Anthracycline Cardiotoxicity: Worrisome Enough to Have You Quaking?

Daniel Bernstein, M.D.
Alfred Woodley Salter and Mabel G. Salter Professor of Pediatrics, Stanford University

In this issue, Gupta and colleagues [1] describe a novel mechanism, mediated through alterations in the RNA-binding protein Quaking (QKI), responsible for anthracycline-mediated cardiotoxicity. Performing global transcriptional profiling in murine hearts exposed to doxorubicin, they identified five differentially expressed RNA binding proteins: four of which were upregulated and one, QKI which was downregulated. QKI expression was decreased both *in vitro* (in rodent cardiomyocytes and in human induced pluripotent stem cell-derived cardiomyocytes [hiPSC-CMs]), as well as *in vivo* in doxorubicin treated mice. To confirm the role of QKI in mediating doxorubicin cardiotoxicity, Gupta showed that QKI siRNA knockdown in primary cardiomyocytes increased doxorubicin-induced apoptosis; in contrast, lentiviral overexpression decreased apoptosis. Translating their findings to an *in vivo* model, they overexpressed *Qki5*, the most abundant cardiac isoform, in murine hearts using AAV9, and showed a decrease in apoptosis as well as improved echocardiographic cardiac function. Finally, to further explore the mechanism of QKI's modulation of cardiotoxicity, they identified QKI-regulated expression of several circular RNAs and demonstrated that inhibition of one, Ttn-derived circular RNA, increased doxorubicin cardiotoxicity. Thus, Gupta et al. have demonstrated a role for the RNA binding protein QKI as a mediator of anthracycline cardiotoxicity, suggesting a potential new target for intervention to prevent this life-threatening complication of anti-cancer treatment.

The anthracyclines are some of the oldest anti-cancer agents in use today. First discovered in the 1950s, anthracyclines such as doxorubicin are still widely used in treating a range of malignancies, and are included in half of breast cancer and two-thirds of all childhood chemotherapy protocols. Anthracyclines have contributed to the dramatic increase in 5-year survival rates for childhood cancer, now greater than 80%. However, as their use became widespread, the occurrence of a dose-dependent cardiotoxicity was recognized. The incidence of clinically significant left ventricular dysfunction (defined as a reduction in ejection fraction of >10% below normal) has been reported as 16%, 32%, and 65% at total doxorubicin doses of 300, 400, and 550 mg/m^2, respectively. Clinical anthracycline

Division of Pediatric Cardiology, 750 Welch Road Suite 325, Palo Alto, CA 94304, 650-723-7913, danb@stanford.edu.
Disclosures: none.

cardiotoxicity can occur early during treatment in about 10% of patients, but more commonly occurs years following cessation of therapy. Recognition of this cardiotoxicity led to attempts to limit total doxorubicin dose to enhance safety, however, even at relatively low exposures (doses of 200–250 mg/m^2), the risk of cardiotoxicity is still in the range of 10%.

There is evidence, however, that anthracycline cardiotoxicity may be even more common. Using echocardiographic methods capable of detecting subtle changes in cardiac contractility, Lipshultz and colleagues showed that the incidence of sub-clinical doxorubicin cardiotoxicity is considerably higher than previously suspected. They found elevated left ventricular wall stress in 59% of long-term survivors of childhood cancer, even at cumulative doses as low as 228 mg/m^2 [2]. Many patients had subtle alterations early after treatment, suggesting that patients exposed to anthracyclines may develop subclinical cardiotoxicity rather than latency between drug exposure and the onset of clinical heart failure, as had been previously suspected. Longer term follow-up studies have shown that many children will experience an initial improvement in fractional shortening within the first 6 years post-chemotherapy, but then a subsequent decline [3]. In contrast to these pediatric studies, the incidence of anthracycline-induced cardiotoxicity in adults has been reported to be lower, suggesting that the immature heart may be at higher risk. However, similar longitudinal echo studies using high-sensitivity indices such as wall stress have not been widely performed in adults. As nearly 1,000,000 patients receive an anthracycline annually in North America, and 1 in 750 adults is a survivor of childhood cancer, anthracycline-induced cardiotoxicity represents a major challenge for those caring for patients with cancer, and is a major focus of the emerging discipline of cardio-oncology.

The mechanism of anthracycline cardiotoxicity, despite over five decades of research, is still incompletely understood. Mechanisms that have been extensively studied include: (1) generation of reactive oxygen species (ROS); (2) degradation of mitochondrial function and activation of pro-apoptotic pathways; and (3) topoisomerase II inhibition causing double stranded break-induced apoptosis and altering transcription. Oxidative stress has been implicated as the central mechanism of doxorubicin cardiotoxicity [4]. Doxorubicin undergoes reduction by oxidoreductases to doxorubicin-semiquinone or doxorubicinol [5]. In the presence of iron, re-oxidation of doxorubicin-semiquinone or doxorubicinol back to doxorubicin leads to O_2^- and H_2O_2 formation, a key rationale for the use of iron chelators such as dexrazoxane as a preventive therapy. Doxorubicin also enhances sarcoplasmic reticulum Ca^{2+} release leading to myofibrillar injury and opening of the mitochondrial permeability transition (MPT) pore [6].

Compared to most other cell types, cardiomyocytes are at especially high risk of anthracycline-induced injury due to their high energy needs and thus their high content of mitochondria, accounting for 30% of cardiomyocyte volume. ROS producing enzymes such as NAD(P)H oxidase are localized in the mitochondria and in closely tethered sarcoplasmic reticulum regions. Besides direct ROS toxicity, mitochondrial-localized production of ROS can induce mitochondrial DNA (mtDNA) mutation and depletion [7]. Doxorubicin also tightly binds to mitochondrial membrane cardiolipin, further inhibiting electron transport chain function.

In cancer cells, doxorubicin binds DNA and topoisomerase II to form a ternary doxorubicin-DNA cleavage complex, a mechanism for induction of cell death pathways. Given that cardiomyocytes are largely non-dividing cells, doxorubicin's effect on topoisomerase was initially thought to be responsible only for its anti-cancer effects, not its cardiotoxicity. However, Zhang et al. recently showed that doxorubicin cardiotoxicity can be attenuated by targeted deletion of Top2β (the dominant topoisomerase isoform in cardiomyocytes). This effect was shown to be mediated by downstream alterations in PGC-1α and PGC-1β [8]. Since the dominant isoform in non-cardiomyocytes (including most cancer cells) is Top2α, this divergence suggests a possible mechanism for selective protection against cardiotoxicity.

The possibility that noncoding RNAs play a role in anthracycline cardiotoxicity presents yet another mechanism and a potential new target for preventive therapeutics. The classical view of RNA as a template for protein synthesis has now given way to a broad landscape of regulatory roles of non-coding RNAs and of RNA binding proteins. Circular RNAs (circRNAs), formed by non-sequential back-splicing of pre-mRNA transcripts, are expressed in multiple cell types including cardiomyocytes, and have been implicated as biomarkers to regulators of development and of cellular remodeling. Werfel et al. [9] identified >9000 candidate circRNAs after exonuclease treatment of hearts from rats (neonatal and adult), mice (sham or after transverse aortic constriction) and humans (both failing and non-failing). CircRNAs were enriched in the cytoplasm compared to linear RNAs, but had a similar level of association with the RISC RNA silencing complex. Intriguingly, dozens of circRNAs were identified arising from the titin (Ttn) gene, which has been shown to undergo complex alternative splicing during cardiac maturation. Werfel's observation of differential regulation of Ttn circRNAs between neonatal and adult rat hearts suggests that circRNAs could play a role in the regulation of titin splicing. Gupta's data in this issue suggest an additional role for Ttn circRNAs in mediating cardiotoxicity/cardioprotection. There is additional data suggesting a role for circRNAs in cardiovascular disease. The circular RNA Cdr1as was shown by Geng et al. [10] to be upregulated after myocardial infarction, and to promote apoptosis via its action as an inhibitory sponge for miR-7a/b. Cdr1as overexpression in mice increased infarct size and expression of PARP and SP1. Du et al. [11] showed that a circRNA from the forkhead family transcription factor Foxo3 was highly expressed in aging hearts and correlated with markers of cell senescence. Relevant to the current manuscript, Circ-Foxo3 overexpression exacerbated doxorubicin cardiotoxicity and silencing circ-Foxo3 attenuated cardiotoxicity.

Quaking (QKI), which belongs to the STAR family of KH domain RNA binding proteins, has been shown to affect pre-mRNA splicing, mRNA turnover and translation, and has been implicated in the regulation of epithelial-mesenchymal transition via its effect on circular RNA formation [12]. Conn et al. found that over one-third of circRNAs were regulated by QKI intronic binding motifs, as addition of these motifs induced de novo formation of circRNA from linear RNA transcripts. Quaking has thus far been implicated in human neurologic diseases including ataxia and schizophrenia and in cancer (glioblastoma) [13]. In the heart, QKI was shown to be protective against ischemia/reperfusion injury by regulating stability of Foxo1 mRNA [14].

Despite our extensive knowledge of the mechanisms of anthracycline cardiotoxicity, this has unfortunately not translated into significant enhancements in clinical care. Current prevention strategies involve limiting total exposure to anthracycline to the lowest possible dose and use of the iron chelator dexrazoxane. However, as a result of the potential for an increased risk of secondary malignancies, the European Medicines Agency has limited the indication for dexrazoxane to adult patients with advanced disease and contraindicated its use in children [15]. Thus, the search for a more effective preventive therapeutic is critical, and the contribution of Gupta et al. suggests a new pathway that is worth investigating. There are, however, several caveats. First, although the *in vitro* data presented by Gupta are convincing, their data *in vivo* are less certain. First, their initial studies using AAV9 *Qki5* overexpression to rescue doxorubicin cardiotoxicity resulted in the opposite effect: a deterioration of cardiac function and increased apoptosis. The authors explained this toxic effect by demonstrating that extremely high levels of *Qki5* were shifting its main subcellular expression from the nucleus to the cytoplasm. A lower dose of *Qki5* was then shown to preserve nuclear localization and to improve cardiac function. If further investigation shows that modulation of QKI is associated with too narrow a therapeutic window, then its clinical efficacy would be in question. Further confounding these results, cardiac function also improved with low dose *Qki5* overexpression alone, in the absence of doxorubicin (Figure 4C), suggesting a possible direct effect of *Qki5* on contractility. Comparing the increase in ejection fraction associated with *Qki5* without doxorubicin to that with doxorubicin, the increases appear to be of similar magnitude, suggesting that the translation of *in vitro* data, no matter how convincing, to *in vivo* models, is never straightforward.

Finally, the vast majority of studies on the mechanisms of anthracycline cardiotoxicity have used either transformed cell lines, mouse or rat cardiomyocytes, or *in vivo* rodent models. Whether these mechanisms can be recapitulated in larger mammals and in humans still remains to be determined. Many antioxidant drugs that have shown promise in preclinical studies have ultimately not been found to be effective. Recently, hiPSC-CMs have been shown to recapitulate the susceptibility to doxorubicin cardiotoxicity in patients with breast cancer [16], demonstrating their utility as a powerful new platform for investigating chemotherapy cardiotoxicity. Gupta's confirmation of the downregulation of QKI in hiPSC-CMs when treated with doxorubicin is an important first step. Future studies should continue to take advantage of this *human* cell platform. Finally, if any new therapeutic proves effective in preventing or attenuating anthracycline cardiotoxicity, we need to be absolutely certain that it does not also reduce the effectiveness of anthracyclines in killing cancer cells, or increase the risk of secondary malignancies.

Acknowledgments

References

1. Gupta SK, Garg A, Bar C, Chatterjee S, Foinquinos A, Milting H, Streckfuss-Bomeke K, Fiedler J, Thum T. Quaking inhibits doxorubicin-mediated cardiotoxicity through regulation of cardiac circular RNA expression. Circulation research. 2018

2. Lipshultz SE, Colan SD, Gelber RD, Perez-Atayde AR, Sallan SE, Sanders SP. Late cardiac effects of doxorubicin therapy for acute lymphoblastic leukemia in childhood. New England Journal of Medicine. 1991; 324:808–15. [PubMed: 1997853]

3. Lipshultz SE, Alvarez JA, Scully RE. Anthracycline associated cardiotoxicity in survivors of childhood cancer. Heart. 2008; 94:525–33. [PubMed: 18347383]

4. Shi Y, Moon M, Dawood S, McManus B, Liu PP. Mechanisms and management of doxorubicin cardiotoxicity. Herz. 2011; 36:296–305. [PubMed: 21656050]

5. Xu X, Persson HL, Richardson DR. Molecular pharmacology of the interaction of anthracyclines with iron. Mol Pharmacol. 2005; 68:261–71. [PubMed: 15883202]

6. Fajardo G, Zhao M, Berry G, Wong LJ, Mochly-Rosen D, Bernstein D. β2-adrenergic receptors mediate cardioprotection through crosstalk with mitochondrial cell death pathways. Journal of molecular and cellular cardiology. 2011; 51:781–9. [PubMed: 21756913]

7. Suliman HB, Carraway MS, Ali AS, Reynolds CM, Welty-Wolf KE, Piantadosi CA. The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. The Journal of clinical investigation. 2007; 117:3730–41. [PubMed: 18037988]

8. Zhang S, Liu X, Bawa-Khalfe T, Lu LS, Lyu YL, Liu LF, Yeh ET. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nat Med. 2012; 18:1639–42. [PubMed: 23104132]

9. Werfel S, Nothjunge S, Schwarzmayr T, Strom TM, Meitinger T, Engelhardt S. Characterization of circular RNAs in human, mouse and rat hearts. Journal of molecular and cellular cardiology. 2016; 98:103–7. [PubMed: 27476877]

10. Geng HH, Li R, Su YM, Xiao J, Pan M, Cai XX, Ji XP. The Circular RNA Cdr1as Promotes Myocardial Infarction by Mediating the Regulation of miR-7a on Its Target Genes Expression. PLoS One. 2016; 11:e0151753. [PubMed: 26998750]

11. Du WW, Yang W, Chen Y, Wu ZK, Foster FS, Yang Z, Li X, Yang BB. Foxo3 circular RNA promotes cardiac senescence by modulating multiple factors associated with stress and senescence responses. Eur Heart J. 2017; 38:1402–1412. [PubMed: 26873092]

12. Conn SJ, Pillman KA, Toubia J, Conn VM, Salmanidis M, Phillips CA, Roslan S, Schreiber AW, Gregory PA, Goodall GJ. The RNA binding protein quaking regulates formation of circRNAs. Cell. 2015; 160:1125–34. [PubMed: 25768908]

13. Chenard CA, Richard S. New implications for the QUAKING RNA binding protein in human disease. Journal of neuroscience research. 2008; 86:233–42. [PubMed: 17787018]

14. Guo W, Jiang T, Lian C, Wang H, Zheng Q, Ma H. QKI deficiency promotes FoxO1 mediated nitrosative stress and endoplasmic reticulum stress contributing to increased vulnerability to ischemic injury in diabetic heart. Journal of molecular and cellular cardiology. 2014; 75:131–40. [PubMed: 25068621]

15. Aminkeng F, Ross CJ, Rassekh SR, Hwang S, Rieder MJ, Bhavsar AP, Smith A, Sanatani S, Gelmon KA, Bernstein D, Hayden MR, Amstutz U, Carleton BC, Group CCPR. Recommendations for genetic testing to reduce the incidence of anthracycline-induced cardiotoxicity. British journal of clinical pharmacology. 2016; 82:683–95. [PubMed: 27197003]

16. Burridge PW, Li YF, Matsa E, Wu H, Ong SG, Sharma A, Holmstrom A, Chang AC, Coronado MJ, Ebert AD, Knowles JW, Telli ML, Witteles RM, Blau HM, Bernstein D, Altman RB, Wu JC. Human induced pluripotent stem cell-derived cardiomyocytes recapitulate the predilection of breast cancer patients to doxorubicin-induced cardiotoxicity. Nat Med. 2016; 22:547–56. [PubMed: 27089514]