Sarco“MiR” friend or foe: a perspective on the mechanisms of doxorubicin-induced cardiomyopathy

Louis A. Saddic, Jochen D. Muehlschlegel

Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA
Correspondence to: Jochen D. Muehlschlegel, MD, MMSc. Department of Anesthesiology, Perioperative, and Pain Medicine, Brigham and Women's Hospital, 75 Francis St., CWNL1, Boston, MA 02115, USA. Email: jmuehlschlegel@partners.org.

Abstract: Anthracyclines are a class of chemotherapeutics used to treat a variety of human cancers including both solid tumors such as breast, ovarian, and lung, as well as malignancies of the blood including leukemia and lymphoma. Despite being extremely effective anti-cancer agents, the application of these drugs is offset by side effects, most notably cardiotoxicity. Many patients treated with doxorubicin (DOX), one of the most common anthracyclines used in oncology, will develop radiographic signs and/or symptoms of cardiomyopathy. Since more and more patients treated with these drugs are surviving their malignancies and manifesting with heart disease, there is particular interest in understanding the mechanisms of anthracycline-induced injury and developing ways to prevent and treat its most feared complication, heart failure. MicroRNAs (miRNAs) are small noncoding RNAs that regulate the expression of mRNAs. Since miRNAs can regulate many mRNAs in a single network they tend to play a crucial role in the pathogenesis of several diseases, including heart failure. Here we present a perspective on a recent work by Roca-Alonso and colleagues who demonstrate a cardioprotective function of the miR-30 family members following DOX-induced cardiac injury. They provide evidence for direct targeting of these miRNAs on key elements of the β-adrenergic pathway and further show that this interaction regulates cardiac function and apoptosis. These experiments deliver fresh insights into the biology of toxin-induced cardiomyopathy and suggest the potential for novel therapeutic targets.

Keywords: Doxorubicin (DOX); heart failure; microRNA (miRNAs); beta-adrenergic pathway

Submitted Apr 20, 2016. Accepted for publication Apr 25, 2016.
doi: 10.21037/atm.2016.05.30
View this article at: http://dx.doi.org/10.21037/atm.2016.05.30

Introduction to the biology of anthracycline toxicity in the heart

Anthracyclines, including doxorubicin (DOX), are among the most effective chemotherapeutic agents used to treat many human cancers. Unfortunately, their use is limited by adverse side effects, most notably dose-dependent cardiotoxicity. These events can manifest acutely following administration of the drug, or more commonly, months to years following cumulative exposure (1). In adults, early and late toxicity typically presents with dilated cardiomyopathy (2). Up to 5–10% of patients exposed to these drugs can have symptomatic or radiographic evidence of heart failure (3). Even more, some studies have demonstrated an increased risk of cardiotoxicity in those patients receiving low doses of DOX (<300 mg/m^2) which were once thought to be considered safe (4). Children, on the other hand, tend to present with dilated cardiomyopathy that progresses to a restrictive pattern (2). Childhood cancer survivors exposed to anthracyclines can have up to a 2–5 fold increased risk of heart failure compared to those patients not exposed to these drugs (5). Given the increasing number of cancer survivors amongst adults and children, anthracycline-induced cardiotoxicity is becoming a prevalent disease despite efforts dedicated to surveillance and prophylactic management.

DOX is thought to deliver its anti-tumor effects primarily through inhibition of the alpha isoform of topoisomerase II (Top2α). The mechanisms of toxicity in the heart seem to be much more complex especially since cardiomyocytes lack

© Annals of Translational Medicine. All rights reserved.

expression of Top2α. These cells do, however, express the beta isoform (Top2β), and cardiomyocyte specific deletion of the gene encoding this protein confers protection against DOX-induced toxicity through inhibition of apoptosis and DNA-damage (6). Another mechanism of DOX-induced cardiotoxicity involves the generation of reactive oxygen species (ROS). This was originally a very attractive mechanism to explain cardiomyocyte injury as these cells have high metabolic rates, are chock full of mitochondria, and express low levels of anti-oxidant enzymes (7). Despite experimental evidence to support this theory, clinical efforts to scavenge free radicals have not been promising (8). Newer mechanisms have focused on the role of DOX in the inhibition of pro-survival pathways such as NRG-1/ErbB (9,10), and the stimulation of inflammation through Toll-like receptors (TLRs) (11).

MicroRNAs (miRNAs) are small noncoding RNA molecules that bind to the 3' un-translated region (UTR) of mRNAs and regulate their expression (12). Recently, miRNAs have been shown to play a critical role in many elements of cardiovascular disease including ischemia (13-15) and heart failure (16-18). Naturally, this led many groups to investigate the potential role of miRNAs in the development of DOX-induced cardiomyopathy (10,19-23). Roca-Alonso and colleagues continued this mission with a comparison of the global changes in miRNA expression in adult rat ventricular cardiomyocytes (ARVCMs) through two models. The first was an acute *in vitro* model of cultured ARVCMs harvested 6 hours after a single dose of DOX. The second was a chronic *in vivo* model where rats were exposed to repeated doses of DOX (cumulative dose of 15 mg/kg) over a two week period followed by harvesting ARVCMs 3 weeks later. A reference model of cardiomyopathy generated from rats with proximal left anterior descending (LAD) artery ligation was also included in the comparison. Three members of the miR-30 family (miR-30a, miR-30d, and miR-30e) were down-regulated in at least two of three models (21). Down-regulation of miR-30b has also been documented in H9C2 rat cardiomyocytes following hypoxia/re-oxygenation (24). Interestingly, other groups that generated cardiac miRNA profiles in animal models exposed to DOX had unique signatures that did not uncover miR-30 family members as being significantly dis-regulated (25,26). Nonetheless, there is quite a bit of variability in the timing of DOX exposure, the genetic background, and the technology used to generate these profiles.

The contribution of miR-30 and the β-adrenergic pathway towards the pathogenesis of heart failure

In cancer biology, miR-30 has been implicated as both an oncogene and a tumor suppressor. Its specific role tends to be cancer type specific (27-29). In the heart, overexpression of miR-30 directly regulated key pro-fibrotic proteins and thus may be associated with preventing the fibrosis characteristic of failing hearts (30). Another group demonstrated that up-regulation of miR-30 in cardiomyocytes blocked the up-regulation of angiotensin II-induced hypertrophy related genes and showed that increasing circulating levels of miR-30 may be used to diagnose myocardial hypertrophy (31).

Roca-Alonso and colleagues attempt to further promote miR-30 as a cardioprotective miRNA through a unique mechanism. Among the list of computationally derived predictive targets of miR-30, this group focused on three proteins in the β-adrenergic pathway (β_1AR, β_2AR, and $G_{1\alpha-2}$) (21). Modulation of contractile function in the heart via the β-adrenergic pathway involves the interaction of β_1AR and β_2AR with stimulatory guanylyl nucleotide binding proteins, Gs. This leads to the activation of adenylyl cyclase, an increase in cyclic AMP (cAMP), activation of protein kinase A (PKA), and the phosphorylation of direct components of the contractile apparatus and elements of the excitation contraction coupling system. β_2AR is also able to interact with inhibitory G proteins, G_i, which block adenylyl cyclase function as well as having the potential to activate the pro-survival phosphoinositide 3 kinase (PI3K)/Akt pathway (32-34). The role of the β-adrenergic pathway in the pathogenesis of heart failure has been well studied (32,34,35) and thus the choice to focus on β_1AR, β_2AR, and $G_{1\alpha-2}$ for further analysis was well conceived.

Chronic adrenergic stimulation in the heart has been shown to elicit cardiotoxicity (36). This effect is thought to be mediated primarily through aberrant activity of β_1AR resulting in calcium overload and cell death (37,38). In failing hearts, there is a down-regulation of β_1AR along with desensitization, whereas the density of β_2AR remains relatively unchanged (39). Transgenic mice overexpressing β_1AR specifically in cardiomyocytes developed fibrosis, hypertrophy, and reduced fractional shortening at least in part due to increased apoptosis (37). Furthermore, administration of beta-blockers is the hallmark of heart failure treatment and prevention including heart failure from DOX-induced cardiomyopathy (8,40,41). On the other hand,

© Annals of Translational Medicine. All rights reserved.

β_2AR is thought to confer cardioprotection (38,42). This pro-survival phenotype is thought to be at least in part associated with its interaction with G_i proteins (43). In human end stage heart failure patients, G_i proteins, particularly the α-2 subunit ($G_{1\alpha-2}$), are up-regulated (44). Down-regulation of $G_{1\alpha-2}$ is associated with apoptosis and worsening heart failure (45-47). Activation of $G_{1\alpha-2}$ is slightly more controversial as one group demonstrated that constitutive activation of $G_{1\alpha-2}$ in a dilated cardiomyopathy and an isoproterenol-induced heart failure mouse model led to worsening hypertrophy and fibrosis, respectively. The authors postulate that the role of $G_{1\alpha-2}$ in fibroblasts as opposed to cardiomyocytes may be the driving force behind these phenotypes (43). To complicate the story even more, recent models have demonstrated reverse phenotypes for the role of β_1AR and β_2AR in heart failure whereby β_1AR is cardioprotective and β_2AR promotes cardiotoxicity (48). As a result, in addition to cell and disease specific contexts, the balance between β_1AR and β_2AR expression may contribute to the relative role of these receptors in the pathogenesis of heart failure.

The cardioprotective potential of miR-30

Since miRNAs typically have multiple mRNA targets, these molecules are poised to regulate the delicate balance of multiple effectors in a single influential pathway such as β-adrenergic signaling. Roca-Alonso and colleagues utilized luciferase assays in H9C2 cells to demonstrate direct binding of a miR-30e mimic to the wild-type 3' UTR of four predicted targets (β_1AR, β_2AR, $G_{1\alpha-2}$, and the pro-apoptotic protein E1B-interacting protein 3-like or BNIP3L) but not to mutant constructs. In addition, these miR-30e mimics were sufficient to attenuate the up-regulation of these targets upon administration of DOX (21). Appropriate controls with random sequence molecules were used throughout their experiments. These results support the author's claim that DOX-induced repression of miR-30 is at least partially responsible for increased expression of β_1AR, β_2AR, $G_{1\alpha-2}$, and BNIP3L.

In order to demonstrate that miR-30 is able to augment the downstream effects of the β-adrenergic pathway, Roca-Alonso and colleagues showed that DOX treatment or administration of a miR-30 sponge vector led to an increase in cAMP, although the magnitude of up-regulation was more prominent in DOX treated cells. This could be due to miR-30 independent mechanisms of cAMP signaling or incomplete miR-30 targeting, which is consistent with the fact that the authors demonstrate close to a 50% knockdown of miR-30e with sponge vectors. Overexpression of miR-30, on the other hand, resulted in decreased cAMP levels (21).

The phenotypic response to miR-30 was assayed through its effects on contractile function and DOX toxicity. Cells overexpressing miR-30 had an attenuation of contractile amplitude in response to increasing concentrations of isoproterenol compared to control cells. With regards to DOX toxicity, over-expression of miR-30 attenuated the increase in caspase activity triggered by DOX, although this attenuation was also incomplete. In addition, down-regulation of miR-30 led to an increased level of caspase activity compared to controls. Furthermore, in the presence of DOX, the intensity of ROS in cardiac cells was decreased with miR-30 overexpression and increased with miR-30 inhibition compared to cells exposed to DOX alone (21). While these findings are intriguing, they do not provide a direct link between miR-30 activity and damage through the β-adrenergic pathway. Future studies manipulating targets of miR-30 will be necessary to draw such conclusions.

Finally, Roca-Alonso and colleagues also uncovered the presence of GATA-6 binding to miR-30 cluster promoters in publically available data from chromatin immunoprecipitation sequencing experiments. They hypothesized that GATA-6 binding to these regions following DOX may mediate down-regulation of miR-30 (21). GATA-6 has already been implicated in cardiac pathology where it seems to be required to mount the cardiac hypertrophic response and prevent heart failure in animal models (49). To substantiate their hypothesis, these authors demonstrated that *GATA-6* is up-regulated acutely following exposure of DOX to cardiac cells in culture and siRNA constructs against *GATA-6* resulted in increased expression of miR-30 family members and decreased expression of miR-30 targets. Even more, in the setting of DOX treatment, knockdown of *GATA-6* resulted in decreased signaling through the apoptotic pathways (21). Nevertheless, experiments demonstrating the attenuation of miR-30 down-regulation after DOX in the absence of GATA-6 are lacking. As a result, other transcription factors could be more important than GATA-6 on the effect of DOX on miR-30. These experiments also beg the question of how GATA-6 is activated following DOX treatment and whether additional targets of GATA-6 could contribute to the damage response. Moreover, if GATA-6 does indeed prove cardioprotective against failing hearts as demonstrated by others, its negative regulation of miR-30 would appear to promote cardiotoxicity. Further studies are needed to untangle the intricacies of these networks.

© Annals of Translational Medicine. All rights reserved.

Roca-Alonso and colleagues provide an interesting model whereby down-regulation of β_1AR, β_2AR, and $G_{1\alpha\text{-}2}$ leads to a cardioprotective phenotype. While this certainly aligns with studies that demonstrate cardiotoxicity of excessive β_1AR, it appears to contradict the cardioprotective role of β_2AR and $G_{1\alpha\text{-}2}$. However, the authors offer an explanation of this apparent contradiction by arguing that the fine tuning of expression characteristic of miRNAs can regulate the feedback loop involving β_1AR, β_2AR, and $G_{1\alpha\text{-}2}$ in such a way to prevent the cardiotoxicity of β_1AR signaling, while still maintaining some of the cardioprotective benefits of β_2AR and $G_{1\alpha\text{-}2}$. In a similar fashion, the beta-blocker Carvedilol has been shown in patients and animal models to be protective against DOX-induced cardiomyopathy despite being characterized as nonselective towards β_1AR and β_2AR (40,41). Most of the experiments for these conclusions were based on *in vitro* cell cultures. Future studies *in vivo* are necessary to determine the long-term phenotypic consequences of miR-30 knockdown and overexpression on DOX-induced cardiomyopathy in order to fully test the therapeutic potential of these miRNAs. Such models can also be used to determine potential additive or synergistic benefit of miR-30 overexpression with beta-blockade.

Conclusions

In a recent report by Roca-Alonso and colleagues, a novel mechanism for DOX-induced injury involving the regulation of the β-adrenergic pathway through miRNAs is uncovered. These findings provide new insight into the complex pathways that govern the damage response and the delicate balance between the expression of key elements in a single pathway. Even more, these studies offer exciting potential for future therapeutic targets of chemotherapy-induced cardiomyopathy.

Acknowledgements

Funding: This work was supported by NIH R01HL118266 to JDM.

Footnote

Provenance: This is a Guest Perspective commissioned by Section Editor Zhijun Han, MD (Department of Laboratory Medicine, Wuxi Second Hospital, Nanjing Medical University, Wuxi, China).

Conflicts of Interest: The authors have no conflicts of interest to declare.

References

1. Volkova M, Russell R 3rd. Anthracycline cardiotoxicity: prevalence, pathogenesis and treatment. Curr Cardiol Rev 2011;7:214-20.
2. Raj S, Franco VI, Lipshultz SE. Anthracycline-induced cardiotoxicity: a review of pathophysiology, diagnosis, and treatment. Curr Treat Options Cardiovasc Med 2014;16:315.
3. Salazar-Mendiguchía J, González-Costello J, Roca J, et al. Anthracycline-mediated cardiomyopathy: basic molecular knowledge for the cardiologist. Arch Cardiol Mex 2014;84:218-23.
4. Swain SM, Whaley FS, Ewer MS. Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. Cancer 2003;97:2869-79.
5. Mulrooney DA, Yeazel MW, Kawashima T, et al. Cardiac outcomes in a cohort of adult survivors of childhood and adolescent cancer: retrospective analysis of the Childhood Cancer Survivor Study cohort. BMJ 2009;339:b4606.
6. Zhang S, Liu X, Bawa-Khalfe T, et al. Identification of the molecular basis of doxorubicin-induced cardiotoxicity. Nat Med 2012;18:1639-42.
7. Ghigo A, Li M, Hirsch E. New signal transduction paradigms in anthracycline-induced cardiotoxicity. Biochim Biophys Acta 2016. [Epub ahead of print].
8. van Dalen EC, Caron HN, Dickinson HO, et al. Cardioprotective interventions for cancer patients receiving anthracyclines. Cochrane Database Syst Rev 2011;(6):CD003917.
9. Vasti C, Hertig CM. Neuregulin-1/erbB activities with focus on the susceptibility of the heart to anthracyclines. World J Cardiol 2014;6:653-62.
10. Horie T, Ono K, Nishi H, et al. Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. Cardiovasc Res 2010;87:656-64.
11. Nozaki N, Shishido T, Takeishi Y, et al. Modulation of doxorubicin-induced cardiac dysfunction in toll-like receptor-2-knockout mice. Circulation 2004;110:2869-74.
12. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281-97.
13. Wang Y, Pan X, Fan Y, et al. Dysregulated expression of microRNAs and mRNAs in myocardial infarction. Am J Transl Res 2015;7:2291-304.

© Annals of Translational Medicine. All rights reserved.

14. Boon RA, Dimmeler S. MicroRNAs in myocardial infarction. Nat Rev Cardiol 2015;12:135-42.
15. Saddic LA, Chang TW, Sigurdsson MI, et al. Integrated microRNA and mRNA responses to acute human left ventricular ischemia. Physiol Genomics 2015;47:455-62.
16. Gurha P. MicroRNAs in cardiovascular disease. Curr Opin Cardiol 2016;31:249-54.
17. Wong LL, Wang J, Liew OW, et al. MicroRNA and Heart Failure. Int J Mol Sci 2016;17.
18. Greco S, Gorospe M, Martelli F. Noncoding RNA in age-related cardiovascular diseases. J Mol Cell Cardiol 2015;83:142-55.
19. Holmgren G, Synnergren J, Andersson CX, et al. MicroRNAs as potential biomarkers for doxorubicin-induced cardiotoxicity. Toxicol In Vitro 2016;34:26-34.
20. Yin Z, Zhao Y, Li H, et al. miR-320a mediates doxorubicin-induced cardiotoxicity by targeting VEGF signal pathway. Aging (Albany NY) 2016;8:192-207.
21. Roca-Alonso L, Castellano L, Mills A, et al. Myocardial MiR-30 downregulation triggered by doxorubicin drives alterations in β-adrenergic signaling and enhances apoptosis. Cell Death Dis 2015;6:e1754.
22. Tong Z, Jiang B, Wu Y, et al. MiR-21 Protected Cardiomyocytes against Doxorubicin-Induced Apoptosis by Targeting BTG2. Int J Mol Sci 2015;16:14511-25.
23. Tony H, Yu K, Qiutang Z. MicroRNA-208a Silencing Attenuates Doxorubicin Induced Myocyte Apoptosis and Cardiac Dysfunction. Oxid Med Cell Longev 2015;2015:597032.
24. Li T, Sun ZL, Xie QY. Protective effect of microRNA-30b on hypoxia/reoxygenation-induced apoptosis in H9C2 cardiomyocytes. Gene 2015;561:268-75.
25. Vacchi-Suzzi C, Bauer Y, Berridge BR, et al. Perturbation of microRNAs in rat heart during chronic doxorubicin treatment. PLoS One 2012;7:e40395.
26. Desai VG, C Kwekel J, Vijay V, et al. Early biomarkers of doxorubicin-induced heart injury in a mouse model. Toxicol Appl Pharmacol 2014;281:221-9.
27. Che S, Sun T, Wang J, et al. miR-30 overexpression promotes glioma stem cells by regulating Jak/STAT3 signaling pathway. Tumour Biol 2015;36:6805-11.
28. Kao CJ, Martiniez A, Shi XB, et al. miR-30 as a tumor suppressor connects EGF/Src signal to ERG and EMT. Oncogene 2014;33:2495-503.
29. Zhong K, Chen K, Han L, Li B. MicroRNA-30b/c inhibits non-small cell lung cancer cell proliferation by targeting Rab18. BMC Cancer 2014;14:703.
30. Duisters RF, Tijsen AJ, Schroen B, et al. miR-133 and miR-30 regulate connective tissue growth factor: implications for a role of microRNAs in myocardial matrix remodeling. Circ Res 2009;104:170-8, 6p following 178.
31. Pan W, Zhong Y, Cheng C, et al. MiR-30-regulated autophagy mediates angiotensin II-induced myocardial hypertrophy. PLoS One 2013;8:e53950.
32. Najafi A, Sequeira V, Kuster DW, et al. β-adrenergic receptor signalling and its functional consequences in the diseased heart. Eur J Clin Invest 2016;46:362-74.
33. Xiao RP. Beta-adrenergic signaling in the heart: dual coupling of the beta2-adrenergic receptor to G(s) and G(i) proteins. Sci STKE 2001;2001:re15.
34. Yang J, Liu Y, Fan X, et al. A pathway and network review on beta-adrenoceptor signaling and beta blockers in cardiac remodeling. Heart Fail Rev 2014;19:799-814.
35. Santulli G, Iaccarino G. Adrenergic signaling in heart failure and cardiovascular aging. Maturitas 2016. [Epub ahead of print].
36. Communal C, Singh K, Pimentel DR, et al. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 1998;98:1329-34.
37. Bisognano JD, Weinberger HD, Bohlmeyer TJ, et al. Myocardial-directed overexpression of the human beta(1)-adrenergic receptor in transgenic mice. J Mol Cell Cardiol 2000;32:817-30.
38. Baker AJ. Adrenergic signaling in heart failure: a balance of toxic and protective effects. Pflugers Arch 2014;466:1139-50.
39. Bristow MR, Ginsburg R, Umans V, et al. Beta 1- and beta 2-adrenergic-receptor subpopulations in nonfailing and failing human ventricular myocardium: coupling of both receptor subtypes to muscle contraction and selective beta 1-receptor down-regulation in heart failure. Circ Res 1986;59:297-309.
40. Tashakori Beheshti A, Mostafavi Toroghi H, Hosseini G, et al. Carvedilol Administration Can Prevent Doxorubicin-Induced Cardiotoxicity: A Double-Blind Randomized Trial. Cardiology 2016;134:47-53.
41. Kalay N, Basar E, Ozdogru I, et al. Protective effects of carvedilol against anthracycline-induced cardiomyopathy. J Am Coll Cardiol 2006;48:2258-62.
42. Patterson AJ, Zhu W, Chow A, et al. Protecting the myocardium: a role for the beta2 adrenergic receptor in the heart. Crit Care Med 2004;32:1041-8.
43. Kaur K, Parra S, Chen R, et al. Gαi2 signaling: friend or foe in cardiac injury and heart failure? Naunyn Schmiedebergs Arch Pharmacol 2012;385:443-53.

© Annals of Translational Medicine. All rights reserved.

44. Eschenhagen T, Mende U, Nose M, et al. Increased messenger RNA level of the inhibitory G protein alpha subunit Gi alpha-2 in human end-stage heart failure. Circ Res 1992;70:688-96.
45. DeGeorge BR Jr, Gao E, Boucher M, et al. Targeted inhibition of cardiomyocyte Gi signaling enhances susceptibility to apoptotic cell death in response to ischemic stress. Circulation 2008;117:1378-87.
46. Foerster K, Groner F, Matthes J, et al. Cardioprotection specific for the G protein Gi2 in chronic adrenergic signaling through beta 2-adrenoceptors. Proc Natl Acad Sci U S A 2003;100:14475-80.
47. Chesley A, Lundberg MS, Asai T, et al. The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase. Circ Res 2000;87:1172-9.
48. Fajardo G, Zhao M, Urashima T, et al. Deletion of the β2-adrenergic receptor prevents the development of cardiomyopathy in mice. J Mol Cell Cardiol 2013;63:155-64.
49. van Berlo JH, Aronow BJ, Molkentin JD. Parsing the roles of the transcription factors GATA-4 and GATA-6 in the adult cardiac hypertrophic response. PLoS One 2013;8:e84591.

Cite this article as: Saddic LA, Muehlschlegel JD. Sarco"MiR" friend or foe: a perspective on the mechanisms of doxorubicin-induced cardiomyopathy. Ann Transl Med 2016;4(10):203. doi: 10.21037/atm.2016.05.30

© Annals of Translational Medicine. All rights reserved.