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Mechanisms of Anthracycline Cardiac Injury: Can we identify strategies for cardio-protection?

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Summary

Anthracycline antibiotics have saved the lives of many cancer victims in the 50 plus years since their discovery. A major limitation of their use is the dose-limiting cardiotoxicity. Efforts focusing on understanding the biochemical basis for anthracycline cardiac effects have provided several strategies currently in clinical use: limit dose exposure; encapsulate anthracyclines in liposomes to reduce myocardial uptake; administer concurrently with the iron chelator dexrazoxane to reduce free iron-catalyzed reactive oxygen species formation; modification of anthracycline structure in an effort to reduce myocardial toxicity. In spite of these efforts, anthracycline-induced heart failure continues to occur with consequences for both morbidity and mortality. Our inability to predict and prevent anthracycline cardiotoxicity is in part due to the fact that the molecular and cellular mechanisms remain controversial and incompletely understood. Studies examining the effects of anthracyclines in cardiac myocytes *in vitro* and small animals *in vivo* have demonstrated several forms of cardiac injury, and it remains unclear how these translate to the clinical setting. Given the clinical evidence that myocyte death occurs after anthracycline exposure in the form of elevations in serum troponin, myocyte cell death appears to be a probable mechanism for anthracycline-induced cardiac injury. Other mechanisms of myocyte injury include the development of cellular ‘sarcopenia’ characterized by disruption of normal sarcomere structure. Anthracyclines suppress expression of several cardiac transcription factors, and this may play a role in the development of myocyte death as well as sarcopenia. Degradation of the giant myofilament protein titin may represent an important proximal step that leads to accelerated myofilament degradation. An interesting interaction has been noted clinically between anthracyclines and newer cancer therapies that target the erbB2 receptor tyrosine kinase. There is now evidence that erbB2 signaling in response to the ligand neuregulin regulates anthracycline uptake into cells via the multidrug-resistance protein. Therefore upregulation of cardiac neuregulin signaling may be one strategy to limit myocardial anthracycline injury. Moreover, assessing an individual’s risk for anthracycline injury may be improved by having some measure of endogenous activity of this and other myocardial protective signals.

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Overview of Anthracycline cardiotoxicity

Anthracycline antibiotics are highly effective and widely used cytotoxic agents with applications in the treatment of multiple cancers. The mechanisms of cytotoxicity of anthracyclines in cancer cells are diverse including (reviewed in [1]): 1) inhibition of both DNA replication and RNA transcription; 2) free radical generation, leading to DNA damage or lipid peroxidation; 3) DNA alkylation; 4) DNA cross-linking; 5) interference with DNA unwinding of DNA strand separation and helicase activity; 6) direct membrane damage due to lipid oxidation; 7) inhibition of topoisomerase II. In response to some or all of these effects, tumor cell growth is inhibited and cells are more likely to die by one or more mechanisms.

A major limitation of anthracycline use is a cumulative dose-dependent cardiac toxicity. Cardiotoxicity has long been considered to occur by mechanisms other than those mediating their antitumor effectiveness, a concept that raises hope for development of strategies for protecting the heart while not diminishing tumor response [2]. Early strategies to prevent cardiac toxicity included reductions in single doses of anthracyclines as well as prolonging the infusion of drug to limit peak serum concentrations [3]. However, in spite of these efforts the cardiotoxicity remains [4].

Multiple mechanisms of anthracycline-induced cardiac cellular injury have been proposed based upon studies in animals and cell culture systems, and it remains unclear which of these are at work in the clinical context of anthracycline use (see Figure). Most mechanisms proposed involve oxidative stress induced by the anthracyclines, though it is not clear why this would result in preferential toxicity to the myocardium. Anthracyclines induce membrane damage via lipid peroxidation in all tissues, including the heart [5]. While formation of reactive oxygen species is induced by the quinone moiety of anthracyclines, oxidative stress can also occur via induction of nitric oxide synthase, leading to nitric oxide and peroxynitrite formation [6]. This mechanism has been linked to nitration and inactivation of key enzymes in the heart including myofibrillar creatine kinase [7]. Anthracyclines also cause impairment of membrane binding, assembly, and enzymatic activity of mitochondrial creatine kinase, though the consequences of this function are yet unclear [8]. In the heart, like other tissue, anthracyclines intercalate into nucleic acids, causing suppression of DNA, RNA and protein synthesis [9]. Some transcriptional regulatory proteins that appear important for regulation of cardiac specific genes are particularly susceptible to anthracyclines [10-12]. Impaired synthesis of myofilament proteins, which in the presence of anthracycline accelerated myofilament degradation [13], leads to a net negative balance of sarcomeric proteins, a condition we have termed 'cardiac sarcopenia'. Myocyte cell death by both apoptosis and necrosis has also been implicated, and the net loss of cells may further contribute to 'cardiac wasting'. Finally, anthracyclines induce changes in adrenergic function and adenylate cyclase [14,15], as well as abnormalities in Ca^{2+} handling [16], all critical for the dynamic regulation of cardiac function. The extent to which each of these contributes to the dose-dependent clinical heart failure in anthracycline treated patient remains controversial.

Anthracyclines induce Myocyte Cell Death

An attractive mechanism for anthracycline-induced cardiotoxicity is the hypothesis that with each exposure to anthracyclines there is myocyte death. Given the limited regenerative capacity of the heart, cumulative toxicity eventually surpasses a threshold of damage that triggers a more generic process of ventricular remodeling common to multiple forms of cardiac injury. Endomyocardial biopsies obtained from human patients within hours after anthracycline exposure shows electron micrographic evidence of mitochondrial swelling and chromatin contraction, hallmarks of apoptosis [17]. Detection of myocyte death by measurement of

released cardiac sarcomeric troponins corroborate that myocyte loss occurs after anthracycline exposure [18].

Experimental studies also demonstrate that anthracyclines can induce myocyte death, and these experimental systems have been utilized to examine potential mechanisms for these effects. Anthracycline treatment of cultured cardiac myocytes induces cell death in a concentration-dependent manner [19,20]. The mechanism of cell death varies with anthracycline concentration with apoptosis occurring at lower and necrosis at higher concentrations [20]. Furthermore, work in animals and humans have demonstrated that apoptotic cell death occurs after *in vivo* exposure to anthracyclines [21-23].

As discussed above, anthracyclines generate cellular oxidative stress, and this may be a proximal step leading to myocyte cell death. Hydroxyl radical formation in the intact heart increases in the presence of anthracyclines and can be inhibited by treatment with superoxide dismutase, catalase and the iron chelator dexrazoxane [24]. Both dexrazoxane and a superoxide dismutase mimetic can suppress anthracycline induced cell death [20]. Overexpression of manganese-SOD in mice demonstrates early protection from mitochondrial damage and decreased markers of myocyte death in the serum [25]. Interestingly the necrosis of myocytes that occurs at high concentrations of anthracyclines is not suppressed by antioxidants.

These experimental results correlate with clinical data. Dexrazoxane is the only drug approved by the FDA to prevent anthracycline-induced heart injury [26]. Dexrazoxane suppresses anthracycline-induced troponin elevation, a marker of myocyte cell death, as well as late cardiotoxicity [27]. That dexrazoxane can also inhibit anthracycline-induced myocyte death *in vitro* supports the concept that oxidative stress induced myocyte apoptosis is a proximal event in the development of anthracycline cardiotoxicity.

Cellular stress, and specifically oxidative stress, activates a host of signaling pathways that regulate cell fate, and these pathways likely modulate the response of the heart to anthracycline exposure. MAPKs deliver extracellular signals from activated receptors to effector proteins and regulate cell processes such as survival and proliferation [28]. In cardiac myocytes, ERK is activated by growth factors/hypertrophic agents as well as oxidant stress (for review see [29]). The SAPKs JNK and p38 are activated by cellular stresses and correlate with cardiac myocyte apoptosis [30]. ROS production can also activate these pathways [31], in particular JNK and p38 [32], supporting the association of anthracyclines and SAPK mediated apoptosis.

The PI-3K/Akt pathway has been shown to regulate the survival of cardiac myocytes from insults such as transient ischemia [33]. Akt is activated in response to many different growth ligands such as insulin-like growth factor-1 (IGF-1)[34-37], and neuregulin [38]. Activation of PI3k/Akt signaling suppresses anthracycline induced apoptosis and inhibition of PI3K/Akt (treatment of LY294002 or transfection of a dominant negative Akt mutant) increases anthracycline-induced apoptosis [38].

Anthracycline induced apoptosis in the heart appears to occur via a mitochondrial pathway involving Bax, cytochrome c and caspase-3 [39,40]. Cytochrome c is normally located in the mitochondria. Production of ROS induces cytochrome c release, suggesting anthracycline-induced ROS generation may trigger apoptosis through this mechanism. Recent studies have demonstrated release of cytochrome c after anthracycline treatment in myocytes [39]. Myopathic hearts from anthracycline-treatment have presented with increased Bax/Bcl-2 ratio [41], similar to what has been seen after ischemia-reperfusion. Activated caspase-3 is also seen on anthracycline-induced cardiomyopathy [42] similar to reperfused rat hearts [43].

Another trigger for apoptosis is likely DNA damage. Anthracyclines are planar and intercalate between base pairs in DNA. This non-covalent interaction inhibits DNA and RNA synthesis

and leads to DNA oxidant damage [44]. DNA damage at levels below that necessary to trigger cell death may lead to cumulative cardiotoxicity via accumulation of DNA mutations. The consequences of cumulative nuclear DNA damage in cardiac myocytes warrant further investigation.

One additional mechanism by which these DNA-damaging effects of anthracyclines could lead to cardiotoxicity is via suppressing expression and/or activity of transcription factors known to modulate sarcomere synthesis as well as cell survival. GATA4 is a member of a zinc finger transcription factor family that regulates myocyte differentiation, sarcomere synthesis, and survival. GATA4 regulates several cardiac specific genes[45,46] as well as antiapoptotic genes [47]. Treatment with anthracyclines down-regulates GATA4 expression in cardiac myocytes and overexpression of GATA4 attenuates anthracycline induced apoptosis [48]. Increasing GATA4 expression by alpha-adrenergic agonists suppresses anthracycline cardiotoxicity[49]. This has led to the hypothesis that pharmacologic activation of alpha-adrenergic receptors may reduce anthracycline cardiac injury. Conceptually this idea is obviously unappealing as the adrenergic system in general is known to promote adverse outcome after cardiac injury. Moreover, carvedilol has α_1 blocking activity and yet is able to reduce anthracycline injury [50]. However, specific activation of α_1 -adrenergic receptors is a potent hypertrophic stimulus, and this may be cardioprotective under some forms of cardiac stress [49]. Hence, prevention of Adriamycin (doxorubicin) toxicity with an α_1 -adrenergic agonist may be worth exploring.

Anthracycline induce sarcomere disruption

Many of the effects of anthracyclines on cardiac cells that have been implicated in the induction of cardiac myocyte cell death may also play a role in the induction of other cellular phenotypes such as disruption of cardiac sarcomeric structure. Ultrastructural changes to myocyte sarcomeres seen in endomyocardial biopsies of anthracycline treated patients as well as rodents include loss of myofibrils, disarray of myofibrils of the sarcomere, dilation of the sarcoplasmic reticulum, swelling of the mitochondria and cytoplasmic vacuolization [51-53]. An early step in the breakdown of sarcomeres after anthracycline exposure is the degradation of titin. Titin is a giant myofilament protein and an integral part of the sarcomere in striated muscle. Titin spans a half-sarcomere, from M-line to Z-disk, and functions to modulate the passive and restoring forces of the myocytes through its elastic elements [54]. Titin serves as a scaffold for assembly of myofilamentous proteins into sarcomeres [55]. Titin integrity is critical for the dynamic regulation of contractile function via length-dependent changes in the sensitivity of actin-myosin cross-bridges to calcium [56]. The loss of intact titin has been demonstrated in ischemic and failing human hearts, implicating titin disruption in the pathogenesis of progressive ventricular dysfunction in these conditions. Anthracycline exposure results in accelerated titin degradation via calpain proteolytic activity. At least in vitro, titin degradation leads to sarcomere disorganization [13].

Accelerated degradation is only part of the equation that will lead to the cardiac “sarcopenia” typical of anthracycline associated heart failure. Suppression of sarcomere protein synthesis by anthracyclines also contributes. The mechanism involved likely includes anthracycline binding to and disrupting nucleic acid synthesis, suppression of critical signaling pathways, as well as suppression of transcription factor activity as discussed above.

In addition to GATA4, a transcriptional regulator that has been implicated but poorly characterized, is cardiac ankyrin repeat protein (CARP, aka Ankrd1). CARP was also named cardiac adriamycin-responsive protein due to the sensitivity of its mRNA to anthracycline treatment [11]. Although CARP function is incompletely understood, its localization to the I-band is essential for normal sarcomere organization [57]. CARP is regulated in part by GATA4 expression [11]. Suppression of CARP expression using siRNA is sufficient to induce

myofibrillar disarray similar to that observed with anthracycline treatment (unpublished data). CARP protein is rapidly degraded in myocytes after anthracycline treatment, similar to the transcriptional coactivator p300 [58]. Thus down-regulation and/or degradation of CARP, GATA4 and p300 may all contribute to the sarcopenia that develops after anthracycline exposure.

Modification of anthracycline toxicity by erbB2 signaling

Newer cancer therapeutics have been developed that target specific oncogenes involved in regulation of cancer growth. One of these, trastuzumab (Herceptin) appears to increase the susceptibility of the heart to anthracyclines, and this experience provides some new insight into the mechanisms of anthracycline cardiotoxicity. Trastuzumab targets the erbB2 (HER2/neu) oncogene, a receptor tyrosine kinase in the erbB family. There are four members, erbB1 (a.k.a. EGFR) to erbB4 [59]. ErbB2 is a ligand-less 185Kd transmembrane protein with a tyrosine kinase in the cytoplasmic domain that is normally activated by heterodimerization with erbB1, erbB3, or erbB4 [60]. ErbB2 is overexpressed in 25-30% of human breast cancer and this is associated with enhanced tumor aggressiveness and a high risk of relapse and death [61]. Several monoclonal antibodies directed against the erbB2 ectodomain that specifically inhibit the growth of tumor cell lines overexpressing erbB2 have been developed. One of them, 4D5 was fully cloned [62] and the Fc portion replaced with human Fc to create trastuzumab [63]. Trastuzumab's antitumor activity against erbB2-positive human breast tumor cells in laboratory models led to clinical trials that ultimately demonstrated a clinical benefit in women with erbB2-overexpressing breast cancers [64]. On the basis of trastuzumab clinical efficacy, this antibody was approved in 1998 for treatment of patients with erbB2 overexpressing metastatic breast cancer.

A fraction of patients treated with trastuzumab develop clinical heart failure with a decrease in cardiac contractile function [64]. This was most notable in patients receiving concurrent anthracyclines, where cardiac dysfunction or symptomatic heart failure was reported at ~4-fold higher rates in patients who received trastuzumab than in those who received anthracycline and cyclophosphamide alone. While initially there was some concern that the effect of trastuzumab on the heart should limit its use [65], more recent clinical trials have demonstrated lower rates of treatment-associated heart failure. Moreover, experience with treatment of those patients who develop heart failure with standard medical therapy suggests that the contractile dysfunction is often reversible [66]. Nevertheless, this experience has triggered further research into the molecular mechanisms of anthracycline-induced cardiotoxicity that may lead to strategies for cardioprotection.

Studies in animals and cell culture have provided some insight into the mechanisms for trastuzumab's effects on the heart. Gene targeting studies in mice have demonstrated that erbB2, the target of trastuzumab, is essential for cardiac development [67], and conditional deletion of erbB2 in mice leads to the development of a dilated cardiomyopathy [68,69]. Thus erbB2 is essential for maintenance of normal cardiac structure and function. In vitro studies with cardiac myocytes provide further insights regarding the cellular and molecular mechanisms for trastuzumab's effects. ErbB2 is expressed in adult cardiac myocytes, along with erbB4, and transmits growth and survival signals in response to the ligand neuregulin-1 [70]. In response to anthracyclines, there is myofilament degradation [13], as well as cell death by both apoptosis and necrosis [20], and both of these effects are suppressed by neuregulin treatment [71]. ErbB2 and erbB4 localize in the transverse tubules of ventricular myocytes [72]. Collectively these data suggest that one role of erbB2/4 signaling is to dynamically regulate sarcomere structure, perhaps in response to stress. In this paradigm, suppression of erbB2 signaling by trastuzumab may accelerate the net breakdown of sarcomeric proteins

induced by anthracyclines, increasing the likelihood of cardiac dysfunction and the clinical syndrome of heart failure.

Strategies to limit Anthracycline Cardiotoxicity

At present, there is no generally accepted method to provide selective protection of the heart from damage induced by anthracyclines. Modification of anthracycline structure has resulted in compounds with reduced cardiotoxicity, allowing for higher dose administration. However, toxicity remains a problem. The iron chelator dexrazoxane reduces the incidence of contractile dysfunction in cancer victims treated with anthracyclines [73]. However, the possibility that dexrazoxane reduces tumor response rates has prevented dexrazoxane administration from becoming a universal practice [74]. Current guidelines from the American Society of Clinical Oncology, Chemotherapy, and Radiotherapy Expert Panel support the use of dexrazoxane in patients who have received high dose of Doxorubicin (Dox, over 300mg/m²) for metastatic breast cancer and who may benefit from continued Dox treatment or who have received over 300mg/m² Dox for treatment of malignancies other than breast cancer or who responded to previous anthracycline-based chemotherapy and may benefit from continued therapy with Epirubicin for advanced breast cancer.

The observation that suppressing erbB2 signaling with trastuzumab increases the likelihood of anthracycline cardiotoxicity leads to the hypothesis that augmentation of erbB2 signaling prior to or at the time of anthracycline exposure would be cardioprotective. Certainly in vitro studies with recombinant neuregulin support this hypothesis [38]. Recent work with the heterozygous neuregulin-1 knockout mouse also supports this hypothesis [75]. Pharmacological strategies such as systemic delivery of neuregulin does reduce cardiotoxicity of anthracyclines in mice [75], though the pleiotrophic growth effects of this ligand, included in some cancers, makes this suboptimal. Other pharmacologic agents to consider along these lines include other growth factors that have demonstrated cytoprotection from anthracyclines such as insulin-like growth factor-1, or cardiotrophin-1[76]. Like neuregulin, however, there are concerns that these agents may promote cancer growth. Obviously the ideal strategy would be one that locally activates neuregulin and/or other cardioprotective ligands or the downstream effector mechanisms in the heart. Local overexpression of downstream effector kinases such as Akt [77] or transcription factors such as GATA4 [78] protect mice heart from anthracyclines. However these tools are still far away from clinical utility.

One strategy yet to be explored is to use endogenous ligands and kinases to create a window of cardioprotection. Focusing on neuregulin, for example, we know that there is robust expression of this ligand in the heart, though it appears to have minimal activity at baseline. The levels of expression of neuregulin indicate that it serves some physiological function beyond cardiac development, though this is yet to be fully understood. If we knew the physiological mechanisms for activation of neuregulin in the heart, one could envision a scenario where subjecting a patient to those conditions prior to anthracycline exposure would create a window of cardioprotection. There is now evidence that erbB2 signaling in response to the ligand neuregulin regulates anthracycline efflux out of the cells via the multidrug-resistance protein [79]. Therefore upregulation of cardiac neuregulin signaling may be one strategy to limit myocardial anthracycline injury.

Based upon our experimental work in rat and human skeletal muscle [80,81], one could hypothesize that physiologic cardiac stress in the form of exercise will increase neuregulin/erbB signaling. In skeletal muscle, neuregulin-1 and the erbB receptors are known to be expressed where they serve a function at the neuromuscular junction to regulate myoblast differentiation as well as formation and maintenance of the post-synaptic endplate [82]. We demonstrated that these proteins are localized in the adult skeletal muscle at the neuromuscular

junction, and that exercise is a potent activator of neuregulin release, with subsequent activation of erbB phosphorylation [80]. The proximal mechanisms for neuregulin release during exercise remain incompletely characterized. Interestingly, an exercise training regimen in people increases the expression of erbB3 receptors in skeletal muscle [81]. Arguably increased erbB3 expression will increase sensitivity of muscle to neuregulin action. The demonstration that neuregulin increases skeletal muscle glucose uptake suggests an interesting hypothesis, that exercise-regulated glucose uptake in skeletal muscle is mediated in part by neuregulin.

Thus an attractive hypothesis is that a period of exercise sufficient to activate neuregulin (and/or other cardioprotective signaling) can protect a cancer patient's heart during anthracycline exposure [83]. Certainly periodic exercise has been known for a long time to reduce risk of ischemic cardiovascular events (e.g. [84,85]). In healthy individuals as well as those with chronic disease, exercise has been shown to improve quality of life and reduce all-cause mortality [86,87]. Epidemiologic studies suggesting that periodic exercise is associated with lower risk of the development of post-menopausal breast cancer [88]. Several recent randomized trials examined whether similar benefits are found in breast cancer patients undergoing conventional adjuvant chemotherapy. The results have provided preliminary evidence that exercise training is a feasible intervention that may attenuate a broad range of deleterious symptoms (e.g., functional decline, fatigue, nausea) associated with cytotoxic therapy, leading to clinically relevant improvements in quality of life [89-91].

The hypothesis that exercise will protect the heart from anthracycline-induced damage has been examined in animals. More than 20 years ago Kanter et al. demonstrated that swim training in rats decreased the histopathologic damage induced by anthracyclines [92]. The authors suggested that the exercise effect they observed in rats occurred via changes in antioxidant activity. They reported increased levels of catalase, SOD and glutathione peroxidase in blood, liver and heart in swim-trained rats [92]. There are many other potential mechanisms by which forced strenuous exercise might protect the heart including the well-known effects of strenuous exercise on expression of heat shock proteins (e.g. [93]), which are cardioprotective against anthracycline cardiotoxicity [94]. Finally the effects of exercise on adrenergic tone might also promote cardioprotection based upon the finding that pharmacologic administration of the alpha-adrenergic agonist phenylephrine is able to prevent anthracycline cardiotoxicity in mice [49]. Whether exercise is protecting the heart of patients being treated with therapies that have known cardiovascular effects including anthracyclines, radiation therapy, and trastuzumab has not been specifically examined.

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Abbreviations

SOD	Superoxide dismutase
ERK	extracellular signal-regulated kinase
SAPK	stress-activated protein kinase
JNK	c-Jun N-terminal kinase
ROS	Reactive oxygen species
PI3K	Phosphoinositide-3-kinase

Akt	protein kinase family, a.k.a. protein kinase B
GATA4	gene name, member of GATA family of zinc-finger transcription factors
IGF	Insulin-like growth factor
CARP	cardiac ankyrin repeat protein and cardiac adriamycin-responsive protein
Dox	Doxorubicin
Nrg	Neuregulin
siRNA	short interfering RNA
erbB2	(a.k.a. HER2 – homology of EGF receptor, neu) erythroblastic leukemia viral oncogene homolog 2

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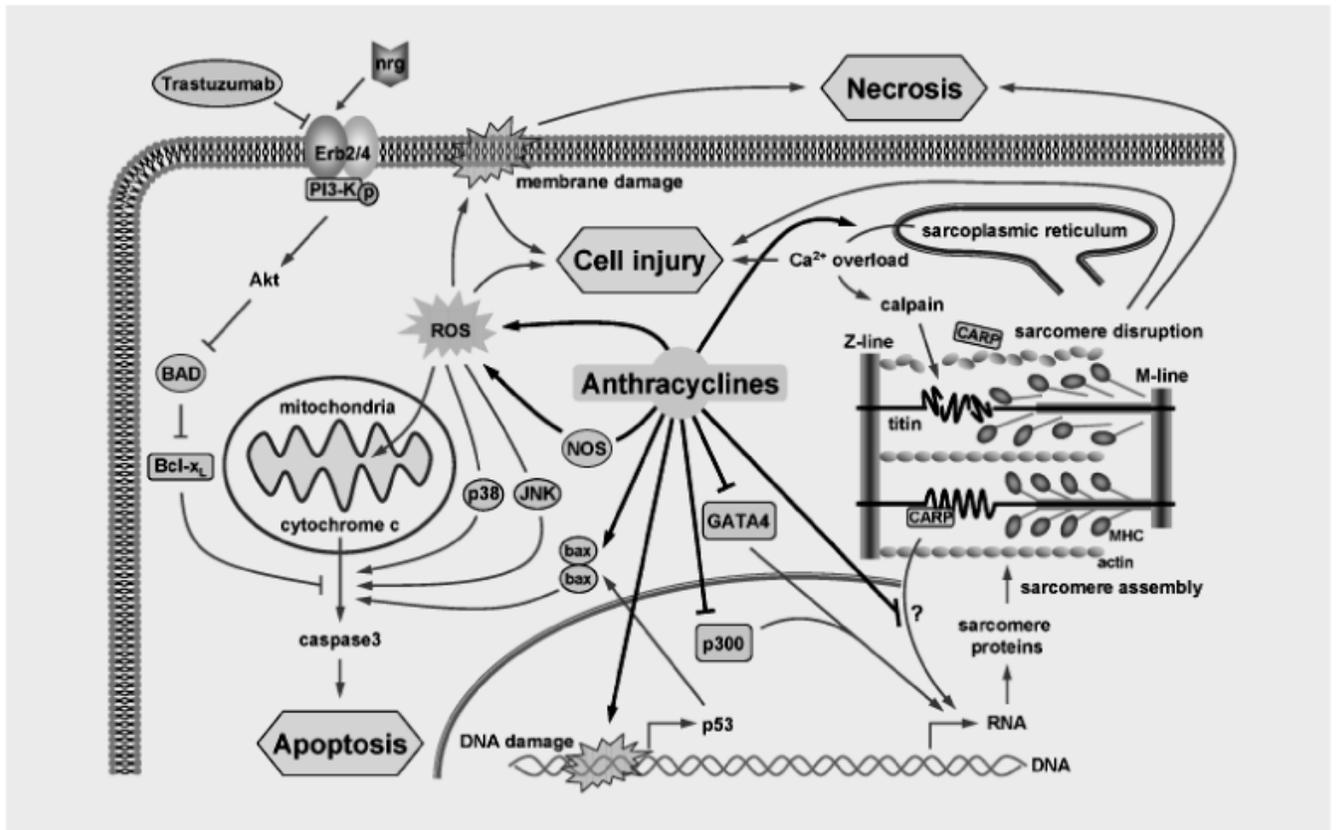


Figure. Anthracyclines promote myocyte injury and death via multiple mechanisms. This is balanced by intracellular signaling (e.g. GATA4, Akt) that is responsive to paracrine factors such as neuregulin (nrg). Trastuzumab (Herceptin) increases the susceptibility to anthracyclines by targeting the erb2 receptor. Anthracyclines also disrupt cardiac sarcomere structure by accelerating the degradation of key sarcomeric proteins (e.g. titin) and inhibit new sarcomere protein synthesis via alteration of transcriptional regulation. See text for details.