Received: 24 October 2022 | Revised: 16 January 2023 | Accepted: 3 April 2023

DOI: 10.1002/cac2.12422

CANCER COMMUNICATIONS Open Access

LETTER TO THE EDITOR

Antitumoral effects of cannabis in *Notch1*-mutated T-cell acute lymphoblastic leukemia

Dear Editor,

In T-cell acute lymphoblastic leukemia (T-ALL), an aggressive hematologic cancer with poor clinical outcomes, more than 50% of cases show NOTCH1-driven transformation [1]. The NOTCH1 receptor signaling pathway is activated through a series of proteolytic cleavages, ultimately causing the release of the active intracellular domain (NICD), which translocates to the nucleus where it promotes transcription of target genes involved in cell growth. The importance of NOTCH1 mutations in T-ALL has generated great interest in the development of anti-NOTCH1 targeted therapies. A new and promising emerging field in cancer treatment is medical cannabis. Accumulating evidence suggests the direct effects of cannabis on tumor progression in cell lines and animal models [2]. Cannabis, and its unique secondary metabolites, known as phytocannabinoids, directly affect the propagation of cancer cells by modulating key cellsignaling pathways [3]. We have previously demonstrated that different cannabis extracts, each containing a unique composition of metabolites, selectively impaired the survival of cancer cell lines depending on a match between the chemical composition of the extract and the characteristics of the specific cancer cell line [4]. In the present work, we set out to investigate whether cannabis extracts with unique phytocannabinoid profiles can selectively facilitate antitumor effects in T-ALL cells that harbor a *Notch1* mutation.

We first performed a preliminary screening in which the viability of four distinct T-ALL cell lines was tested when treated with 15 extracts from different cannabis chemovars (Figure 1A). The composition of each extract was analyzed using ultra high-performance liquid chromatography with an ultraviolet detector (UHPLC/UV) method we developed [5], and substantial differences were found in their content of phytocannabinoids (Extracts 1-15, Supplementary Table S1). The four T-ALL cancer cell lines varied in their sensitivity to the cytotoxic effect of the extracts. Extract 12 was found to dramatically impair the viability of MOLT-4 and CCRF-CEM cells and to a lesser extent the viability of Jurkat and Loucy cells (Figure 1A). Extract 12 is a cannabidiol (CBD)-rich extract containing high amounts of this major non-psychoactive phytocannabinoid. Moreover, Extract 12 was found to be significantly more potent against MOLT-4 and CCRF-CEM cell lines than other high-CBD extracts (Extracts 16-19, Supplementary Table S1) or pure CBD at the same concentration as in the extract (Figure 1B). These findings suggested that a unique combination of secondary metabolites potentiates the cytotoxic effects observed for Extract 12 but not for CBD alone. To assess whether the reduction in viability is due to increased apoptosis, we examined the protein levels of cleaved caspase-3 (Figure 1C) and performed Annexin-V and propidium iodide (PI) staining (Figure 1D-E). Treatment with Extract 12 led to significantly enhanced apoptosis of MOLT-4 and CCRF-CEM cells compared to Jurkat or Loucy cells. Analyzing the genetic differences between these cells led us to conjecture that the antitumor effect of Extract 12 is dependent on abnormal NOTCH1 signaling, as MOLT-4 and CCRF-CEM cell lines harbor *Notch1* mutations while Jurkat and Loucy do not. By testing three additional cell lines, HPB-ALL and DND-41 that harbor *Notch1* mutation and wild-type *PTEN* and T-ALL1 which harbors wild-type *Notch1* and wild-type *PTEN*, we confirmed that the cytotoxic effect of Extract 12 was not mediated via *PTEN*-related mutations as only HPB-ALL and DND-41 cells demonstrated significantly increased apoptosis upon Extract 12 treatment (Supplementary Figure S1A).

Abbreviations: ANOVA, Analysis of variance; c-Cas-3, Cleaved Caspase-3; CBD, Cannabidiol; CRISPR/Cas9, Clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease9; FITC, Fluorescein isothiocyanate; gRNA, Guide RNA; HES1, Hairy and Enhancer of Split 1; NICD, Notch1 intracellular domain; NOD/Scid, Nonobese diabetic - severe combined immunodeficiency; PI, propidium iodide; PTEN, Phosphatase and tensin homolog; qPCR, Quantitative (a.k.a. real-time) polymerase chain reaction; SEM, Standard error of the mean; T-ALL, T-cell acute lymphoblastic leukemia; UHPLC/UV, Ultrahigh-performance liquid chromatography with an ultraviolet detector.

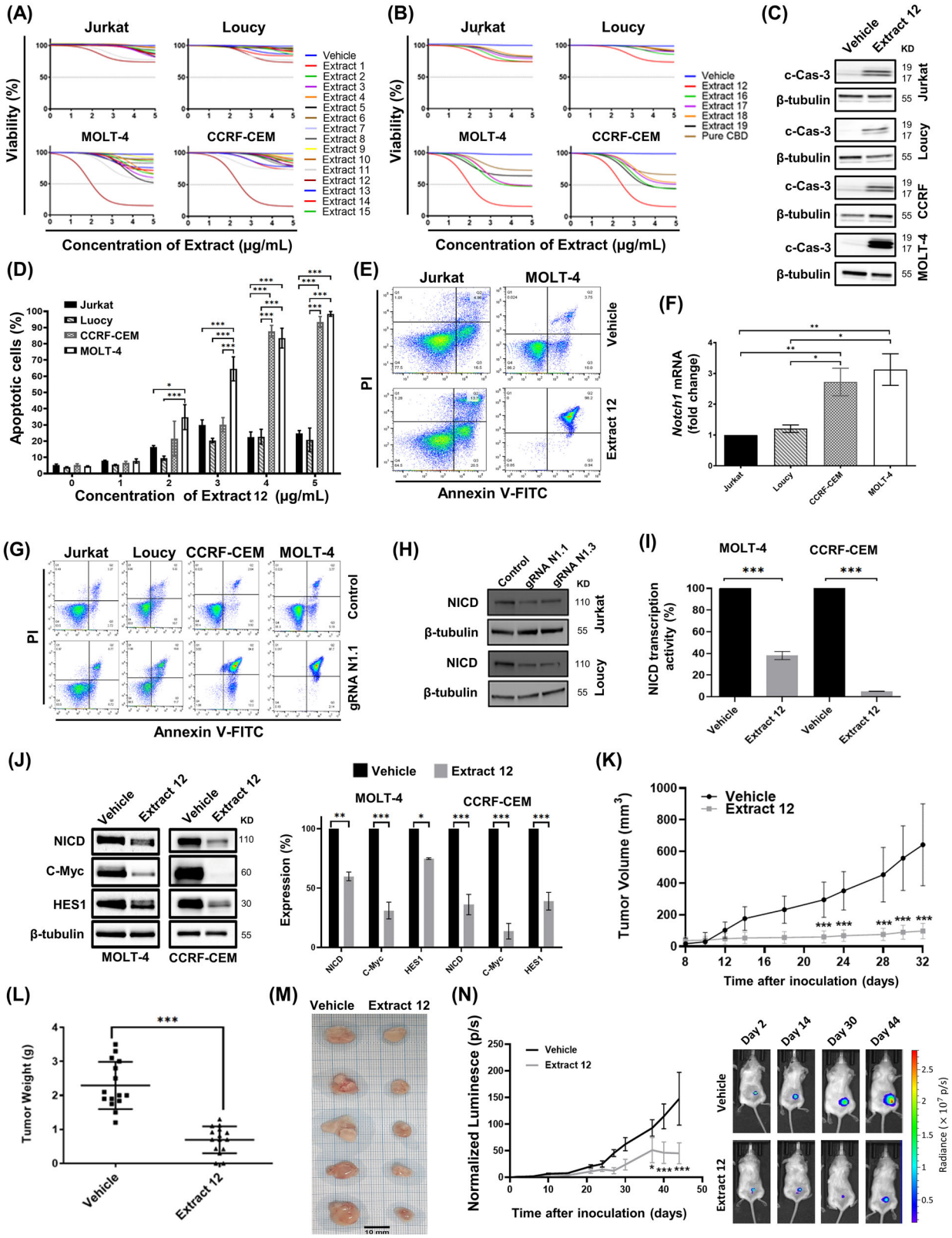

FIGURE 1 Cannabis Extract 12 induces apoptosis in NOTCH1-transformed T-ALL cell lines and inhibits cancer progression in vivo. Dose-dependent AlamarBlue© viability assays were performed on Loucy, Jurkat, CCRF-CEM and MOLT-4 cell lines following 24 h treatment with either (A) cannabis Extracts 1-15, (B) five high-CBD cannabis extracts (Extracts 12 and 16-19) or pure CBD in the same concentration as in Extract 12. The assays were performed at concentrations of 1 to 5 μg/mL in triplicates ($n = 3$). (C) A representative blot of cleaved caspase-3 (c-Cas-3) in the four T-ALL cell lines following treatment with either vehicle or Extract 12 (5 μg/mL) for 3 h, with β-tubulin as the loading control ($n = 3$). (D) Analysis of apoptosis via Annexin V/PI staining using flow cytometry in the four T-ALL cell lines following 24 h treatment

To test the hypothesis that NOTCH1 mutations make T-ALL susceptible to the cytotoxic effects of Extract 12, we first measured the mRNA expression of *Notch1* in the four T-ALL cell lines, confirming that *Notch1* expression was significantly higher in MOLT-4 and CCRF-CEM cells (Figure 1F). Next, we used CRISPR-Cas9 to knockout *Notch1*. Knocking out *Notch1* resulted in apoptotic cell death of CCRF-CEM and MOLT-4 cells which harbor a *Notch1* mutation, but not of Loucy or Jurkat cells which are *Notch1* wild-type (Figure 1G and Supplementary Figure S1B). We confirmed in Jurkat and Loucy cells which survived the CRISPR/Cas9 manipulation that NICD expression was reduced (Figure 1H). These results demonstrate the dependency of MOLT-4 and CCRF-CEM cells on NOTCH1 expression. Next, we tested whether Extract 12 directly affects the activity of NOTCH1 in MOLT-4 and CCRF-CEM cells. A designated reporter assay showed that Extract 12 treatment resulted in a reduction of 62% and 95% of NICD transcription activity in the nucleus of MOLT-4 and CCRF-CEM cell lines, respectively (Figure 1I). Additionally, we showed by immunoblotting that the levels of the NICD protein and its downstream targets, c-Myc and Hairy and Enhancer of Split 1 (HES1), key proteins in NOTCH1 signaling, were significantly reduced following treatment with Extract 12 to 59%, 31% and 74%, respectively, in MOLT-4 cells and 36%, 13% and 38%, respectively, in CCRF-CEM cells (Figure 1J). Overexpression of the NICD in MOLT-4 cells was able to rescue them from Extract 12-induced apoptosis (Supplementary Figure S1C-F). The full-length immature form of NOTCH1 accumulated in the membrane upon treatment with Extract 12 (Supplementary Figure S1G-I).

Next, the antitumor properties of Extract 12 on tumor growth in vivo were tested. NOD/Scid mice were engrafted subcutaneously with MOLT-4 cells. After two days, the mice were randomly divided into two groups and treated with either vehicle or Extract 12 every other day for four weeks. Tumor volume was significantly smaller in the Extract 12-treated group compared to the vehicle-treated group already after three weeks (Figure 1K). At the endpoint of the experiment, excised tumors from Extract 12-treated mice weighed significantly less than tumors from the vehicle-treated group (Figure 1L-M). This experimental setup was repeated with MOLT-4-Luc cells and tumors were tracked by bioluminescence images acquired over time. The bioluminescence signal of MOLT-4-Luc tumors was detectable as early as 2 days post-injection, and its intensity increased over time to reach approximately 108 photons emitted/s per tumor on day 44. Treatment with Extract 12 significantly inhibited tumor growth, as indicated by lower luminescence intensity (Figure 1N). When the subcutaneous tumors were allowed to develop for two weeks before initiating Extract 12 treatment (Supplementary Figure S2A), immunofluorescence staining of DAPI and NICD demonstrated that the expression levels of NICD were much lower in tumors from the Extract 12-treated group compared to the vehicle-treated group (Supplementary Figure S2B). The volumes and weights of

with either vehicle or Extract 12 (1-5 μg/mL). (E) A representative flow cytometry dot plot of MOLT-4 and Jurkat cell lines following 24 h treatment with either vehicle or Extract 12 (5 μg/mL). (F) *Notch1* mRNA expression in T-ALL cell lines by qPCR. (G) Representative dot plots of apoptosis assessed by Annexin V/PI staining using flow cytometry following CRISPR-Cas9 knockout of *Notch1* in Jurkat, Loucy, CCRF-CEM and MOLT-4 cell lines. (H) Representative NICD blots with β-tubulin as the loading control after *Notch*1 knockout in Jurkat and Loucy cells. (I) MOLT-4 and CCRF-CEM cells were infected with Cignal Lenti RBP-Jk Reporter, and following 3 h treatment with vehicle or Extract 12 (5 μg/mL), luciferase intensity was measured indicating NICD transcription activity ($n = 3$). (J) MOLT-4 and CCRF-CEM cell lines were treated for 3 h with vehicle or Extract 12 (5 μg/mL), and the expression of NICD, c-Myc and HES-1 was evaluated via Western blotting with β-tubulin as the loading control. The intensity analysis of three independent experiments is presented on the *right*. (K) Female NOD/Scid mice ($n = 5$/group, three independent repeats) were engrafted subcutaneously with 1×10^6 MOLT-4 cells. After two days, the mice were randomly divided into two groups and alternate-day treated intraperitoneally with either vehicle or Extract 12 (150 mg/kg). Ectopic tumor volume was measured using a vernier caliper and calculated according to the formula (length $\times$ width^2) $\times$ 0.5. The average difference between vehicle- and Extract 12-treated groups is presented and was statistically analyzed by Bonferroni's multiple comparisons test. (L) Excised tumors were weighed ($n = 15$), and the difference between groups was statistically analyzed by unpaired Student's *t*-test. (M) Representative photograph of excised tumors. (N) Bioluminescence-based tumor quantification of MOLT-4-Luc cells injected subcutaneously to mice ($n = 5$) that were treated intraperitoneally with either vehicle or Extract 12 for six weeks. Luminescence is calculated as total photon flux relative to vehicle control on day 2, and differences were statistically analyzed by unpaired Student's *t*-test; representative images captured by IVIS imaging system are presented on the *right*.

Data are presented as mean ± SEM and statistically analyzed by two-way ANOVA unless otherwise stated (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Abbreviations: ANOVA, Analysis of variance; CBD, Cannabidiol; c-Cas-3, cleaved caspase-3; CRISPR-Cas9, Clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9; FITC, Fluorescein isothiocyanate; gRNA, Guide RNA; HES1, hairy and enhancer of split-1; IVIS, in vivo imaging system; Luc, Luciferase; NICD, Notch1 intracellular domain; NOD/SCID, Nonobese diabetic/severe combined immunodeficiency; PI, Propidium iodide; qPCR, Quantitative polymerase chain reaction; SEM, standard error of the mean; T-ALL, T-cell acute lymphoblastic leukemia.

tumors were also significantly lower, while the average weight of the mice was similar, and in this setup as well the differences were apparent approximately 3 weeks after treatment initiation (Supplementary Figure S2C-F).

In summary, targeting NOTCH1 signaling has generated much interest for its therapeutic potential. However, so far, efforts to develop such treatments have been unsuccessful [6]. The cannabis plant contains over 140 phytocannabinoids, many of which are presumed to have pharmacological properties, and accumulating evidence suggests anticancer capabilities [4]. Here, we identified a specific CBD-rich extract that selectively induced apoptosis in NOTCH1-mutated T-ALL cells. Although CBD by itself was able to induce cell death, the whole extract was more effective, suggesting that other metabolites from the plant are required to achieve full potency. We have previously demonstrated this phenomenon in a mouse model of epilepsy, where CBD-rich extracts with equal amounts of CBD but varying concentrations of other minor compounds led to diverse anticonvulsant effects [5]. A possible mechanism previously suggested to explain the difference between the effects of purified phytocannabinoids versus full-spectrum extracts is the "entourage effect" [7], where one compound may enhance the activity and efficacy of another on the same target. While this synergy is well-established for endogenous cannabinoids of the endocannabinoid system [8], only very few studies demonstrated this phenomenon for phytocannabinoids [9]. Cannabis is already being prescribed to cancer patients for its palliative qualities; however, the huge variety between different chemovars in their composition is disregarded [10]. Matching an effective extract to certain cancer subtypes will ultimately lead to personalized cancer treatments and medications that not only treat symptoms but also treat the disease. As dysregulation of NOTCH1 signaling has been found in various cancers other than T-ALL and in non-cancerous diseases, our findings suggest a novel therapeutic strategy for the effective treatment of a variety of malignancies.

DECLARATIONS

AUTHOR CONTRIBUTIONS

Conceptualization: E.B. and D.M., Methodology: E.B. and P.B., Investigation: E.B., A.G., P.B., I.L.H. and I.S.L., Analysis and interpretation of data: E.B., A.G., G.M.L., S.P., H.N.K., P.B. and I.L.H., Writing – original draft: E.B. and D.M., Writing – review & editing: E.B., P.B., G.M.L., S.P., H.N.K. and D.M., Study supervision: D.M.

ACKNOWLEDGMENTS

We thank the Israel Cancer Research Fund (ICRF) for their financial support of this work and Ohad Guberman for the extraction and sample preparation of the cannabis extracts.

CONFLICT OF INTEREST STATEMENT

D.M. is a scientific advisor and shareholder at Cannasoul Analytics, where his activity is unrelated to the current study. All other authors declare that they have no conflicts of interest.

FUNDING INFORMATION

Israel Cancer Research Fund grant 21-112-PG

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All in vivo mouse experiments in this study have been conducted in accordance with the guidelines and approved by the Technion Administrative Panel of Laboratory Animal Care (IL_0470317).

CONSENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY STATEMENT

The data underlying this study is available from the corresponding author on reasonable request.

Elazar Besser[1]
Anat Gelfand[1]
Gil Moshe Lewitus[1]
Hila Novak-Kotzer[1]
Shiri Procaccia[1]
Paula Berman[1]
Igal Louria-Hayon[1]
Inbar Shreiber-Livne[1]
Yishai Ofran[2]
David Meiri[1]

[1]*The Laboratory of Cancer Biology and Cannabinoid Research, Faculty of Biology, Technion-Israel Institute of Technology, Haifa, Israel*
[2]*Department of Hematology and Bone Marrow Transplantation, Rambam Health Care Campus, Haifa, Israel*

Correspondence
David Meiri, The Laboratory of Cancer Biology and Cannabinoid Research, Faculty of Biology, Technion-Israel Institute of Technology, Haifa 3200003, Israel.
Email: dmeiri@technion.ac.il

ORCID

Elazar Besser https://orcid.org/0000-0002-5700-0947

REFERENCES

1. Vadillo E, Dorantes-Acosta E, Pelayo R, Schnoor M. T cell acute lymphoblastic leukemia (T-ALL): New insights into the cellu-

lar origins and infiltration mechanisms common and unique among hematologic malignancies. Blood Rev [Internet]. 2018 Jan 1 [cited 2019 Mar 30];32(1):36–51. Available from: https://pubmed.ncbi.nlm.nih.gov/28830639/

2. Velasco G, Sánchez C, Guzmán M. Towards the use of cannabinoids as antitumour agents. Nat Rev Cancer [Internet]. 2012 Jun 4 [cited 2020 Feb 23];12(6):436–44. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22555283
3. McAllister SD, Soroceanu L, Desprez P-YY. The Antitumor Activity of Plant-Derived Non-Psychoactive Cannabinoids. J Neuroimmune Pharmacol [Internet]. 2015 Jun [cited 2020 Apr 23];10(2):255–67. Available from: http://link.springer.com/10.1007/s11481-015-9608-y
4. Baram L, Peled E, Berman P, Yellin B, Besser E, Benami M, et al. The heterogeneity and complexity of *Cannabis* extracts as antitumor agents. Oncotarget [Internet]. 2019 Jun 25 [cited 2019 Jul 11];10(41):4091–106. Available from: http://www.oncotarget.com/fulltext/26983
5. Berman P, Futoran K, Lewitus GM, Mukha D, Benami M, Shlomi T, et al. A new ESI-LC/MS approach for comprehensive metabolic profiling of phytocannabinoids in Cannabis. Sci Rep [Internet]. 2018 Dec 24 [cited 2019 Jul 11];8(1):14280. Available from: http://www.nature.com/articles/s41598-018-32651-4
6. Sorrentino C, Cuneo A, Roti G. Therapeutic Targeting of Notch Signaling Pathway in Hematological Malignancies. Mediterr J Hematol Infect Dis [Internet]. 2019 [cited 2019 Jul 28];11(1):e2019037. Available from: http://www.ncbi.nlm.nih.gov/pubmed/31308913
7. Russo EB. Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol. 2011;163(7):1344–64.
8. Di Marzo V. New approaches and challenges to targeting the endocannabinoid system. Nature Reviews Drug Discovery. Nature Publishing Group; 2018;17:623–39.
9. Blasco-Benito S, Seijo-Vila M, Caro-Villalobos M, Tundidor I, Andradas C, García-Taboada E, et al. Appraising the "entourage effect": Antitumor action of a pure cannabinoid versus a botanical drug preparation in preclinical models of breast cancer. Biochem Pharmacol [Internet]. 2018 Nov 1 [cited 2019 Aug 10];157:285–93. Available from: https://www.sciencedirect.com/science/article/pii/S0006295218302387?via%3Dihub
10. Procaccia S, Lewitus GM, Feder CL, Shapira A, Berman P, Meiri D. Cannabis for Medical Use: Versatile Plant Rather Than a Single Drug. Front Pharmacol. 2022;13:894960.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.