNST cells (MPNST-724), whereas similar potency was observed for deucravacitinib and baricitinib in sporadic MPNST-724 cells (Fig. 4E and F).

MEK inhibition acts synergistically with TYK2 inhibition in MPNST

On the basis of our data demonstrating that inhibition of TYK2 stimulates the MEK/ERK pathway in what may be a compensatory mechanism for the cancer cells (Figs. 2 and 3), we investigated whether adding a MEK inhibitor improved the efficacy of TYK2 inhibition in MPNST. Single-agent treatment of JW23.3, JH-2-002, and MPNST-724 cells with an investigational MEK1/2 inhibitor, mirdametininib (PD-0325901; ref. 36), dose-dependently reduced cell

confluence percentage over 3 days, with an IC_{50} of 0.22 to 1.22 $\mu\text{mol/L}$ (Supplementary Table S4). When mirdametininib was combined with the TYK2 inhibitor, deucravacitinib, in JW23.3 cells, the two drugs synergistically inhibited cell proliferation and increased apoptosis over either drug alone, with significant mean synergy scores of approximately 10 (Fig. 5). Deucravacitinib and mirdametininib also acted synergistically in JH-2-002 cells (Supplementary Fig. S8) and MPNST-724 cells (Supplementary Fig. S9). The synergistic actions of TYK2 and MEK inhibitors *in vitro* suggest that this combination would allow effective treatment *in vivo* with lower doses of each drug.

Combination of TYK2 and MEK inhibition reduces MPNST tumor growth *in vivo*

On the basis of these data, we examined the combination of TYK2 and MEK inhibitors in mice with MPNST cell line xenograft tumors (Fig. 6A). In immunocompetent mice implanted with murine JW23.3 MPNST cells, treatment with mirdametininib, a MEK inhibitor, or deucravacitinib, a TYK2 inhibitor, significantly reduced tumors to nearly half the volume of vehicle control (Fig. 6B). Consistent with *in vitro* synergy studies, the combination of mirdametininib and deucravacitinib decreased tumor growth to less than one-third of control, and was significantly more effective than either drug alone (Fig. 6B). In line with this, the drug combination of mirdametininib and deucravacitinib also inhibited human WU-386 MPNST PDX tumor growth significantly and was more effective than either of the single agents (Fig. 6C). For treatment with mirdametininib or deucravacitinib on their own, there was a nonsignificant trend toward decreased tumor size (Fig. 6C). In a third tumor model using human JH-2-002 MPNST cell line xenografts, combination TYK2 and MEK inhibition significantly reduced tumor growth compared with control or either drug alone

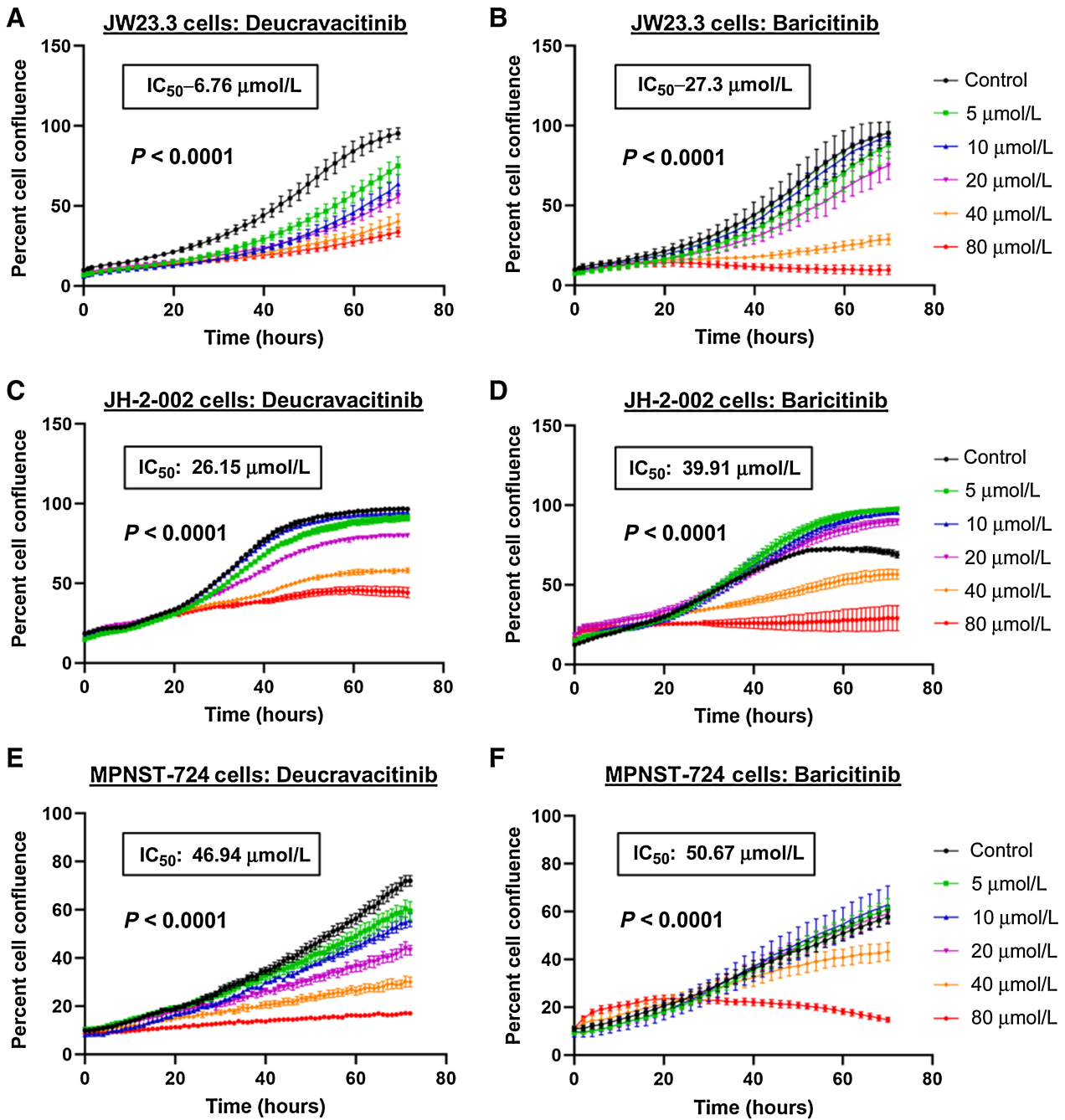


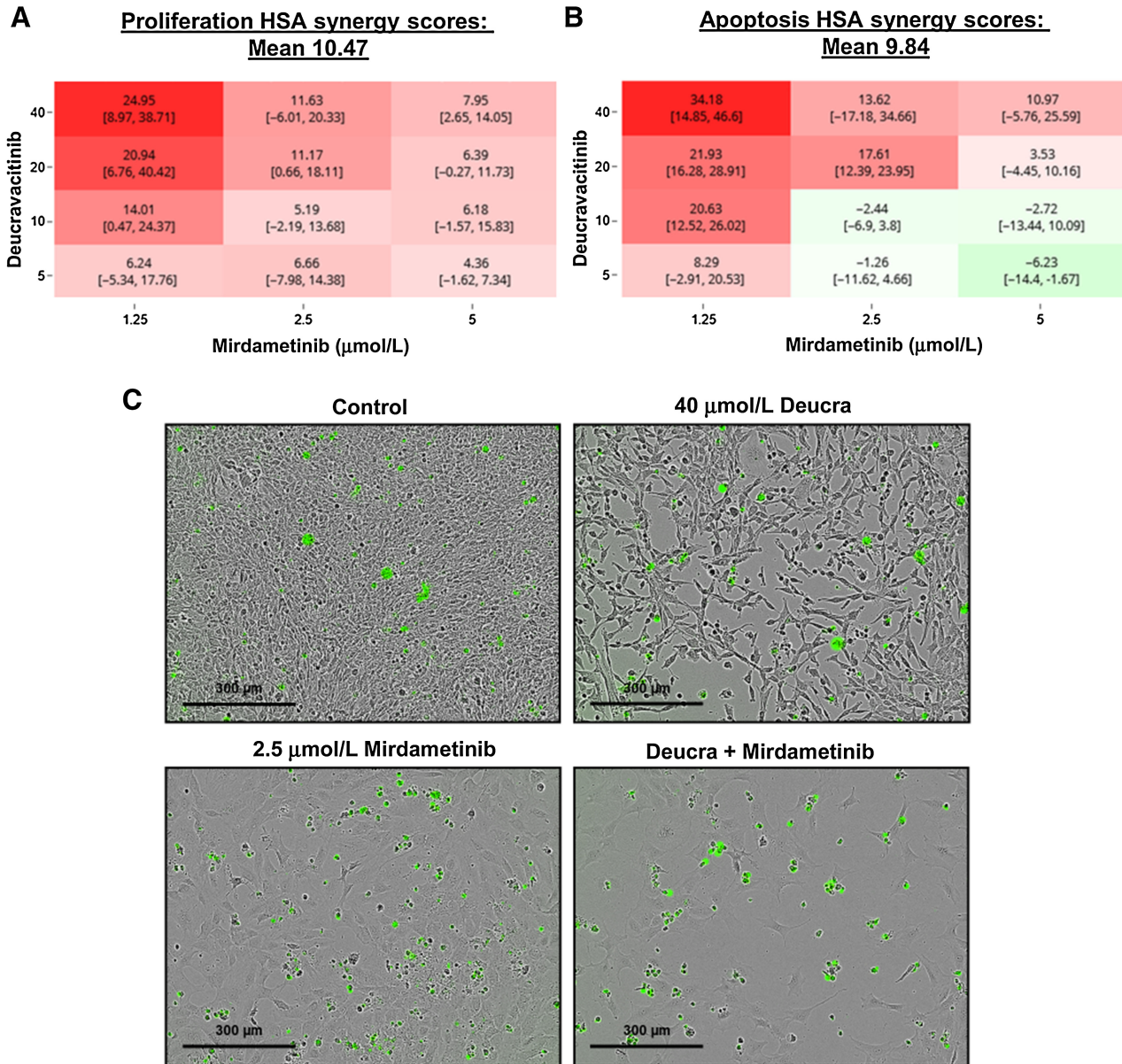
Figure 4.

The specific TYK2 inhibitor deucravacitinib (BMS-986165) decreases MPNST cell proliferation at lower doses. The MPNST cell lines JW23.3 (A and B), JH-2-002 (C and D), and MPNST-724 (E and F) were treated with the indicated doses of the specific TYK2 inhibitor deucravacitinib or the pan-JAK inhibitor baricitinib for 3 days in IncuCyte live cell proliferation assays.

(Fig. 6D). Mice given the mirdametinib/deucravacitinib drug combination did not significantly lose weight or show adverse health effects in any of the mouse models (Supplementary Fig. S10). Thus, the combination of drugs inhibiting TYK2 and MEK acted synergistically to improve the therapeutic efficacy in both murine and human MPNST models.

Discussion

Therapeutic options are limited for MPNST, thus necessitating development of novel treatment strategies. Previous work in our lab identified high expression of TYK2 in more than two-thirds of MPNST samples, suggesting that this protein could be a potential drug target for a high proportion of MPNST (4, 7). There are several key findings

**Figure 5.**

Inhibitors of TYK2 (deucravacitinib) and MEK (mirdametininib) act synergistically to reduce proliferation and increase apoptosis in JW23.3 MPNST cells. Cell confluence and apoptosis were analyzed by the InCuCyte assay after 48-hour incubation with drugs. Synergy was analyzed using Synergy Finder software by the HSA method for (A) inhibition of cell proliferation or (B) apoptosis, and mean synergy score is reported ($P < 0.05$ vs. drug alone). Intensity of red color indicates synergy score for each dose combination, whereas green indicates antagonism. C, Representative images of JW23.3 cells treated for 48 hours. YOYO-1 green fluorescence indicates apoptotic cells.

in our current study. First, multiple pharmacologic inhibitors of TYK2 were shown to reduce proliferation and increase apoptosis in a panel of murine and human MPNST cell lines. Deucravacitinib (BMS-986165), a highly specific second-generation TYK2 inhibitor, targets the JH2 pseudokinase domain of the TYK2 protein and is FDA-approved for the treatment of plaque psoriasis (37). Unlike first-generation TYK2 inhibitors directed against the catalytic JH1 domain, which shares overlapping homology among all JAKs, deucravacitinib does not block JAK1–3 at clinically relevant doses (38). In plaque psoriasis, the actions of deucravacitinib are mediated through inhibition of type I IFN, IL12,

and IL23 signal transduction (38). In MPNST, it remains unclear as to what upstream proteins are relevant.

Because of their immunosuppressive properties, selective inhibitors of JAKs (JAK1–3 and TYK2) were initially utilized for treatment of autoimmune diseases (15). However, these drugs are also being explored in the oncology space. JAK inhibitors, including ruxolitinib, fedratinib, and momelotinib, have been approved as therapeutics for hematologic cancers, and the JAK2 inhibitor AZD1480 is in clinical trials for solid cancers (39). In addition, first- and second-generation TYK2 inhibitors (e.g., SAR-20347, SAR-20351, and NDI-031301) show

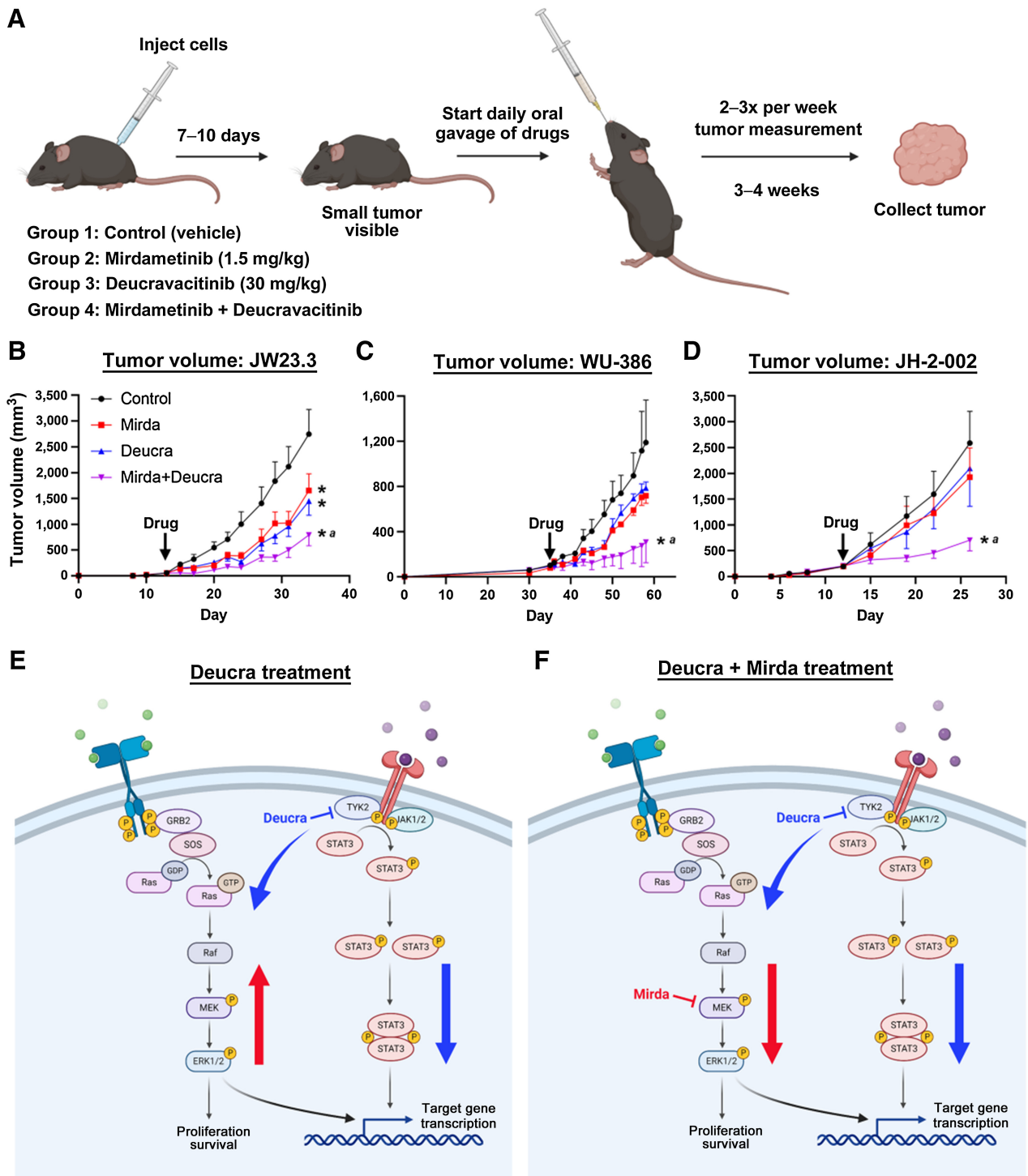


Figure 6.

The combination of drugs inhibiting TYK2 and MEK block MPNST tumor growth in mice. **A**, Schematic diagram of treatment paradigm. Mice with JW23.3 MPNST xenograft tumors ($n = 6$ per group; **B**), WU-386 MPNST PDX tumors ($n = 3$ per group; **C**), or JH-2-002 MPNST xenograft tumors ($n = 5$ per group; **D**) were treated daily with 1.5 mg/kg mirdametininib (Mirda), 30 mg/kg deucravacitinib (Deucra, BMS-986165), the combination of drugs, or vehicle control for 3 weeks or until tumors reached the maximum allowed volume. *, $P < 0.05$ vs. vehicle control; ^a, $P < 0.05$ for drug combination vs. drugs alone. **E** and **F**, Diagram of TYK2/STAT3 and MEK/ERK pathways after treatment with Deucra and/or Mirda in MPNST cells. Illustrations were created with BioRender.com.

promise in preclinical studies for treatment of blood and solid tumor malignancies (19, 20). However, regulators have recently limited use of some inhibitors directed against JAK1–3 after reports of serious adverse events, including blood clots, cardiac events, and cancer, thus increasing interest in development of specific TYK2 inhibitors (16). Because deucravacitinib does not bind to other JAKs, it should have a safer side effect profile compared with inhibitors directed against JAK1–3 (37, 38).

Second, in this study, we demonstrated that TYK2 inhibition decreased pSTAT3 levels while stimulating activation of the MEK/ERK pathway in what is likely a compensatory survival mechanism for the cancer cells. This is in line with studies in other types of cancer, in which intrinsic or acquired resistance over time to JAKinib/TYKinib can result in treatment failure and poor outcomes (21, 22). Drug resistance is common in patients with hematologic malignancies treated with ruxolitinib, a pan-JAK inhibitor, for 2 to 3 years (23, 40, 41). Similarly, leukemia cells can develop resistance with protracted exposure to cerdulatinib, a pan-JAK/TYK2 inhibitor (22). Insensitivity to JAK and TYK2 inhibitors may be the result of heterodimerization with other JAK family members, subsequently acquired mutations in the JAK/TYK2 kinase domain that interfere with drug binding, activation of other signaling pathways (i.e., RAS, MAPK, and Akt pathways), or mutations in epigenetic regulatory genes (21, 42–44). In an effort to overcome resistance to TYK2 and JAK inhibitors, combination therapies have been investigated, including with histone deacetylase inhibitor (HDACi), Hsp90 inhibitors, chemotherapy drugs, MEK inhibitors, mTOR inhibitor, or a second JAK inhibitor (21, 22, 24, 25, 45, 46).

In this study, we examined signaling pathways downstream of TYK2 and changes in global gene expression in MPNST cells to identify additional targets for possible combination drug therapy. The mechanism of TYK2 signaling was evaluated via several complementary methods, including Western blot analysis for protein activation, RNA-seq for global gene expression, and a high throughput qPCR array for expression of genes known to be downstream of JAK/STAT family members. These results detected significant changes in genes and proteins involved in cell cycle, inflammation, immune function, and cancer signaling. At the protein level, TYK2 inhibitor drugs lowered STAT3 activation, while increasing ERK1/2 activation at 1 to 48 hours (Fig. 3). This indicates rapid signal transduction at the protein level, as well as long-term, sustained gene expression changes affecting the MEK/ERK pathway. However, the exact signaling molecules mediating the direct crosstalk of TYK2 inhibition to elevate pERK1/2 in the short-term is unclear. In addition, the MEK/ERK pathway is complex and regulated by multiple feedback mechanisms to regulate activation state and output, and levels of ERK1/2 phosphorylation are therefore not adequate predictive biomarkers of steady-state activation (27). MEK/ERK gene expression signatures generated using the MEK inhibitors mirdametininib and selumetinib are reliable biomarkers of ERK transcriptional output and therefore pathway activation (27, 29). Consistent with these MEK/ERK pathway gene signatures, our qPCR array and RNA-seq data show that TYK2 inhibitors (i.e., WU-12, WU-76, and deucravacitinib) stimulated gene expression in the MEK/ERK pathway, including *SPRY4*, *SPRED1*, *CCDN1*, *Map3k2/3*, *MAP2k2/4*, *Map2*, and *Dusp4* (Fig. 2). Others report that treatment with JAK inhibitors can induce the MEK/ERK pathway in myeloproliferative neoplasms and melanoma in preclinical *in vitro* and *in vivo* studies (47, 48). Conversely, MEK inhibitors increase the JAK/STAT pathway in melanoma cells, indicating crosstalk between the two pathways, and co-incubation of a JAK inhibitor

with a MEK inhibitor greatly improves treatment efficacy in melanoma (48). MEK inhibitors, including selumetinib and mirdametininib, are used clinically for benign PN (3). However, preclinical models demonstrate that MPNST develop resistance to kinase inhibitors, including MEK inhibitors, with long-term treatment (28). Despite promising preclinical studies, single agents, including MEK inhibitors, mTOR inhibitors, and Hsp90 inhibitors, have had limited success treating MPNST in patients, likely due to adaptive survival responses (28, 49). Indeed, in our current study, single-agent therapy with a MEK inhibitor, mirdametininib, only moderately reduced tumor growth *in vivo* (Fig. 6).

Finally, addition of mirdametininib, an investigational MEK inhibitor, synergistically enhanced the efficacy of deucravacitinib, a TYK2 inhibitor, in MPNST cells *in vitro* and on MPNST tumor growth in three *in vivo* mouse models (Figs. 5 and 6). A schematic diagram of the proposed signaling actions of TYK2 and/or MEK inhibitors in MPNST is shown in Fig. 6E and F. Development of drug combination strategies aims to improve therapeutic efficacy in patients with MPNST, resulting in longer survival and increasing the treatment options available for this aggressive cancer (28, 50). Taken together, these data provide the preclinical rationale for the development of a phase I clinical trial of deucravacitinib and mirdametininib in patients with NF1-associated MPNSTs.

Authors' Disclosures

D.C. Borchering reports grants from Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP), St. Louis Men's Group Against Cancer, and The Doris Duke Charitable Foundation and non-financial support from SpringWorks Therapeutics, Inc. during the conduct of the study; in addition, D.C. Borchering has U.S. Provisional Patent Applications Nos. 63/277,331 and 18/053,935 pending. K. He reports grants from Department of Defense during the conduct of the study. A. Gothra reports grants from Washington University School of Medicine during the conduct of the study. P. Ruminski reports grants from Department of Defense during the conduct of the study. C.A. Pratilas reports grants from Novartis and personal fees from Day One Therapeutics and Kura Oncology outside the submitted work; in addition, C.A. Pratilas has patent 63184422 pending. A.C. Hirbe reports personal fees from Springworks Therapeutics during the conduct of the study as well as personal fees from AstraZenica/Alexion and grants from Tango Therapeutics outside the submitted work; in addition, A.C. Hirbe has a patent for US provisional application Serial No. 63/277,331, filed on November 9, 2021, pending. No disclosures were reported by the other authors.

Authors' Contributions

D.C. Borchering: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing. **N.V. Amin:** Formal analysis, validation, investigation, visualization. **K. He:** Formal analysis, validation, investigation, visualization. **X. Zhang:** Resources, investigation. **Y. Lyu:** Software, formal analysis, visualization. **C. Dehner:** Formal analysis, validation, investigation, visualization. **H. Bhatia:** Resources, writing—original draft, writing—review and editing. **A. Gothra:** Formal analysis, investigation, visualization. **L. Daud:** Formal analysis, investigation, visualization. **P. Ruminski:** Resources, writing—review and editing. **C.A. Pratilas:** Resources, writing—review and editing. **K. Pollard:** Resources, writing—review and editing. **T. Sundby:** Resources, writing—review and editing. **B.C. Widemann:** Resources, writing—review and editing. **A.C. Hirbe:** Conceptualization, supervision, funding acquisition, methodology, writing—original draft, project administration, writing—review and editing.

Acknowledgments

This work was funded by a New Investigator Award through the Neurofibromatosis Research Program (NFRP) from the Department of Defense Office of the Congressionally Directed Medical Research Programs (CDMRP; W81XWH-20-1-0148 to A.C. Hirbe), the St. Louis Men's Group Against Cancer (to A.C. Hirbe), The Doris Duke Charitable Foundation (to A.C. Hirbe), and the

Neurofibromatosis Therapeutic Acceleration Program (NTAP; to C.A. Pratilas). Mirdametinib for this work was provided by SpringWorks Therapeutics, Inc. under an investigator-initiated research agreement with Washington University. We would like to acknowledge the Genome Technology Access Center (GTAC) and the Genome Engineering & Stem Cell Center (GESCC) at Washington University in St. Louis for processing RNA-sequencing samples and providing CRISPR-Cas9 gRNAs for KO studies, respectively. We thank the Alvin J. Siteman Cancer Center at Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis for use of the Siteman Flow Cytometry for FACS. The Siteman Cancer Center was supported in part by an NCI Cancer Center Support Grant No. P30 CA091842. We also acknowledge BioRender.com for use of figure illustration software.

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Note

Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Received December 1, 2022; revised January 23, 2023; accepted February 15, 2023; published first February 16, 2023.

References

- Zhang X, Murray B, Mo G, Shern JF. The role of polycomb repressive complex in malignant peripheral nerve sheath tumor. *Genes (Basel)* 2020;11:287.
- Cai Z, Tang X, Liang H, Yang R, Yan T, Guo W. Prognosis and risk factors for malignant peripheral nerve sheath tumor: a systematic review and meta-analysis. *World J Surg Oncol* 2020;18:257.
- Wu LMN, Lu QR. Therapeutic targets for malignant peripheral nerve sheath tumors. *Future Neurology* 2019;14:FNL7.
- Hirbe AC, Kaushal M, Sharma MK, Dahiya S, Pekmezci M, Perry A, et al. Clinical genomic profiling identifies TYK2 mutation and overexpression in patients with neurofibromatosis type 1-associated malignant peripheral nerve sheath tumors. *Cancer* 2017;123:1194–201.
- Borcherding DC, He K, Amin NV, Hirbe AC. TYK2 in cancer metastases: genomic and proteomic discovery. *Cancers (Basel)* 2021;13:4171.
- Carpenter RL, Lo HW. STAT3 target genes relevant to human cancers. *Cancers (Basel)* 2014;6:897–925.
- Qin W, Godec A, Zhang X, Zhu C, Shao J, Tao Y, et al. TYK2 promotes malignant peripheral nerve sheath tumor progression through inhibition of cell death. *Cancer Med* 2019;8:5232–41.
- Sanda T, Tyner JW, Gutierrez A, Ngo VN, Glover J, Chang BH, et al. TYK2-STAT1-BCL2 pathway dependence in T-cell acute lymphoblastic leukemia. *Cancer Discov* 2013;3:564–77.
- Velusamy T, Kiel MJ, Sahasrabudhe AA, Rolland D, Dixon CA, Bailey NG, et al. A novel recurrent NPM1-TYK2 gene fusion in cutaneous CD30-positive lymphoproliferative disorders. *Blood* 2014;124:3768–71.
- Organ SL, Tong J, Taylor P, St-Germain JR, Navab R, Moran MF, et al. Quantitative phospho-proteomic profiling of hepatocyte growth factor (HGF)-MET signaling in colorectal cancer. *J Proteome Res* 2011;10:3200–11.
- Song XC, Fu G, Yang X, Jiang Z, Wang Y, Zhou GW. Protein expression profiling of breast cancer cells by dissociable antibody microarray (DAMA) staining. *Mol Cell Proteomics* 2008;7:163–9.
- Zhu X, Lv J, Yu L, Zhu X, Wu J, Zou S, et al. Proteomic identification of differentially-expressed proteins in squamous cervical cancer. *Gynecol Oncol* 2009;112:248–56.
- Drake JM, Graham NA, Lee JK, Stoyanova T, Faltermeier CM, Sud S, et al. Metastatic castration-resistant prostate cancer reveals intrapatient similarity and interpatient heterogeneity of therapeutic kinase targets. *Proc Natl Acad Sci U S A* 2013;110:E4762–9.
- Groisberg R, Hong DS, Holla V, Janku F, Piha-Paul S, Ravi V, et al. Clinical genomic profiling to identify actionable alterations for investigational therapies in patients with diverse sarcomas. *Oncotarget* 2017;8:39254–67.
- Taylor PC. Clinical efficacy of launched JAK inhibitors in rheumatoid arthritis. *Rheumatology (Oxford)* 2019;58(Suppl 1):i17–26.
- Agrawal M, Kim ES, Colombel JF. JAK inhibitors safety in ulcerative colitis: practical implications. *J Crohns Colitis* 2020;14(Supplement_2):S755–S60.
- Harrison C, Kiladjan J-J, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovska V, et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. *New Engl J Med* 2012;366:787–98.
- Harrison CN, Schaap N, Vannucchi AM, Kiladjan J-J, Jourdan E, Silver RT, et al. Fedratinib in patients with myelofibrosis previously treated with ruxolitinib: an updated analysis of the JAKAR2A2 study using stringent criteria for ruxolitinib failure. *Am J Hematol* 2020;95:594–603.
- Akahane K, Li Z, Etchin J, Berezovskaya A, Gjini E, Masse CE, et al. Anti-leukaemic activity of the TYK2 selective inhibitor NDI-031301 in T-cell acute lymphoblastic leukaemia. *Br J Haematol* 2017;177:271–82.
- Reader J, Williams N, Bojdo J, Worthington J, Mitchell T. Abstract C086: Immunotherapeutic effects of the TYK2 inhibitor SAR-20351 in syngeneic tumor models. *Mol Cancer Ther* 2019;18(12 Supplement):C086–C.
- Greenfield G, McPherson S, Mills K, McMullin MF. The ruxolitinib effect: understanding how molecular pathogenesis and epigenetic dysregulation impact therapeutic efficacy in myeloproliferative neoplasms. *J Transl Med* 2018;16:360.
- Tavakoli Shirazi P, Eadie LN, Page EC, Heatley SL, Bruning JB, White DL. Constitutive JAK/STAT signaling is the primary mechanism of resistance to JAKi in TYK2-rearranged acute lymphoblastic leukemia. *Cancer Lett* 2021;512:28–37.
- Cervantes F, Vannucchi AM, Kiladjan JJ, Al-Ali HK, Sirulnik A, Stalbovska V, et al. Three-year efficacy, safety, and survival findings from COMFORT-II, a phase 3 study comparing ruxolitinib with best available therapy for myelofibrosis. *Blood* 2013;122:4047–53.
- Levine RL, Koppikar P, Marubayashi S, Bhagwat N, Taldone T, Park CY, et al. Combination therapy using JAK2 and HSP90 inhibitors increased efficacy in myelofibrosis in vivo. *Blood* 2012;120:805.
- Giordano G, Parcesepe P, D'Andrea MR, Coppola L, Di Raimo T, Remo A, et al. JAK/Stat5-mediated subtype-specific lymphocyte antigen 6 complex, locus G6D (LY6G6D) expression drives mismatch repair proficient colorectal cancer. *J Exp Clin Cancer Res* 2019;38:28.
- Endo M, Yamamoto H, Setsu N, Kohashi K, Takahashi Y, Ishii T, et al. Prognostic significance of AKT/mTOR and MAPK pathways and antitumor effect of mTOR inhibitor in NF1-related and sporadic malignant peripheral nerve sheath tumors. *Clin Cancer Res* 2013;19:450–61.
- Dry JR, Pavey S, Pratilas CA, Harbron C, Runswick S, Hodgson D, et al. Transcriptional pathway signatures predict MEK addiction and response to selumetinib (AZD6244). *Cancer Res* 2010;70:2264–73.
- Wang J, Pollard K, Calizo A, Pratilas CA. Activation of receptor tyrosine kinases mediates acquired resistance to MEK inhibition in malignant peripheral nerve sheath tumors. *Cancer Res* 2021;81:747–62.
- Pratilas CA, Taylor BS, Ye Q, Viale A, Sander C, Solit DB, et al. (V600E)BRAF is associated with disabled feedback inhibition of RAF-MEK signaling and elevated transcriptional output of the pathway. *Proc Natl Acad Sci U S A* 2009;106:4519–24.
- Gross AM, Wolters PL, Dombi E, Baldwin A, Whitcomb P, Fisher MJ, et al. Selumetinib in children with inoperable plexiform neurofibromas. *N Engl J Med* 2020;382:1430–42.
- Pollard K, Banerjee J, Doan X, Wang J, Guo X, Allaway R, et al. A clinically and genomically annotated nerve sheath tumor biospecimen repository. *Sci Data* 2020;7:184.
- Dehner C, Moon CI, Zhang X, Zhou Z, Miller C, Xu H, et al. Chromosome 8 gain is associated with high-grade transformation in MPNST. *JCI Insight* 2021;6:e146351.
- Hirbe AC, Dahiya S, Miller CA, Li T, Fulton RS, Zhang X, et al. Whole exome sequencing reveals the order of genetic changes during malignant transformation and metastasis in a single patient with NF1-plexiform neurofibroma. *Clin Cancer Res* 2015;21:4201–11.
- Ianevski A, Giri AK, Aittokallio T. SynergyFinder 2.0: visual analytics of multi-drug combination synergies. *Nucleic Acids Res* 2020;48:W488–W93.
- Townsend EC, Murakami MA, Christodoulou A, Christie AL, Koster J, DeSouza TA, et al. The public repository of xenografts enables discovery and randomized phase II-like trials in mice. *Cancer Cell* 2016;29:574–86.

36. Brown AP, Carlson TC, Loi CM, Graziano MJ. Pharmacodynamic and toxicokinetic evaluation of the novel MEK inhibitor, PD0325901, in the rat following oral and intravenous administration. *Cancer Chemother Pharmacol* 2007;59:671–9.
37. Armstrong AW, Gooderham M, Warren RB, Papp KA, Strober B, Thaçi D, et al. Deucravacitinib versus placebo and apremilast in moderate to severe plaque psoriasis: efficacy and safety results from the 52-week, randomized, double-blinded, placebo-controlled phase 3 POETYK PSO-1 trial. *J Am Acad Dermatol* 2023;88:29–39.
38. Wroblewski ST, Moslin R, Lin S, Zhang Y, Spergel S, Kempson J, et al. Highly selective inhibition of tyrosine kinase 2 (TYK2) for the treatment of autoimmune diseases: discovery of the allosteric inhibitor BMS-986165. *J Med Chem* 2019;62:8973–95.
39. Qureshy Z, Johnson DE, Grandis JR. Targeting the JAK/STAT pathway in solid tumors. *J Cancer Metastasis Treat* 2020;6:27.
40. Cervantes F, Pereira A. Does ruxolitinib prolong the survival of patients with myelofibrosis? *Blood* 2017;129:832–7.
41. Andreoli A, Verger E, Robin M, Raffoux E, Zini JM, Rousselot P, et al. Clinical resistance to ruxolitinib is more frequent in patients without MPN-associated mutations and is rarely due to mutations in the JAK2 kinase drug-binding domain. *Blood* 2013;122.
42. Koppikar P, Bhagwat N, Kilpivaara O, Manshouri T, Adli M, Hricik T, et al. Heterodimeric JAK-STAT activation as a mechanism of persistence to JAK2 inhibitor therapy. *Nature* 2012;489:155–9.
43. Bhagwat N, Levine RL, Koppikar P. Sensitivity and resistance of JAK2 inhibitors to myeloproliferative neoplasms. *Int J Hematol* 2013;97:695–702.
44. Hornakova T, Springuel L, Devreux J, Dusa A, Constantinescu SN, Knoops L, et al. Oncogenic JAK1 and JAK2-activating mutations resistant to ATP-competitive inhibitors. *Haematologica* 2011;96:845–53.
45. Chakraborty SN, Leng X, Perazzona B, Sun X, Lin YH, Arlinghaus RB. Combination of JAK2 and HSP90 inhibitors: an effective therapeutic option in drug-resistant chronic myelogenous leukemia. *Genes Cancer* 2016;7:201–8.
46. Harrison CN, Schaap N, Mesa RA. Management of myelofibrosis after ruxolitinib failure. *Ann Hematol* 2020;99:1177–91.
47. Stivala S, Codilupi T, Brkic S, Baerenwaldt A, Ghosh N, Hao-Shen H, et al. Targeting compensatory MEK/ERK activation increases JAK inhibitor efficacy in myeloproliferative neoplasms. *J Clin Invest* 2019;129:1596–611.
48. Zhao K, Lu Y, Chen Y, Cheng J, Zhang W. Dual inhibition of MAPK and JAK2/STAT3 pathways is critical for the treatment of BRAF mutant melanoma. *Mol Ther Oncolytics* 2020;18:100–8.
49. Kim A, Lu Y, Okuno SH, Reinke D, Maertens O, Perentesis J, et al. Targeting refractory sarcomas and malignant peripheral nerve sheath tumors in a phase I/II study of sirolimus in combination with ganetespib (SARC023). *Sarcoma* 2020;2020:5784876.
50. González-Muñoz T, Kim A, Ratner N, Peinado H. The need for new treatments targeting MPNST: the potential of strategies combining MEK inhibitors with antiangiogenic agents. *Clin Cancer Res* 2022;28:3185–95.

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

Plans for the next reporting period (6 month no cost extension: Year 4):

Specific Aim 1: We will determine whether TYK2 is a prognostic biomarker for MPNST.

Major Task 1: Are TYK2 expression levels associated with overall survival, time to metastasis, and response to therapy in patients with MPNST?

Plans: This major task has been completed.

Major Task 2: Does TYK2 expression distinguish atypical neurofibromas from MPNSTs?

Plans: This major task has been completed.

Specific Aim 2: We will delineate the mechanism of action of TYK2 in MPNST cells.

Major Task 3: Determine whether STAT-3 mediated activation of Bcl-2 is responsible for TYK-2 mediated protection from cell death.

Plans: We are in the process of expanding and validating additional colonies of human JH-2-002 MPNST cells transfected by CRISPR/Cas9 for knockout of *TYK2* and/or *STAT3*. We will continue IncuCyte cell proliferation and apoptosis assays with the *TYK2*, *STAT3* and *TYK2/STAT3* knockout cells. We will also examine other downstream proteins (e.g. other STATs) as well as upstream cytokine ligands (e.g. interleukins and/or interferons) that are involved in TYK2 signaling and downstream effects in MPNST. This may identify additional drug targets for MPNST therapeutics. While we have focused on proliferative and cell survival effects of TYK2 in MPNST, we may later evaluate cell death mechanisms with apoptosis assays, including Bcl-2 knockout, TUNEL assay, cleaved caspase-3 Western blotting, and flow cytometry for Annexin-V/propidium iodide staining.

Major Task 4: Explore the impact of TYK2 downregulation on signaling nodes known to be important in MPNST pathogenesis.

Plans: We will continue to explore the interaction of TYK2 signaling (e.g. STAT3) and MPNST known signaling nodes (e.g. the MEK/ERK and mTOR/S6K pathways) by Western blot in cells deficient for *TYK2*, *STAT3* or both genes (CRISPR/Cas9 KO) in the JW23.3 and 2-002 cell-lines compared to control cells. In addition, we are currently evaluating the effect of the specific TYK2 inhibitor, deucravacitinib (BMS-986165), on the phosphorylation of STAT3, ERK, and S6-kinase in JW23.3 and JH 2-002 cells.

Major Task 5: Determine the impact of TYK2 downregulation on the global expression profile of MPNSTs.

Plans: This major task has been completed.

Specific Aim 3: Evaluate pharmacologic inhibition of TYK2 in MPNSTs *in vivo*.

Major Task 6. Does pharmacologic inhibition of TYK2 lead to decreased tumor viability *in vitro*?

Plans: This major task has been completed early during Year 1, and was expanded to include a clinically relevant and specific TYK2 inhibitor, deucravacitinib (BMS-986165), in Years 2-3.

Major Task 7. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in MPNST PDX lines?

Plans: This major task has been completed.

Major Task 8. Does pharmacologic inhibition of TYK2 lead to decreased tumor growth and/or metastasis *in vivo* in mice with an intact immune system?

Plans: This major task has been completed.