An In Vitro Model for Identifying Cardiac Side Effects of Anesthetics

Alex C. Y. Chang, PhD,*†‡ Andrew C. H. Chang, BSc,*† Luka Nicin, BSc,* Gerhard J. Weber, MD,§ Colin Holbrook, BSc,* M. Frances Davies, PhD,‖¶ Helen M. Blau, PhD,*† and Edward J. Bertaccini, MD‖¶

The understanding of anesthetic side effects on the heart has been hindered by the lack of sophisticated clinical models. Using micropatterned human-induced pluripotent stem cell–derived cardiomyocytes, we obtained cardiac muscle depressant profiles for propofol, etomidate, and our newly identified anesthetic compound KSEB01-S2. Propofol was the strongest depressant among the 3 compounds tested, exhibiting the largest decrease in contraction velocity, depression rate, and beating frequency. Interestingly, KSEB01-S2 behaved similarly to etomidate, suggesting a better cardiac safety profile. Our results provide a proof-of-concept for using human-induced pluripotent stem cell–derived cardiomyocytes as an in vitro platform for future drug design. (Anesth Analg XXX;XXX:00–00)

Although their mechanism of action remains to be elucidated, anesthetics have been used widely around the world to safely relieve surgical suffering for over 170 years. Propofol (1-hydroxyl-2,6-diisopropylbenzene [PFL]) is currently one of the most well-known intravenous anesthetics used but has known cardiovascular side effects. Etomidate, a more hemodynamically stable anesthetic acting through the γ-aminobutyric acid type A receptors ($GABA_A$Rs), poses a toxicity profile that suppresses corticosteroid synthesis in the adrenal cortex. Using in silico docking algorithms and our validated model of the $GABA_A$R, we have previously identified a novel anesthetic compound, KSEB01-S2,[1–3] that is a highly potent anesthetic, but has a predicted cardiovascular depressant profile that is similar to etomidate and which has been chemically modified to abolish corticosteroid deficiencies. Before induced pluripotent stem cell technology, it was impossible to profile cardiovascular depressant levels in a human cardiomyocyte. Recent advances in bioengineering have allowed testing of small compounds on geometrically more mature human-induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs).[4] Using micropatterned hiPSC-CMs, we tested cardiac side effects of the aforementioned compounds. Our results show that we can identify the level of anesthetic side effects at physiological concentrations. Our findings strongly support the use of this hiPSC-CM platform for the future characterization of anesthetics.

METHODS

Culture of hiPSCs and hiPSC-CM

hiPSC lines were generated as part of the Stanford Cardiovascular Institute Biobank initiative. The culturing of the established hiPSC lines in our laboratory was reviewed and approved by the Stanford Stem Cell Research Oversight committee (#602). hiPSCs derived from 2 male (22 and 45 years of age) and 2 female patients' (42 and 48 years of age) peripheral blood mononuclear cells were grown on Matrigel-coated plates (Corning, NY) using chemically defined Nutristem (Stemgent, Cambridge, MA) medium as previously described (Figure, panel A).[5,6] The medium was changed daily, and cells were passaged every 4 days using Accutase (Thermo Scientific, Carlsbad, CA) with the addition of rock inhibitor 5 μM (1R,4r)-4-((R)-1-aminoethyl)-N-(pyridin-4-yl)cyclohexanecarboxamide dihydrochloride (Y-27632 2HCl) (Selleck Chem, Houston, TX). hiPSCs were grown to 70%–90% confluence and subsequently differentiated into beating cardiomyocytes (Figure, panel A).[5,6] On days 1 and 3, 4–6 μM 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile (CHIR-99021) (Wnt activator; Selleck Chem) and 5 μM 4-(1,3,3a,4,7,7a-Hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl)-N-8-quinolinyl-Benzamide (IWR-1) (Wnt inhibitor; Sigma, St Louis, MO) were added to Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with insulin-free B27 (Life Technologies, Waltham, MA), respectively. On day 5, the differentiating hiPSCs were treated with RPMI-1640 insulin-free B27 alone. On days 7 and 9, the medium was changed to RPMI-1640

From the *Baxter Laboratory for Stem Cell Biology, Department of Microbiology and Immunology, Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, California; †Stanford Cardiovascular Institute, Stanford University School of Medicine, Stanford, California; ‡Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, California; §Division of Cardiology, University of Washington, Seattle, Washington; ‖Department of Anesthesia, Stanford University School of Medicine, Stanford, California; and ¶Palo Alto Veterans Affairs Health Care System, Palo Alto, California.

Accepted for publication July 26, 2018.

Funding: This research was supported by the Canadian Institutes of Health Research Fellowship (201411MFE-338745-169197 to A.C.Y.C.); the Baxter Foundation and National Institutes of Health (AG044815 and AR063963 to H.M.B.); and Stanford University Department of Anesthesia Field of anesthesia Investigator Departmental benefit Leverage for external research dollars (FIDL) Grant, Stanford University SPARK Drug Discovery Program, and Stanford University Children's Health Research Initiative to M.F.D. and E.J.B.

The authors declare no conflicts of interest.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website (www.anesthesia-analgesia.org).

Reprints will not be available from the authors.

Address correspondence to Alex C. Y. Chang, PhD, Stanford University, 269 Campus Dr, CCSR Building, Room 3200, Stanford, CA 94305-5175. Address e-mail to acychang@stanford.edu; and Edward J. Bertaccini, MD, Department of Anesthesia, 112A Palo Alto Veterans Affairs Health Care System, Palo Alto Division, 3801 Miranda Ave, Palo Alto, CA 94304. Address e-mail to edward.Bertaccini@va.gov.

DOI: 10.1213/ANE.0000000000003757

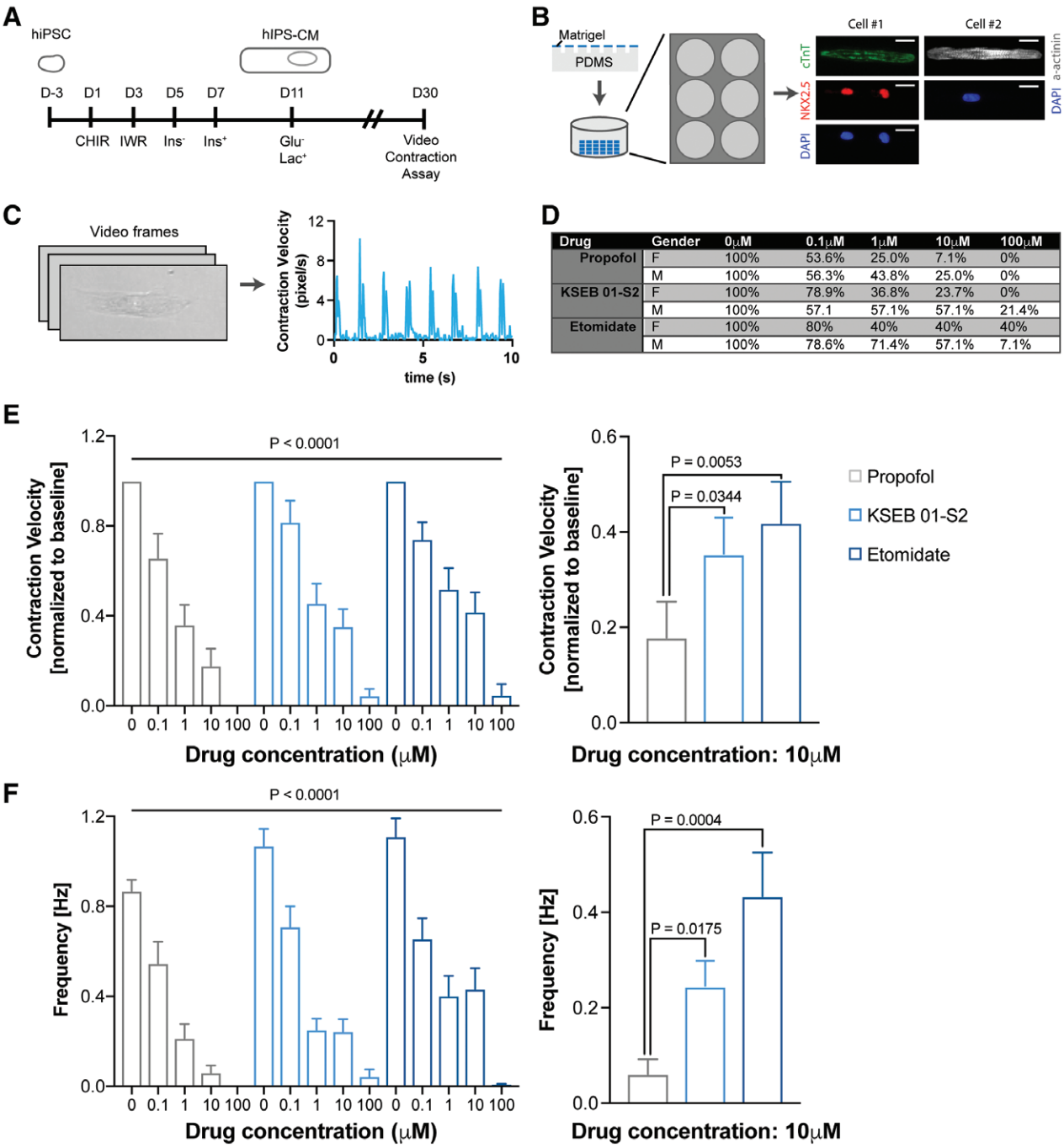

Figure. Cardiotoxicity profiling of KSEB01-S2 using biopatterned hiPSC-CMs. A, Patient hiPSC lines were used to differentiate into hiPSC-CMs. B, Single hiPSC-CM was seeded onto microprinted Matrigel-patterned plates. Representative hiPSC-CMs stained with cardiac markers troponin-T, NKX2.5, α-actinin, and nuclear DAPI (blue) (scale bars, 10 μm). C, hiPSC-CM contraction video analysis and representative tracing of the average contraction speed versus time for a single-patterned hiPSC-CM. Dropoff rate (D), average contraction velocity (E), and beating frequency of propofol (F) (n = 43), KSEB01-S2 (n = 52), and etomidate (n = 39) treated hiPSC-CMs and plotted as mean ± SEM. CHIR indicates CHIR-99021; cTnT, cardiac troponin t; DAPI, 4′,6-diamidino-2-phenylindole; F, female; Glu⁻, glucose free; hiPSC, human-induced pluripotent stem cell; hiPSC-CM, human-induced pluripotent stem cell–derived cardiomyocyte; Ins⁻, insulin free; Ins⁺, with insulin; IWR, IWR-1; KSEB01-S2, compound designation; Lac⁺, lactate containing; M, male; NKX2.5, NK2 Homeobox 5; PDMS, polydimethylsiloxane; SEM, standard error of the mean.

medium supplemented with insulin-containing B27. It has been shown that glucose deprivation allows removal of fibroblasts that arise during differentiation while lactate supplementation enhances metabolic maturation of hiPSC-CMs.[6] Beating hiPSC-CMs were purified and maintained in glucose-free conditions using RPMI-1640 glucose-free medium supplemented with B27 supplement and lactate from day 11 onward until day 25 for seeding.

Microprinting

Elastomeric microstamps of polydimethylsiloxane-182 (Sylgard 182; Electron Microscopy Sciences, Hatfield,

PA) containing rectangles of 17 × 119 μm^2 were cleaned, prechilled, and coated with 100 μL of diluted Matrigel (Matrigel:F10 medium, 1:10) for ≥2 hours at 4°C. Matrigel was aspirated and the patterns were air-dried. Patterns were carefully microprinted onto glass-bottom 6-well plates (Cellvis, Mountain View, CA; Figure, panel B). Day 25 hiPSC-CMs were lifted and resuspended into single cells using Accutase (Innovative Cell Technologies, San Diego, CA) and were then seeded onto microprinted 6-wells at 10,000–50,000 cells/mL. The hiPSC-CMs were allowed to attach and recover for 5 days before contractility was assessed.

Measurement of hiPSC-CM Contractility

Videos of beating micropatterned single hiPSC-CMs were captured on a Keyence BZ-X710 microscope (Itasca, IL) using a 40×/0.6 numerical aperture objective in oblique illumination/standard capture mode at 960 × 720 resolution at 29 fps for 10 seconds (Supplemental Digital Content, Video 1, http://links.lww.com/AA/C562). hiPSC-CMs were maintained at 37°C and 5% carbon dioxide (CO_2) (Tokai Hit Incubation System; Tokai Hit, Shizuoka, Japan). Propofol, KSEB01-S2, and etomidate were dissolved in dimethyl sulfoxide for 100 μM stock solutions and diluted with lactate medium to generate working solutions (0.1, 1, and 10 μM). Quantification of hiPSC-CM contractions was performed in a blinded fashion using Matlab-based motion-tracking software (Matlab, Natick, MA) as previously described (Figure, panel C).[7] Contraction velocities and frequencies of hiPSC-CMs were averaged over the duration of each recording normalized to untreated baseline. Dropout rates at various drug conditions were calculated as the percentage of beating hiPSC-CMs normalized to the number of beating hiPSC-CMs at baseline.

Sample Size and Statistical Methods

Statistical differences between the compound profiles were determined using a nonparametric Kruskal-Wallis test with multiple comparisons with uncorrected Dunn tests. All data are shown as the mean ± standard error of the mean. Significant differences were defined as a P value of <.05. Based on empirically determined acquisition times, a total of 50 cells per condition (2 independent hiPSC-CM differentiation to minimize batch differences) were targeted. A priori statistical power was 94.2% when a 2-tailed test with a 50% decrease in signal, sample size of 50, standard deviation of 1, and a 5% α error level was assumed.

RESULTS

We previously identified a novel anesthetic KSEB01-S2 by using in silico flexible docking targeting $GABA_AR$.[8] In tadpoles, KSEB01-S2 exhibits an half maximal effective concentration (EC50) of about 500 nM.[8] We were interested in testing anesthetic side effects in human cardiomyocytes. To determine the side effects of propofol, etomidate, and KSEB01-S2, we measured changes in contraction frequencies and velocities of micropatterned single hiPSC-CMs in response to incremental drug doses (0, 0.1, 1, 10, and 100 μM; Figure, panels B, C). With incrementing concentrations, the drugs' negative inotropic and chronotropic effects on hiPSC-CMs increased (Figure, panel D). Consistent with patient clinical data, the observed contraction suppression was most pronounced with propofol and least with etomidate. In accordance with dropoff rates as well, we observed a decrease in average contraction velocity by propofol (0.66 ± 0.11, 0.36 ± 0.09, 0.18 ± 0.08, and 0.00 ± 0.00), but to a lesser degree by KSEB01-S2 (0.82 ± 0.10, 0.46 ± 0.09, 0.35 ± 0.08, and 0.05 ± 0.02), and etomidate (0.74 ± 0.08, 0.52 ± 0.09, 0.42 ± 0.09, and 0.05 ± 0.05) treated hiPSC-CMs at 0.1, 1, 10, and 100 μM, respectively (Figure, panel E). Moreover, we observed a slower beating frequency in propofol (0.87 ± 0.05, 0.55 ± 0.10, 0.21 ± 0.06, 0.06 ± 0.03, and 0.00 ± 0.00) compared to KSEB01-S2 (1.07 ± 0.07, 0.71 ± 0.07, 0.25 ± 0.05, 0.24 ± 0.05, and 0.04 ± 0.03) or etomidate (1.11 ± 0.08, 0.66 ± 0.09, 0.40 ± 0.09, 0.43 ± 0.09, and 0.01 ± 0.01) treated hiPSC-CMs at 0, 0.1, 1, 10, and 100 μM, respectively (Figure, panel F).

DISCUSSION

All currently used intravenous anesthetic agents are associated with an entire spectrum of undesirable side effects, most notably cardiovascular instabilities. These side effects can be poorly tolerated in many surgical patients without proper intervention, but especially in very young children who possess immature physiological compensatory mechanisms, as well as in the elderly with confounding comorbidities and otherwise exhausted compensatory mechanisms. However necessary such animal studies are in the pipeline of drug development, performing analyses in rats and higher mammals is both time consuming and expensive. hiPSC-CM technology has been successfully used for studying aberrant electrophysiological conditions such as long QT[9] and used in disease model genetic cardiomyopathies[10,11]; it is also currently being developed under the Comprehensive In Vitro Proarrhythmia Assay initiative as the new cardiotoxicity standard.[12] In this study, we show the use of an efficient hiPSC-CM platform to assess and predict the potential for anesthetic side effects within a class of intravenous general anesthetics. It has been shown that cell shape and aspect ratio can greatly influence hiPSC-CM contractile maturation.[13] Our microprinting[13] coupled video contraction[7] hiPSC-CM platform affords us the ability to track the side effect responses of prescribed anesthetics on a single hiPSC-CM. Although we only tested a limited number of healthy hiPSC-CMs in this study, it is foreseeable that cohorts consisting of varying disease states, ethnicity, and ages could be tested for anesthetic side effects. At physiological concentrations, our results demonstrate that propofol exhibits more undesirable side effects compared to etomidate and KSEB01-S2. As demonstrated before, hiPSC-CM is a very powerful platform for disease modeling[10,11] and cardiotoxicity testing.[5] Our results serve as a proof-of-concept that now affords the ability to interrogate the signaling pathway behind anesthetic side effects, the unique opportunity to directly characterize specific cardiac physiological responses of currently prescribed drugs using a purely in vitro methodology, as well as test new lead compounds in the drug development pipeline.

ACKNOWLEDGMENTS

The authors thank Noëlie Cayla for compound preparation.

DISCLOSURES

Name: Alex C. Y. Chang, PhD.
Contribution: This author helped conceive and design the research, optimize video analysis algorithm, perform research, analyze the data, and write the manuscript.

Name: Andrew C. H. Chang, BSc.
Contribution: This author helped perform research, analyze the data, and write the manuscript.
Name: Luka Nicin, BSc.
Contribution: This author helped perform research, analyze the data, and write the manuscript.
Name: Gerhard J. Weber, MD.
Contribution: This author helped conceive and design the research, optimize video analysis algorithm, perform research, analyze the data, and write the manuscript.
Name: Colin Holbrook, BSc.
Contribution: This author helped optimize video analysis algorithm, analyze the data, and write the manuscript.
Name: M. Frances Davies, PhD.
Contribution: This author helped conceive and design the research, analyze the data, and write the manuscript.
Name: Helen M. Blau, PhD.
Contribution: This author helped conceive and design the research, analyze the data, and write the manuscript.
Name: Edward J. Bertaccini, MD.
Contribution: This author helped conceive and design the research, analyze the data, and write the manuscript.
This manuscript was handled by: Ken B. Johnson, MD.

REFERENCES

1. Bertaccini EJ, Yoluk O, Lindahl ER, Trudell JR. Assessment of homology templates and an anesthetic binding site within the γ-aminobutyric acid receptor. *Anesthesiology*. 2013;119:1087–1095.
2. Bertaccini EJ, Davies MF. The Board of Trustees of the Leland Stanford Junior University, Affairs TUSGARBTDOV. Novel methods, compounds, and compositions for anesthesia. Patent #US4053604A; 2016.
3. Cayla N, Dagne B, Davies M, Gross E, BacIver M, Bertaccini EJ. *Teasing Apart the Desired Effects of Anesthetics From Unwanted Side Effects at GABAA Receptors*. Boston, MA: International Society for Anaesthetic Pharmacology; 2017. Available at: https://www.isaponline.org/application/files/6015/0817/9040/ISAP_Syllabus_17.pdf. Accessed August 21, 2018.
4. Ribeiro AJS, Ang YS, Fu JD, et al. Contractility of single cardiomyocytes differentiated from pluripotent stem cells depends on physiological shape and substrate stiffness. *Proc Natl Acad Sci U S A*. 2015;112:12705–12710.
5. Burridge PW, Li YF, Matsa E, et al. Human induced pluripotent stem cell-derived cardiomyocytes recapitulate the predilection of breast cancer patients to doxorubicin-induced cardiotoxicity. *Nat Med*. 2016;22:547–556.
6. Tohyama S, Hattori F, Sano M, et al. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell*. 2013;12:127–137.
7. Huebsch N, Loskill P, Mandegar MA, et al. Automated video-based analysis of contractility and calcium flux in human-induced pluripotent stem cell-derived cardiomyocytes cultured over different spatial scales. *Tissue Eng Part C Methods*. 2015;21:467–479.
8. Bertaccini EJ, Davies MF, Cayla N, et al. *Molecular Modeling Leads to a New Class of Intravenous Anesthetics*. San Diego, CA: American Society of Anesthesiologists; 2015. Available at: http://www.asaabstracts.com/strands/asaabstracts/abstract.htm?year=2015&index=2&absnum=3745. Accessed August 21, 2018.
9. Liang P, Lan F, Lee AS, et al. Drug screening using a library of human induced pluripotent stem cell-derived cardiomyocytes reveals disease-specific patterns of cardiotoxicity. *Circulation*. 2013;127:1677–1691.
10. Sun N, Yazawa M, Liu J, et al. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Sci Transl Med*. 2012;4:130ra47–7.
11. Lan F, Lee AS, Liang P, XXXet al. Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell*. 2013;12:101–113.
12. Millard D, Dang Q, Shi H, et al. Cross-site reliability of human induced pluripotent stem-cell derived cardiomyocyte based safety assays using microelectrode arrays: results from a blinded CiPA pilot study. *Toxicol Sci*. 2018;84:111.
13. Ribeiro AJS, Schwab O, Mandegar MA, et al. Multi-imaging method to assay the contractile mechanical output of micropatterned human iPSC-derived cardiac myocytes. *Circ Res*. 2017;120:1572–1583.