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# CARDIOTOXICITY RELATED TO
# OLD AND NEW ANTICANCER DRUGS

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## 1) INTRODUCTION

Cardiovascular diseases and cancer respectively represent the first and second cause of death in industrialized countries. These two conditions may become synergistic if we consider the cardiovascular complications of anti-cancer therapy.

During the past three decades, the development of effective screening and treatment strategies for many cancers has resulted in an enormous population of long-term cancer survivors: it is currently estimated that five year survival rates involve over the 70% of the children diagnosed with cancer, and 60% of the adult population[1]. To treat cancer as a manageable, or even curable, disease, similarly to other chronic conditions, is currently an important emerging concept to focus our attention to the life quality of cancer survivors. For all these reasons in the last years recognition of the importance of cardiotoxic side effects has increased.

It has been suggested that the effects should be classified as non-reversible (type I) and reversible (type II), with the former associated with ultrastructural changes on myocardial biopsy[2]. Yet the dysfunction caused by 'type II' agents is not always reversible, the distinction is not always binary and the clinical presentation is perhaps just as relevant when identifying causative agents and initiating appropriate therapy.

The clinical manifestations of cardiotoxic effects on myocardium are different.

*Khwaja et al* have classified the cardiac complications of the effects of the potentially cardiotoxic agents used in the treatment of neoplastic disease and their presenting syndrome in 4 clinical appearance: 1) heart failure, 2) cardiac ischemia, 3) arrhythmias and 4) hypertension.

**CLINICAL MANIFESTATION**

1) **Heart failure (HF)**

*Defining chemotherapy induced HF.*

Left ventricle (LV) dysfunction in the setting of cancer is most commonly systolic and results in HF with a reduced ejection fraction (EF) (HF-REF). The current European Society of Cardiology (ESC) guidelines require three conditions to be met for a diagnosis of HF-REF: a patient should have a reduced EF, along with signs and also symptoms of HF. For a diagnosis of HF with preserved EF (HF-PEF) four conditions are required: the LVEF should be normal or only mildly impaired, there should be evidence of relevant structural heart disease, and a patient should have sign and symptoms of HF. In contrast, the latest American college Foundation/American heart Association (ACCF/AHA) guidelines propose that diagnosis of HF-REF can be made when LVEF is <40% and a patient has symptoms of HF. For HF-PEF a minimum LVEF of <40% is required along with symptoms of HF.

| ACCF/AHA stages of HF[7] | | NYHA classification[6] | | CTCAE V.4.03[8] | |
|---|---|---|---|---|---|
| A | At high risk for HF but without structural heart disease or symptoms of HF | None | | 1 | Asymptomatic with laboratory (eg, BNP) or cardiac imaging abnormalities |
| B | Structural heart disease but without signs or symptoms of HF | I | No limitation of physical activity. Ordinary physical activity does not cause symptoms of HF | | |
| C | Structural heart disease with prior or current symptoms of HF | II | Slight limitation of physical activity. Comfortable at rest, but less than ordinary activity results in symptoms of HF | 2 | Symptoms with mild to moderate activity or exertion |
| | | III | Notable limitation of physical activity. Comfortable at rest, but less than ordinary activity causes symptoms of HF | 3 | Severe with symptoms at rest or with minimal activity or exertion; intervention indicated |
| D | Refractory HF requiring specialised interventions | IV | Unable to carry on any physical activity without symptoms of HF, or symptoms of HF at rest | 4 | Life threatening consequences; urgent intervention indicated (eg, continuous intravenous therapy or mechanical haemodynamic support) |
| | | | | 5 | Death |

ACCF, American College of Cardiology Foundation; AHA, American Heart Association; BNP, B-type natriuretic peptide; CTCAE, Common Terminology Criteria for Adverse Events; HF, heart failure; NYHA, New York Heart Association.

*Image 1. A comparison of classification of HF. (ref. Cardiac complications and manifestations of chemotherapy for cancer, Muhammed Zeeshan Khawaja et al., Heart 2013;**0**:1–8.)*

*Assessing LV function.*

The issue is further complicated by the variety of methods used to measure LVEF and other parameters of LV function. The accepted mainstay of the assessment of LV systolic function is 2D transthoracic echocardiography, due in part to its accessibility, even if 3D echocardiography seems to reduce the inter operator variability. Transthoracic echocardiography also has the additional benefit of being able to assess early diastolic dysfunction, myocardial tissue Doppler velocities, and myocardial deformation properties, all of which may be important in predicting which patients will eventually develop overt failure. These additional echocardiographic parameters can identify subtle cardiotoxic effects before frank deterioration in LVEF, though their significance is not clear. Where image quality is suboptimal or precise serial measurements are required, LV assessment using 3D cardiac MRI (CMR) is of value, though as yet there are no data on the impact of

more advanced CMR techniques such as myocardial tagging, phase contrast velocity imaging, etc.

In addition to imaging, cardiac biomarkers have shown potential in being able to identify patients with chemotherapy-associated cardiomyopathy. While the use of B-type natriuretic peptide (BNP) for cardiotoxicity in anthracycline use is limited due to its weak association in LVEF changes, troponin I has been demonstrated to be useful in risk stratifying patients receiving high dose chemotherapy regimens for future cardiac events, mainly driven by HF and asymptomatic LV dysfunction. Nevertheless, no cardiac biomarker has so far shown to have specificity in the prediction of chemotherapy related cardiac dysfunction.

*Drugs implicated in HF*

*DOXO-anthra*

Anthracyclines are among the most commonly used chemotherapeutic agents. Drugs such as doxorubicin and epirubicin are effective in the treatment of both solid tumours such as breast and ovarian cancers, and haematological malignancies such as lymphoma and leukaemia.

Despite improvements in mortality after childhood cancer, long-term survivors have an eightfold greater rate of cardiac death than the population at large. Much of this is considered to be related to the type I cardiotoxic effects of anthracycline use.

Symptomatic HF secondary to doxorubicin-induced cardiomyopathy has a high mortality rate of up to 60% at 2 years. Cardiotoxicity can manifest as an acute, reversible impairment of myocardial systolic function during or immediately after the initial infusion— though this

is rare. Cessation of therapy usually improves myocardial function, though this is governed by the cumulative dose experienced by patients, similar to the more frequent presentations of early and late onset progressive cardiotoxicity. These are defined as occurring within 12 months (early) of treatment or beyond 12 months (late). Early toxicity has been noted to occur in up to 2.1% of children treated with anthracyclines, with a peak incidence at 3–4 months.
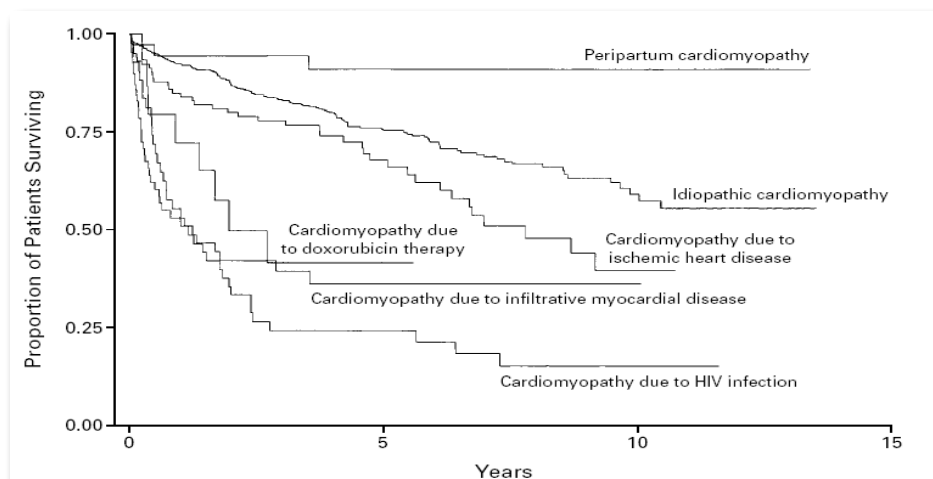


*Image 2. Adjusted Kaplan–Meier Estimates of Survival According to the Underlying Cause of Cardiomyopathy. Only idiopathic cardiomyopathy and cardiomyopathy due to causes for which survival was significantly different from that in patients with idiopathic cardiomyopathy are shown. Felker et al. N England Journ Med 2000*

The risk is significantly increased by higher cumulative doses, especially >550 mg/m2. This risk increases with the addition of other factors such as younger age, female sex, and thoracic radiotherapy.  More recently the concomitant use of trastuzumab has been shown to potentiate these effects in adults. Late cardiotoxicity manifesting as HF has been thought to occur in 4–5% of patients up to two decades after treatment, so a history of childhood cancer in younger adults presenting with HF is an important risk factor for cardiotoxicity.

The risk of late cardiotoxicity in adulthood is further increased by factors such as cumulative anthracycline dose, prior cardiac radiation exposure (eg, due to radiotherapy), and concomitant cardiovascular disease—suggesting that the myocardium of these patients may be more susceptible to developing HF in the face of conventional risk factors as they age. The pathophysiology of anthracycline cardiotoxicity will be discussed below.

Other chemotherapeutic agents associated with type I irreversible damage include alkylating agents (eg, cyclophosphamide and cisplatin) and inhibitors of microtubule polymerisation (eg, paclitaxel).


*Trastuzumab*.

Trastuzumab is a monoclonal antibody active against the human epidermal growth factor receptor (HER2) overexpressed in over a quarter of breast cancers. A significant increase in cardiotoxicity manifesting as a cardiomyopathy has been shown using this drug, with 19% higher when added to the combination of an anthracycline and cyclophosphamide, and 12% higher when added to paclitaxel alone [3]. Subsequent trials of adjuvant chemotherapy in HER2 positive breast cancer have demonstrated that sequential administration of agents reduces the incidence of this synergistic effect. This trastuzumab-induced cardiomyopathy is potentially reversible and it is thus a type II agent. The long-term effects of such a

(temporary) degradation of LV function remain to be seen. The effects of HER2 targeted agents may be due to interference with the neuregulin–HER cascade in the heart, which is thought to promote myocardial regeneration after stressors such as myocardial infarction and to compensate for the maladaptive changes in HF. HER2 knockout mice have been shown to develop a dilated cardiomyopathy without ventricular apoptosis but rather myofibril hypertrophy.

*Angiogenesis inhibitors*

Bevacizumab is a recombinant humanised monoclonal antibody against the vascular endothelial growth factor (VEGF) receptor, while sorafenib and sunitinib are small molecule tyrosine kinase inhibitors (TKI) targeting the VEGF signalling pathway. They have been shown to have beneficial effects in the treatment of a variety of solid malignancies including advanced breast, colon, renal, and ovarian cancers. Bevacizumab is associated with low rates of cardiac dysfunction which exhibits type II reversible features. [4]

Of the two TKI agents, sunitinib seems to be the most cardiotoxic with HF or LV dysfunction seen in over 6% of patients, in association with pre-existing coronary artery disease and hypertension. [5] All three agents are associated with induction of hypertension. The mechanism of cardiomyopathy in these agents is not yet known. VEGF is involved in myocardial angiogenesis and endothelial function and may protect against oxidative stress; anti-VEGF agents perhaps disrupt this process or the mechanism may be related to the pro-hypertensive effects (which are thought to be related to impaired vasodilatation and nitric oxide (NO) synthesis).

*TKI*.   The advent of target therapy has vastly improved the treatment and prognosis of multiple types of cancer. The goal of these agents is to improve specificity in the elimination of tumor cells while minimizing toxicity on healthy cells. Despite this goal, unexpected cardiotoxicity has arisen for many target compounds. For example Imatinib mesilate, a bcr-abl, PDGF, SCF and c-kit inhibitor for the treatment of chronic myelogenous leukemia, is associated with left ventricular dysfunction and severe congestive heart failure in patients.

Two types of toxicity account for the majority of the effects of TKI on cardiomyocytes: on-target toxicity and off-target toxicity. With on target toxicity, the drug target blocks a signaling pathway critical to both cancer cell growth and cardiac cell homeostasis, leading to the destruction of the cancer cell as well as cardiac dysfunction. The importance of receptor tyrosine kinases in cell function and the overlap of critical signaling pathways in both cell types lead to the development of on target toxicity. With off-target toxicity, cardiac damage is intended caused by a drug that blocks an unintended kinase pathway. Since most TKi agents target the conserved ATP binding pocket, they inherently nonselective, leading the promiscuity of kinase inhibition and the potential for off target toxicity. In addition the event of multi targeted drugs that block multiple pathways increases the likelihood for cardiac cell damage from off-target toxicity. The cardiotoxicological properties of small molecule kinase inhibitors will typically be due to (intended or unintended) kinase inhibition, although off-target effects on non-kinases could have a role. In some cases, on-target (intended) inhibition of a kinase which is appealing from a tumour progression and/or angiogenesis perspective could also be physiologically crucial in the heart and/or the vasculature. Off-target kinase inhibitor-mediated cardiotoxicity is a consequence of the inherent challenge of selectivity of most ATP-competitive kinase inhibitors. This leads to unintended inhibition of multiple kinases beyond the intended target kinase (or kinases). Although these off-target

kinases may or may not have a role in tumour progression, they may be essential to the function of the heart. Exacerbating this form of toxicity is the current trend of intentional 'multi-targeting' (inhibiting multiple kinases with a single drug). Members of this class of agents typi-cally target factors driving both tumour progression and angiogenesis, and targets linked to angiogenesis include vascular endothelial growth factor receptor 2 (veGFR2), placenta growth factor (PlGF), and platelet-derived growth factor receptor-β (PDGFRβ). It has become increasingly apparent that agents targeting VEGFRs, particularly veGFR2, induce significant hypertension (see below). Indeed, this appears to be a class effect of these agents, be they small molecules or monoclonal antibodies (mAbs), and can require at least temporary cessation of treatment. This additional stress of hypertension can be particularly problematic in patients with an already compromised cardiac reserve or advanced coronary artery disease. An additional issue that is likely to exacerbate kinase inhibitor toxicity is pathway targeting, that is, the targeting of multiple kinases within a specific pathway to achieve maximal suppression of activity of that pathway. For example, inhibition of multiple components of the phosphoinositide 3-kinase (PI3K)–AKT pathway appears to be a viable strategy in a number of cancers. However, this pathway also maintains cardiomyocyte homeostasis and protects cardiomyocytes from injury and death. Thus, the potential cardiovascular toxicity of compounds that inhibit the PI3K–AKT pathway should be carefully examined. A third mechanism by which small molecule kinase inhibitors could mediate toxicity would be through the inhibition of non-kinase targets. Any enzyme, that requires ATP to perform its various functions, could be inhibited by small molecule kinase inhibitors. Thus, even if one knew the complete selectivity profile of a drug against all kinases, it would be impossible (at least at this point in time) to have a full selectivity pro-file against the non-kinase targets. [6]

2) **cardiac ischemia**

Use of the pyrimidine analogues 5-fluorouracil (5-FU) and capecitabine (a 5-FU precursor administered orally) has been associated with similar cardiotoxic effects, mainly coronary vasospasm[7] in approximately 3% of patients. Symptoms usually resolve with withdrawal of the drug, which is easier with the intravenous delivery of 5-FU. Recurrence is likely, especially in the presence of existing coronary disease, and so alternative therapies should be explored.

3) **hypertension**

The anti-VEGF agents have variable hypertensive effects and the mechanism is not clear. As well as endothelial and NO impairment, reduction in capillary density and increased vascular resistance is thought to play a role. Careful identification of 'at-risk' patients with comorbidities and management of hypertension according to current guidelines is advised.

*Sunitinib*. Sunitinib is an example of complexities inherent in identifying mechanisms of kinase inhibitor toxicity which appears to have both on- and off-target effects contributing to cardiotoxicity[6]. It has been presumed that a major contributor to the deterioration in left ventricular function is sunitinib-induced hypertension. In support of this idea, sunitinib-treated mice did not develop cardiomyocyte apoptosis until an increase in blood pressure was induced. A number of studies in various mouse models have also shown that angiogenesis (which is mediated in the heart by both veGFR2 and PDGFRβ, targets of sunitinib) is key to maintaining cardiac homeostasis in the setting of a pressure load or

ischaemia [8,9,10]. Although this indicates that sunitinib-induced hypertension has an important role in cardiotoxicity, several patients who developed cardiotoxicity on sunitinib did not have hypertension, suggesting that additional mechanisms may be responsible. Although no head-to-head cardiotoxicity study has been conducted with veGFR antagonists, the cardiotoxicity of sunitinib does appear to be greater than the other agents[11]. Even though this could be due to the greater potency of sunitinib in inhibiting veGFR2, this difference suggests the possibility of additional off-target effects. Identifying the mechanisms of sunitinib toxicity is more difficult than for other more selective kinase inhibitors, as sunitinib inhibits at least 50 kinase targets at therapeutically relevant concentrations in *in vitro* assays[12]. Hints to the mechanisms were found in transmission electron micrographs of endomyocardial biopsies from a patient who presented with profound sunitinib-associated heart failure. The biopsies showed marked mitochondrial swelling indicative of the opening of the mitochondrial permeability transition pore, a response that typically leads to a necrotic form of cell death. This swelling appeared to be so widespread that it seemed likely that energy (ATP) homeostasis may have been compromised. Studies in neonatal rat ventricular myocytes (NRvms) also confirmed that energy compromise does have a key role in sunitinib-mediated toxicity, but this decrease in available energy did not lead to activation of AmP-activated protein kinase, the central regula-tor of the response of the cell to energy perturbation. Given what we know about the role of veGFRs and PDGFRβ in the heart's response to stressors, inhibition of these kinases most probably contributes to the on-target toxicity associated with sunitinib and the other multi-targeted agents. This toxicity is mediated via adverse effects on the vasculature that, at least in animal models, lead to loss of capillaries, impaired blood flow and regional ischaemia, especially in the setting of cardiac stress [8,9,10]. These processes are undoubtedly mechanistic contributors to the hypertension

that is characteristic of kinase inhibitors. Remarkably, it appears that hypertension is a 'biomarker' of the anticancer efficacy of the multi- targeted agents as several studies have shown that cancer patients who develop hypertension have better outcomes than those who do not [13,14,15]. That said, it is important to underline that hypertension is an off-target consequence and probably plays no part in the killing of tumour cells; it should therefore be aggressively treated.

## 4) QT interval prolongation / arrythmias

While most electrophysiological sequelae of cancer treatment are relatively benign, such as paclitaxel associated bradycardia or anthracycline induced ectopy, QT interval prolongation is potentially life threatening. QTc interval prolongation has been noted with the use of newer TKIs such as vandetanib (up to 20%) and nilotinib—though seemingly at very low risk of torsades de pointes—and of course in electrolyte imbalances, which are common during cancer therapy. However, it is most usually associated with the use of arsenic trioxide (ATO) in relapsed acute promyeloid leukaemia[16]. In these patients a careful risk/benefit analysis should be undertaken and discussed with the patient, as ATO is the sole effective agent in this fatal disease.

By providing detailed information on drug-related cardiovascular complications and new insights on the pathogenetic mechanisms of cardiotoxicity of antineoplastic therapy, an attempt was made to open innovative prospectives to impact on an emerging relevant clinical issue. The recently introduced, although timely known, concept that the heart is not a post mitotic organ [17,18] and contains a stem cell pool responsible for myocardial cell

turnover [19,20] may offer a new way in understanding cardiotoxicity. Specifically, beyond cardiomyocytes or parenchymal cells, an alternative cellular target of anticancer therapy is proposed to explain a major clinical problem in oncology. The demonstration that the adult mammalian heart possesses a cell turnover regulated by primitive cells suggests that this cell population may be implicated in the onset and development of cardiovascular effects of anti-cancer strategies.

## 2) ISSUE

Focalizing our attention on doxorubicin and on the older TKI Imatinib, we advanced the hypothesis that a potential mechanism of drug related toxicity could be the ablation or suppression of the proliferation of the stem/progenitor cell compartment of the heart. Given the limited ability of adult cardiomyocytes to reenter the cell cycle and proliferate, and the acute nature of cardiac injury, intrinsic mechanisms to repair the heart are not robust. Although these cardiac stem/progenitor cell populations are relatively quiescent, stress can induce these cells to generate significant numbers of new cardiomyocytes. The physiological significance of the replication of small subsets of progenitor cells is still under debate, but it is possible that these cell populations are collateral targets of some of the small molecule kinase inhibitor anticancer agents.

In fact, adverse effects of cancer therapeutics on stem and progenitor cells may be exacerbated by kinase inhibitor-mediated effects on energy metabolism in the adult cardiomyocyte, as noted for sunitinib.

For example, treatment of juvenile mice with the anthracycline doxorubicin impairs cardiac progenitor cell function and vascularization, leading to cardiotoxicity as the mice age. This is similar to the effect observed in children treated with doxorubicin who developed heart failure later in life [21].

Experimentally, infusion of cardiac stem cells can rescue rats from the cardiotoxicity of doxorubicin, leading the authors of the study to hypothesize that cardiac stem cells are a key target of doxorubicin- induced cardiotoxicity [22].

In other studies, inhibition in mice of KIT[23], the receptor for stem cell factor, resulted in enhanced cardiomyocyte proliferation and better-preserved left ventricular function when

the mice were subjected to pressure overload. Although beneficial in the short term, chronic inhibition of KIT in this model raised concerns about long-term maintenance of the progenitor pool in the heart. Although the biological significance of these findings in humans is not known, concerns regarding the effects of kinase inhibitors on stem and progenitor cells or on immature cardiomyocytes that are capable of entering the cell cycle should continue to be an active area of research.
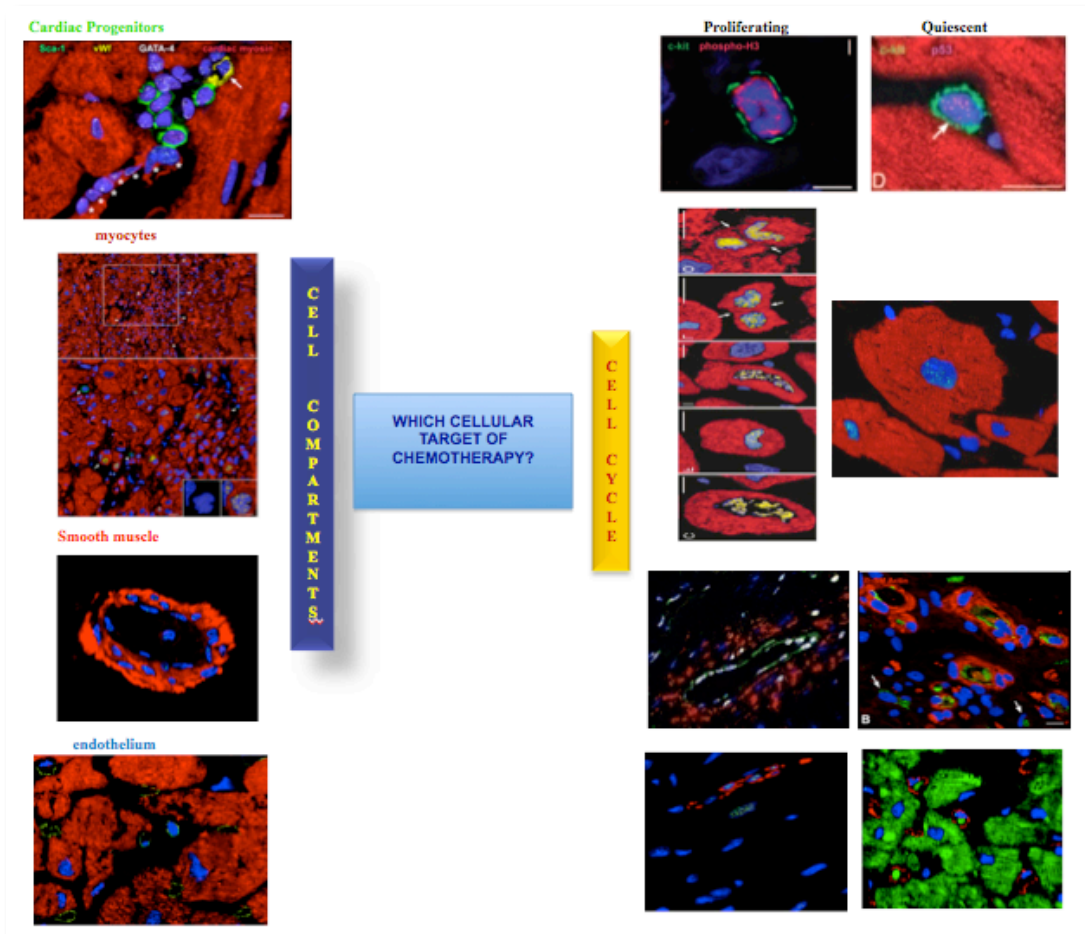
*Image 3. Schematic representation of possible cellular target of chemotherapy: different cell compartment of the heart could be affected, and in during the different phases of cell cycle. Cardiac progenitors could lso be affected, leading to an impairment in damage repairing.*

## 3) MATERIAL AND METHODS

**HUMAN**

**HUMAN SAMPLES**

Human Myocardial samples were collected from heart of patients who showed clinical sign of drug related cardiovascular complications. The characteristics of patients are briefly described below.

*Patients with drug related cardiomyopathy*

Case 1- In a caucasian women Non Hodgkin Lymphoma (NHL) involving the left lung was diagnosed at the age of 17. She was successfully treated with Doxo containing regimens reaching (cumulative dose 350 mg/m2) in association with local radiotherapy (36 Gy). Being free from the disease, at the age of 23 she delivered a healthy boy without post-partum complications. At the age of 43, she started to display symptoms of exertion dyspnoea and moderate fatigue although no medications were prescribed. Following a brief period in good performance status, a progressive impairment of cardiac function took place leading to overt heart failure requiring several pharmacological interventions and hospitalizations. She came to our observation at the age of 47 in bad clinical conditions. PET and CT scan showed a complete collapse of the left lung and severe pericarditis (400 ml) that was partially resolved by diuretic therapy (fig. 1A, B, C and D). During hospitalization exacerbation of heart failure and atrial fibrillation were present. The clinical condition worsened, with a severe hypokinesia of antero-lateral segment of left ventricle. A

bronchoscopy showed fibrotic retraction of left lung, with occlusion of superior and inferior bronchus. After a short period of intensive care the patient died at the age of 48. The autoptic examination showed a complete collapse of the left lung and severe calcific pericarditis (fig. 1E and F).

Case 2- A 48 years old women affected by relapsed acute myeloid leukemia died for cardiovascular complications 2 years after anthracycline containing regimen.

Case 3 – A 53 years old women who died during anthracycline containing regimens for relapsed acute myeloid leukemia.

Case 4 - A 60 years old man affected by NHL. He was treated with anthracyclines containing chemotherapy (3 mega-CHOP and MAD) plus BEAM conditioning regimen for subsequent autologous bone marrow transplantation. He died acutely during chemotherapy while in waiting list for allogeneic bone marrow transplantation.

Case 5- A 50 years old woman affected by NHL, treated with DOXO containing regimen, died acutely in the Intensive Therapy Unit during the third course of chemotherapy. The macroscopic analysis at autopsy showed cardiac hypertrophy.

Case 6 - A 2 years old girl affected by coccigeal teratocarcinoma was treated with chemotherapy containing DOXO, She developed a dilative cardiomyopathy nearly 20 years later. At the age of 26 years the patient underwent heart transplantation for severe congestive heart failure (CHF). Samples of the explanted heart were analyzed.

Case 7 - Men suffering from gastrointestinal stromal tumor (GIST) diagnosed at the age of 63 years, after hospitalization at the Department of Digestive Endoscopy for normocytic anemia secondary to gastric mesenchymal lesion ulcerated and bleeding. Following the stabilization of the parameters were performed a CT scan that showed a lesion of 70x70x75 mm at the lesser curvature, with necrotic aspects , and two hepatic lesions. The patient underwent surgery for total gastrectomy - pancreatectomy - splenectomy and cholecystectomy – and then treatment with IM (dose of 600 mg/day) was started. At the time of diagnosis anamnesis showed the absence of cardiovascular disease, even negative family history for this type of diseases. At the age of 68 years, the patient was hospitalized in the department of medical clinic with a diagnosis of acute hepatitis, possibly from iatrogenic effect of Imatinib. During hospitalization hemorrhagic shock (intermediate cause of death), multi- organ failure and DIC (causes of death terminals) occured, leading the patient to death. At the autopsy myocardial infarction of the posterior wall of the left ventricle and scarring of myocardial infarction were reported with coronary disease of moderate severity .

*Control sample*

Four samples of hearts obtained from autopsies of patients who died in the absence of cardiovascular disease and cancer treatments were used as control sample

• Control Case 1:  87 year old female died because of sub-arachnoid haemorrhage .

• Control Case 2: 56 year old female died for pulmonary edema and emphysema, chronic bronchitis in patients with cirrhosis and chronic renal failure.

• Control Case 3:  77 year old male died as a result of previous empyema, hemothorax.

Case-control 4: 45 year old female who died for an heart attack and ileal extensive necrotic hemorrhagic

**HUMAN CPC (hCPC)**

Samples were obteined from left Ventricular and atrial myocardial fragments of patients affected by severe aortic stenosis, undergoing aortic valve replacement. 0.5-1gr of myocardium obtained from the outflow tract proximal to the aortic valve were dissected and placed in a sterile transfer solution composed by Iscove Modified Dulbecco's Medium (IMDM, Sigma, Italy) supplemented with 1% Penicillin-Streptomycin (P/S, Sigma, Italy). Myocardial fragments were minced and seeded onto the surface of Laminin-coated Petri dishes (Corning, USA) containing 10 ml of IMDM supplemented with 1% P/S and 1% Insulin-Transferrin-Sodium Selenite (I/T/S, Sigma, Italy). Subsequently, a cover slip was applied and samples placed at 37°C-5% CO2. By this approach cells are allowed to outgrow from the fragments. Once reached confluence cells were detached by Trypsin-EDTA (Try, Cambrex, Italy), fragments eliminated, and cells were seeded in full growth medium composed by IMDM, 10% Fetal Bovine Serum (FBS, Sigma, Italy), 1% P/S, 1% I/T/S and 10 ng/ml Basic-Fibroblast Growth Factor (b-FGF, Sigma, Italy) for their amplification. At each passage, a cell aliquot was cryopreserved in a medium composed of FBS supplemented with 1% Dimethylsulphoxide (DMSO, Sigma, Italy).

The cells were seeded in "chamber slides " to the concentration of 5000 cells/cm$^2$.

**LYMPHATIC ENDOTHELIAL CELLS**

Samples were taken from artery and the pulmonary parenchyma macroscopically spared from 12 patients enrolled in another study in process at the Department of Biochemical Sciences , Biotechnology and Translational ( S.Bi.Bi.T. ) of Parma without any evidence of cardiomyopathy. Fresh samples obtained were simultaneously used for the isolation of endothelial populations.

Fragments of about 1-2 gr were obtained from the excised tissue under laminar flow biological hood. The fragments were processed separately following the same protocol obtained from published work and suitably modified. The biological material was chopped finely with surgical scissors and the fragments were incubated with shaking at 37 ° C in a solution of 1mg/ml collagenase / dispase for 75 minutes. The product of digestion was filtered with 15 ml of saline solution, through a nylon membrane with a pore size of 100 micron. The cell suspension obtained was centrifuged for 5 minutes at 240g. The pellet was then re-suspended in growth medium EGM- MV (LONZA , Basel , Switzerland ) to which were added 10% FBS and VEGF -C ( 30ng/ml , RELIATECH , Wolfenbuttel, Germany) and seeded in plates 6 wells pre- collagenate .

After 24 hours the debris and non-adherent cells (red blood cells, white blood cells, dead cells) were removed by 2 washes with PBS and the cell culture was added to the soil fresh EGM-MV, supplemented with 10 % FBS and 30NG/ml of VEGF -C. The cell culture was observed daily with an inverted optical microscope, and the medium was replaced twice a week. After five days the growth of a mixed morphologically recognizable population of cells was observed, consisting mainly of fibroblasts, stromal cells, epithelial cells and endothelial cells. Paramagnetic microbeads commercially available have been used to separate endothelial cells from lung tissue. Briefly, the mixed population of cells of the

primary seeding was trypsinized and re-suspended in 60μl of EGM - MV, in which were added 20μl of FcR Blocking Reagent and after 5 seconds stirring with 20μl of paramagnetic microbeads coated with anti-CD31 (MYLTENYI BIOTEC). After 20 minutes of incubation at 4 ° C, the cell suspension was transferred into a column MS Column subjected to a magnetic field generated by OctoMACS Separator TM and diluted with 2ml of EGM - MV . The recovered cells represent the negative selection, ie all those cells that do not express the endothelial marker CD31. Subsequently, the column was removed from the magnet and eluted with 2ml of EGM - MV to recover the fraction positive cell for the endothelial marker CD31. The positive selection was sown in a 25cm$^2$ flask pre-collagenated with soil EGM- MV 10% FBS supplemented with 30ng/ml of VEGF-C. After 5-7 days a homogeneous cell monolayer with typical endothelial morphology has obtained.

Because the lung parenchyma is perfused by both the vascular and lymphatic system, we further selected the primary CD31+ culture with podoplanin, the specific marker of lymphatic line. The CD31+ endothelial cells, obtained from the microcirculation of human lung (HLuBEC), once reached a confluence of 80%, were detached using trypsin/EDTA and centrifuged at 240g for 5 minutes. The pellet was re-suspended in 100 uL of PBS supplemented with 0.5 % FBS and incubated for 25 minutes at 4 ° C with 2μg of antibody mouse anti - Human podoplanin (RELIATECH). Therefore, a wash with 3 ml of PBS followed by centrifugation at 240g for 5 minuteswas performed. The pellet was re-suspended in 30μl of PBS 0.5 % FBS and incubated for 15 minutes at 4 ° C with 15μl of anti- mouse IgG microbeads (MYLTENYI BIOTEC) . After incubation, the cell suspension was transferred into a column MS Column exposed to a magnetic field generated by OctoMACS SeparatorTM and eluted with 2ml of PBS 0.5 % FBS . The cells recovered below the post, in a 15ml tube, represent the CD31+/Podoplanin- selection .

The column was then removed from the magnet and eluted with 2ml of PBS 0.5 % FBS to recover the fraction of cell positive for the lymphatic endothelial marker podoplanin.

The human lung lymphatic endothelial cells CD31+/Podoplanin+ (Lung Human Lymphatic Endothelial Cell: HLuLEC) were seeded in 75cm2 plates pre- collagenated with soil EGM-MV 10% FBS and supplemented with 30ng/ml of VEGF-C . All cells were cultured at 37 ° C in an atmosphere with 5 % $CO_2$. The cells were in part cryopreserved in nitrogen and in part were used for subsequent phenotypic analysis. In particular, the purity of the primary culture was evaluated by immunocytochemistry using antibodies rabbit anti-human Prox-1 and mouse anti-human podoplanin and using a functional assay of tubulogenesis on Matrigel.

### ENDOTHELIAL CELLS FROM PULMONARY ARTERY

The excised lung was observed under laminar flow biological hood and when possible branches of the pulmonary artery were sampled. Sections of 5-10mm in length of arteries of different caliber were processed separately. Connective tissue was removed from each piece of artery. A digestion of 20 minutes at room temperature with a solution of 1mg/ml collagenase/dispase (Roche, Monza (MB), Italy) preheated to 37 ° C was performed. The undigested tissue was removed and the cell suspension was centrifuged at 240g for 5 minutes at room temperature. The pellet was re-suspended in 2ml of EGM-MV/MV2 (LONZA) and seeded into plates pre-collagenated in 6 wells. The trend of cell growth was followed daily with an inverted optical microscope, and the ground has been completely replaced 2 times per week. After 5-7 days a mixed population of fibroblasts and endothelial cells was observed. In order to isolate endothelial cells, a selection by CD31 microbeads kit

(Miltenyi Biotec) was carried out, following the protocol suggested by the company and previously described by our laboratory for other endothelial cell lines. CD31 + endothelial cells of human lung artery (blood endothelial cells : BEC ) were cultured at 37 ° C in a humid atmosphere with 5% CO2. The cells were in part cryopreserved and in part used for subsequent phenotypic investigations. In particular, the purity of the primary culture was assayed by immunocytochemistry for the CD31 antigen and by tubulogenesis assay on Matrigel to assess their functional ability to form tubes in vitro.

## MCF7

MCF-7 is a breast cancer cell line isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established in 1973 by Herbert Soule and co-workers. This cell line retained several characteristics of differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic estrogen receptors and the capability of forming domes. Treatment with anti-estrogens can modulate the secretion of insulin-like growth factor binding proteins. PIK3CA helical mutations were identified in MCF-7, but with low AKT activation. Tumor necrosis factor alpha and doxorubicin inhibit the growth of MCF-7 breast cancer cells.

## K562

K562 cells were the first human immortalised myelogenous leukemia line to be established. K562 cells are of the erythroleukemia type, and the line is derived from a 53 years old female CML patient in blast crisis. The cells are non-adherent and rounded, are positive for

the bcr/abl fusion gene, and bear some proteomic resemblance to both undifferentiated granulocytes and erythrocytes.

In culture they exhibit much less clumping than many other suspension lines, presumably due to the downregulation of surface adhesion molecules by bcr/abl. K562s can spontaneously develop characteristics similar to early-stage erythrocytes, granulocytes and monocytes and are easily killed by natural killer cells as they lack the MHC complex required to inhibit NK activity. In addition to the Philadelphia chromosome they also exhibit a second reciprocal translocation between the long arm of chromosome 15 with chromosome 17. This cell line is a target for IM.


## MORPHOLOGICAL INVESTIGATION

Human heart samples were obtained from explanted organs for transplantation or from autopsies of the cases previously described. The material for the histological and immunohistochemical (IHC) was immediately fixed in 10% buffered formalin and then processed following the ordinary protocol of processing histological materials, which include dehydration, clearing and paraffin embedding. From each sample serial sections of 5μm thick were obtained, stained with hematoxylin and eosin, Masson's trichrome or used for immunohistochemical staining .

It was then carried out a morphological investigation in order to assess the suitability of the preparation, the diagnostic definition and perform morphometric analysis.


**Immunohistochemistry.** Tissue sections of the human heart, 2 μm in thickness, were employed for immunolabeling and fluorescence microscopic analysis.

The incidence of stem cells (hCPC) were studied by using specific anti-c-kit (Polyclonal rabbit, DAKO) primary antibody, the stem cell factor (SCF) receptor. Sections of human samples were incubated with antibody anti c-kit (CD117; rabbit polyclonal, 1:10, 90'a 37°C, DAKO), then with antibody anti-rabbit (FITC, 1:20, 60'a 37°C, SIGMA ALDRICH). Cd117 is also expressed by mastocytes, the same sections were incubated with tryptase (TR), an enzyme not present in stem cells, but present in mastocytes. The incubation with anti tryptase antibody (mouse monoclonal, 1:200, o.n. 4°C, ABD SEROTEC, Kidlington, Oxford, UK) was revealed by fluorescence (anti-mouse TRITC, 1:20, 60'a 37°C, SIGMA ALDRICH). The c-Kit $^{pos}$/TR$^{neg}$ cells were considered stem cells and thei distribution and number (in percentage and density) was evaluated.

Cycling myocardial cells and mitotic index were quantified using anti- Phospho Histone-H3 (PH-H3, Polyclonal rabbit, Upstate) and anti-mini-chromosome maintenance protein 5 (MCM5, Monoclonal Mouse, Santa Cruz). The primary antibody against phH3 (PH-H3, rabbit polyclonal, 1:100 o.n. a 4°C, UPSTATE, Lake Placid, USA) revealed by secondary antibody anti rabbit was and primary antibody against mini chromosome maintenance protein 5 (MCM5, mouse monoclonal, 1:30 o.n. a 4°C, ACCURATE, Westbury, USA) revealed by secondary antibody anti mouse were used. Myocytes were identified by α-sarcomeric actin. MCM5$^{pos}$/α-SARC$^{pos}$ and PH-H3$^{pos}$/α-SARC$^{pos}$ cells represented the cycling and mitotic myocyte, respectively.

DNA double strand breaks and apoptotic death of myocardial cells were analysed by γ-H2AX (Polyclonal rabbit, Bethyl) and TUNEL assay (Roche Diagnostics), respectively.

Myocytes were identified by α-sarcomeric actin (α-SARC, Monoclonal Mouse, 1:100 per 90' a 37°C Sigma-Aldrich), endothelial cells by von Willebrand factor (vWF, Polyclonal rabbit 1:30 per 90' a 37°C, Sigma-Aldrich) , smooth muscle cells by α-smooth muscle actin

(SMA, Monoclonal Mouse, Sigma-Aldrich). anti-lymphatic endothelial cells by LYVE (rabbit policlonal , 1:50 on at 4 ° C , Abcam , Cambridge, UK ). FITC, TRITC-, Cy5-conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson Laboratory) were used to detect different epitopes. Nuclei were recognized by DAPI (4',6-diamidine-2-phenyndole, Sigma) staining.

**Collagen accumulation:** Five-micrometer-thick human heart sections were stained with Masson's trichrome and analyzed by optical microscopy (magnification 250X) in order to evaluate: (i) the volume fraction of fibrosis, and (ii) the numerical density and average cross-sectional area of fibrotic foci. According to a procedure previously described[24], for each section, a quantitative evaluation of the volume fraction of myocytes and fibrotic tissue was performed in 60 adjacent fields from myocardium. The measurement was obtained with the aid of a grid defining a tissue area of 0.160 mm$^2$ and containing 42 sampling points each covering an area of 0.0038 mm$^2$. To define the volume fraction of fibrosis, the number of points overlying myocardial fibrosis was counted and expressed as percentage of the total number of points explored. The transversal section of fibrotic foci was evaluated by a software for image analysis (Image Pro-Plus 4.0, Media Cybernetics, Bethesda, MD, USA) connected with microscope. The diameter of each fibrotic focus has been measured. The transversal area of myocyte was analyzed, measuring the diameter through nuclei, in the myocytes transversally oriented.

**Fluorescence In Situ Hybridization:** Chimerism in human samples were documented by fluorescence *in situ* hybridization (FISH) to detect human X and Y chromosomes using human specific CEP X alpha-satellite and CEP Y satellite III DNA probes (Vysis, USA)

**Detection of ematic and lymphatic vessels**

An immunofluorescent analysis with antibodies anti-α smooth muscle actin (α-SMA, mouse monoclonal 1:200 60' at 37°C, SIGMA ALDRICH), anti-endothelial cells (vW factor, rabbit policlonal, 1:20 60' at 37°C, DAKO) and anti-lymphatic endothelial cells (LYVE, rabbit policlonal, 1:50 on at 4°C, Abcam , Cambridge, UK) was performed. With a secondary anti-mouse antibody for α -SMA (1:20 60 ' at 37°C, developed in Donkey), anti -rabbit (1:20 60' at 37°C developed in Donkey) for Factor VW and LYVE was detected the positivity.

A quantification of vessels was performed by the three markers using a fluorescence microscope (Leica DMI6000B , 630X final magnification ).

**In vitro treatment with DOXO**

hCPC were exposed to concentration of DOXO (Adriblastina, Pharmacia & Upjohn, Division of Pfizer Inc, NY, USA) of 0.1 μM, 0.5 μM, 1 μM, 10 μM, and 50 μM, for 12, 24 and 48 hours.  The different concentration of DOXO are in accord to the concentration revealed at the steady state or at the plasmatic peak in patient treated in vivo with the drug.[25]

The same experiment was performed with MCF7, an immortalized breast cancer cell line, that is considered the specific target of DOXO. Concentrations of 0.1 μM, 0.5 μM, 1 μM e 10 μM were tested.[26, 27, 28]

**In vitro treatment with Imatinib**

After 24 and 48 hours, the seeded cells, as described above, were exposed to Imatinib at a concentration of 5 μM (IM5) or 50 μM (IM50) and endothelial cells at concentrations of 1μM , 5μM, 25μM and 50μM.

The same procedure was performed on K562 (ATCC, Manassas, VA, USA) at the concentration of IM 50 μM and 5 μM, an immortalized cell line positive for Bcr-Abl, isolated from a patient with leukemia chronic myelogenous, considered to be the target of choice for IM. [29, 30, 31 32]

**Cell Viability Assay.**

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to determine cytotoxicity of the drug, analyzing the number of living cells, through mitochondrial activity. Yellow MTT is reduced to purple formazan when mitochondrial reductase enzymes are active. This conversion is directly related to the number of living cells. Optical density (OD) was measured at 540 nm with spectrophotometer (MULTISKAN EX, Thermo Electron Corporation, Vantaa, Finlandia)

**Comet Assay**

  The Comet assay or single cell gel electrophoresis ( SCGE ) is a relatively simple and sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. It has become a new tool in genetic toxicology, useful to assess both in vitro and in vivo genotoxic damage induced by a variety of physical or chemical agents. It evaluates the integrity of the DNA by measuring the damage in relation to the presence of

fragmented DNA outside of the core of the nucleus, after electrophoresis.[33] The technique of electrophoresis on microgel was introduced in 1984 by Ostling and Johanson as a method to measure the single-strand breaks, that determined the relaxation of supercoiled DNA.[34] A modified version was published by Singh and colleagues in 1988. The idea was to combine the gel electrophoresis with fluorescence microscopy to visualize the migration of the DNA fragments. If DNA is broken, there is a relaxation of the molecule and the ends of the fragments, migrate to the anode during electrophoresis, because of their negative charge, giving the typical comet morphology. If DNA is not the damaged, the lack of free ends and the size of the fragments make the migration impossible. The determination of DNA migrating allows an assessment of the amount of broken DNA in every single cell. The head of the comet contains the DNA at higher molecular weight and the tail of the comet contains the fragments that migrate towards the anode. Head and tail are measured by analysis of digitized images using dedicated software[35.] Several modifications of the protocol allows the identification of single- and double-strand breaks, cross-links, damage to DNA bases and repair .

**TUNEL Assay**

Terminal deoxynucleotidyltransferase (TdT)–mediated dUTP nick end labeling (TUNEL) assay was used for the detection of apoptosis with ApoAlert DNA fragmentation kit (Clontech).

**Caspase-3 Activity**

Cells were washed with ice-cold PBS and lysed with cell lysis buffer (ApoAlert Caspase-3 Colorimetric Assay kit, Clontech Laboratories). Samples were incubated on ice and centrifuged at 12,000 g for 10 min at 4°C. Caspase-3 activity in the supernatant was measured in a microplate reader (Bio-Rad), using DEVD-*p*-nitroanilide as a substrate.

**CPC Proliferation**

One hour before the end of the experiment, CPCs were incubated with BrdU (10 μM). Cell proliferation was determined by the number of BrdU-positive CPCs.36 CPCs in mitosis were identified by phospho-H3 antibody (Upstate).

**Western Blotting**

Cells were lysed in buffer containing 0.1% Triton X100 and a cocktail of protease inhibitors (Sigma-Aldrich). Protein concentration was measured by Bradford assay (Bio-Rad). Protein extracts were then separated on 8-12% SDS-PAGE and transferred onto PVDF membrane (Amersham). Antibody binding was visualized by ECL (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce Biotechnology) and OD of the bands was analyzed with Molecular Analysis software (Bio-Rad). Loading conditions were determined by the expression of β-actin (Sigma-Aldrich). Primary antibodies against cyclin D1 (Zymed), cdk4, cyclin B1, p16INK4a, cdc2, p21Cip, p27Kip1, Bax (Santa Cruz Biotechnology), phospho-cdc2, Rb, phospho-Rb, p53, phospho-p53Ser15, phospho-p53Ser20, ATM kinase, Bad (Cell Signaling), Mn-SOD, Cu/Zn-SOD (Upstate) and catalase (Sigma-Aldrich) were used. Peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) were employed.

**CPCs and Oxidative Stress**

Oxidative stress in CPC was determined by immunolabeling with 8-OH-dG mouse monoclonal antibody 4 (QED Bioscience).

**Evaluation of oxidative stress with diclorofluorescinadiidrodiacetato (DCFH -DA)**

The evaluation was carried out through the use of DCFH -DA, an apolar and non-fluorescent molecule that can cross the membrane of the cell. Once inside the cytoplasm, it is degraded by cellular esterases that remove the two acetate groups , transforming it into a polar molecule but still not fluorescent, the diclorodiidrofluorescina ( DCFH ) . This molecule is highly reactive against ROS by reacting with them and turns into dichlorofluorescein , highly fluorescent .

Were seeded 1 x $10^5$ cells in 1 ml wells and incubated at 37 ° C for 24h . At the end of incubation time, the cells were placed in contact with of DCFH -DA 10μM diluted in PBS pH 7.4 , and incubated for 30 ' at 37 ° C to allow the DCFH -DA permeate inside cells. The excess of DCFH -DA is washed and re-suspended in culture medium and the treatment performed .

For this experiment the cells ( CPCs and K562 ) were treated for 3h with IM 1-5 uM and 25 uM with Menadione. The cell suspension is then subjected to lysis ( 50 mM Tris- HCL + 0.5 % TritonX , pH7.4 -7.5 ; ell dissociation solution, SIGMA ) , centrifuged and the supernatant loaded into wells in a multi-well black plate. Using spectrophotometer it is possible to assess the fluorescence emission (excitation 488 nm , emission 525 nm) which will be proportional to the contents of ROS .

The data are presented in terms of % increase calculated with the following formula: 100 * [ (Media treated / untreated Media ) -1 ] .

**Antioxidant Enzyme Activity**

Protein extracts from control and DOXO-treated CPCs were prepared by sonication and centrifugation at 2,500 g for 10 min.

**SOD Activity**

The activity of Cu/Zn-SOD and Mn-SOD was measured by employing a superoxide dismutase kit (R&D). Samples were incubated with a xanthine/xanthine oxidase solution. The generation of superoxide ions converts NBT to NBT-diformazan. SOD present in the sample decreases superoxide ion concentration reducing the rate of NBT-diformazan formation. The extent of reduction in the appearance of NBTdiformazan is a measure of SOD activity present in the cell lysates.[6] The absorbance of non reduced NBT was measured at 560 nm.

**Catalase activity**

This parameter was measured according to the modified method developed by Cohen.[7] Cell lysates were incubated for 5 min with 6 mM $H_2O_2$ at room temperature. The reaction was quenched by the addition of 0.6 N $H_2SO_4$ and 10 mM $FeSO_4$. The colorimetric reaction of the formed products was obtained by adding potassium thiocyanate (KSCN). The absorbance of the ferrithiocyanate product was measured at 490 nm.

**Quantitative RT-PCR Array**

The transcriptional profile of CPCs in the absence and presence of DOXO was assessed by quantitative RT-PCR array in 5 cases. This quantitative analysis involved a restricted panel of genes with SYBR Green-optimized primer assays. Arrays containing a panel of stem cell related genes (SuperArray) and custom-designed panels of lineage-related and senescenc eassociated genes were performed. In each case, 3 x $10^6$ CPCs were sent to the SuperArray facility (SABiosciences) for RNA isolation, hybridization and data analysis.

**Effect of treatment on cell growth**

For endothelial cells and the hCPC growth curve has been drawn in response to each treatment, capturing 10 microimages by camera (Olympus DP21), connected to an inverted microscope (Olympus CK40) at 4X or 10x magnification, every day from the start of treatment for 2 weeks.

The concentrations used for the treatment were: for DOXO 0.1 uM, 0.5 uM, 1 uM for IM and 1 uM, 5 uM, 25 uM, 50 uM.

**Angiogenesis assay on Matrigel**

Matrigel is a biological matrix in which angiogenic phenomena can be simulated in vitro. This ability can be evaluated microscopically, verifying the ability of endothelial cells to form tubular structures similar to vessel networks, under stimulation of growth factors.

The endothelial cell lines generated in our laboratory were trypsinized and $1x10^4$ cells were seeded on 96-well plate (Corning) previously coated with 50μl of Matrigel and maintained in their culture medium at 37 ° C in humid atmosphere at 95% and $CO_2$ at 5%. The plate

was observed by inverted light microscope after 24 h in order to detect the formation of tubes and the images were acquired.

Endothelial cells were treated in part with DOXO (0.1 m M and 0.5 m M) and in part with imatinib ( 1 m M and 5 m M). Untreated endothelial cell was used as control.

The plate was observed under the inverted light microscope after 48 h to eventually detect the formation of tubes. Images were acquired and processed with image pro -plus.

**Wound-healing assay**

W*ound-healing assay* evaluates the *in vitro* migration ability of HLuAEC e HLuLEC.

$5 \times 10^4$ cells were seeded in 24-well plates. Once reached confluence, the cells were incubated with different doses of DOXO (0.1 m m M and 0.5 M) and IM (1 m m M and 5 M). The test consists in causing damage to the cell monolayer with a tip of 1000 mL, to assess the ability of the cells to heal the rift observing the culture at different times (T0, 24 and 48 h) with an inverted optical microscope. The images were captured using digital camera.

**Autophagy**

Autophagy is a conserved proteolytic mechanism required for maintaining cellular homesostasis and is observed when cells are exposed to stress, such as lack of oxygen or starvation.

To date, LC3, a mammalian homolog of yeast Atg8, is the only reliable marker of autophagosomes.

Autophagic process has been studied to assess whether it could be a possible mechanism of the DOXO or Imatinib induced cardiotoxicity. MCF7 and K562 have been used as control for the method.

*RT-PCR*

The quantification of expression of genes involved in autophagy (ATM , ATR) was performed by "Real Time PCR ". This is a PCR reaction in which, for each amplification, the probe bound to the cDNA substrate is destroyed by the polymerase, producing fluorescence release that is recorded as a synthesis signal .

The evaluation of the different concentrations of the treatment is performed by comparing the fluorescence data obtained for the genes of interest compared to the control, normalizing the data with respect to a reference house-keeping gene : ACTB ( actin B)

**RAT STUDIES**

**IN VIVO STUDIES**

Two experimental models were prepared to investigate the mechanism of onset of cardiotoxicity. The investigations were approved by the Veterinary Committee of the University of Naples and Parma and were in accordance to the national ethical guidelines (Italian Ministry of Health, Legislative Decree 116, January 27, 1992) and to the Guide for the Care and the use of laboratory animals (NIH publication no. 85-23, revised 1996).

**DOXO treatment**

Female 344 Fischer rats (n=160) at 3 months of age (body weight 180±10 g) received 6 i.p. injections of 2.5 mg/kg b.w. of DOXO over a period of 2 weeks to reach a cumulative dose of 15 mg/kg b.w. Control rats were injected with saline. Animals were sacrificed at 3 weeks and 6 weeks after the first injection of DOXO. In a third group of animals, cells were delivered intramyocardially. Under anesthesia with ketamine (100 mg/kg b.w., i.p.) and acepromazine (1 mg/kg b.w., i.p.), rats were intubated, thoracotomy was performed and the heart was exposed. A total of $5 \times 10^4$ cells were injected in 4 different sites in the left ventricular myocardium. Cells were suspended in PBS with polystyrene microspheres conjugated with rhodamine (Molecular Probes) for identification of the cells and of the sites of injection on the tissue[37]. Control rats were injected with PBS. BrdU was injected twice a day (50 mg/kg b.w., i.p.) and added to the drinking water (1 mg/ml) to identify newly formed cells [38,39]. Rats were then sacrificed 3 weeks after surgery, i.e., 6 weeks after the first injection of DOXO.

**IM treatment**

Capsules of IM were dissolved with prolonged stirring in sterile water at pH = 2 with HCl 10 N. The suspension was filtered and stored in aliquots at -80 ° C. At the time of administration the drug was diluted in function of the desired concentration (injection volume 0.7-1.2 ml) and the pH was brought to a value of 6.2 with addition of sodium hydroxide (NaOH) 10N.

8 weeks old male Wistar rats (Rattus norvegicus) weighing 200-250 g were randomized in 4 experimental group subjected to intraperitoneal (ip) injection of different doses of IM three times per week for three weeks.

(1) n = 8 animals were treated with 50mg/kg = IM50

(2) n = 10 animals were treated of 100mg/kg = IM 100

(3) n= 4 animals were treated with 200 mg/Kg = IM200

(4) n = 8 animals were injected with equal volume of saline as control = CTRL

A 100 % mortality was seen in female rats treated

A 100% mortality was seen in rats treated with IM200.

The rats were anesthetized with droperidol and fentanyl citrate (Leptofen , 1.5 mg / kg, im ; Farmitalia Carlo Erba , Italy)  and subjected to measurement of hemodynamic parameters 3 weeks after initiation of treatment. The animals were then sacrificed and the heart taken as previously described for analysis.

**Echocardiography and Hemodynamics**

Echocardiographic parameters were collected with a high resolution Micro-Ultrasound system System (Vevo 770, VisualSonics Inc.) equipped with a 25-MHz linear transducer. Body temperature was maintained at ~37°C with a heating pad. Serial M-mode echocardiographic images were taken in the short axis view at the level of the papillary muscles. Left ventricular diameter and posterior wall thickness were measured in diastole and systole and fractional shortening and ejection fraction were calculated [40.]

At sacrifice, hemodynamic parameters were collected. Animals were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.), and the right carotid artery was cannulated with a microtip pressure transducer (SPR-612, Millar Instruments) connected to an A/D converter (iWorx 214) and a computer system. The catheter was advanced into the LV cavity for the evaluation of LV pressures and + and − dP/dt in the closed-chest preparation.[41]

**Anatomical parameters**

**Fixation of the Heart.** After the collection of hemodynamic measurements, the abdominal aorta was cannulated with a polyethylene catheter filled with phosphate buffer (0.2 M, pH 7.4) and heparin (100 IU/ml). In rapid succession, the heart was arrested in diastole by injection of cadmium chloride (100 mM) through the aortic catheter, the thorax was opened, perfusion with phosphate-buffered formalin was started, and the right atrium was cut to allow drainage. Perfusion pressure was adjusted to the mean arterial pressure. 42. The left ventricular chamber was filled with fixative from a pressure reservoir set at a height equivalent to the in vivo measured LVEDP. This was accomplished by inserting a 25G3/4 Vacutainer (Becton Dickinson) into the LV chamber. After fixation, the heart was dissected and the weights of the right ventricle and LV inclusive of the interventricular septum were obtained. After measuring the major longitudinal axis, the LV was sectioned serially into five rings perpendicular to the longitudinal axis of the heart, and the thickness of the free wall and septum was determined (ImagePro software). Similarly, the transverse diameter of the LV chamber was evaluated. The transverse and longitudinal diameters were employed to compute chamber volume [43]. Tissue specimens were embedded in paraffin and histological sections were obtained for immunohistochemistry.[44]

**Morphometric analysis with collagen quantification**

A quantitative assessment of the volume fraction of myocytes and fibrotic tissue was performed for each section, according to a procedure described in human related section.

**Immunohistochemistry**

Histological sections obtained from each group were analyzed by fluorescence microscopy for the immunohistochemical evaluation.

- Cardiomyocytes, lymphatic and arteriolar density were identified by immunofluorescence, incubating the sections of LV with the primary antibodies, respectively: α-sarcomeric actin (anti- α SARC, 1:30 on at 4 ° C + 30 ' at 37° C, SIGMA ALDRICH), anti- LYVE1, (rabbit polyclonal, 1:50 on at 4° C + 30 ' at 37° C , Abcam , UK) and α-smooth muscle actin (α - SMA , mouse monoclonal), 1:200 on at 4° C, DAKO.

- Apoptotic cell death in the myocardium was quantified by TUNEL assay (ROCHE DIAGNOSTICS , Italy).

- DNA damage was evaluated in terms of double-strand breaks in DNA detected by primary antibody rabbit polyclonal anti-histone range H2 (γ-H2AX, (1:50 on at 4° C , BETHIL , Montgomery, USA)

- Cell proliferation was evaluated by anti -phospho - histone H3 ( H3 - PH , rabbit polyclonal , 1:50 on at 4 ° C , Upstate, Lake Placid , USA) followed by incubation with specific secondary antibodies conjugated to a fluorochrome .

- cell coupling was established by the presence of connexin 43 (Sigma-Aldrich).

- Cycling cells were identified by Ki-67 (Vector) and BrdU (Roche) labeling.

- The incidence of cells c-Kit pos in LV evaluated by immunofluorescence using the primary antibody to SCF R / c- kit ( rabbit polyclonal , 1 20 on at 4 ° C , SANTA CRUZ, Santa Cruz , USA).

Secondary antibodies conjugated with fluorochromes FITC-TRITC, Cy5 (JACKSON LABORATORY, Baltimore, MD, USA) were employed to detect primary antibodies.

Nuclei were recognized with DAPI staining (4 ' ,6- diamidine -2- phenyndole , SIGMA , Italy).

**IN VITRO STUDIES**

**CARDIOMYOCYTES AND CPC ISOLATION**

CPC isolation was obtained from rat according to the protocol described by Beltrami et al.

Once cleaned of adipose tissue, beating heart has been hung to a perfusion Langhendorff type apparatus by cannulation of the ascending aorta. The coronary vessels were perfused first with phosphate buffer, then with a solution of collagenase type II at a temperature of 33-34 ° C. At the end of the enzymatic digestion, the heart was minced and put under stirring at 37 ° C to allow the mechanical digestion of tissue. The suspensions obtained were washed and subjected to centrifugation to obtain a separation of the different cell populations. The pellet obtained was centrifuged in Percoll gradient (SIGMA) to obtain a pure population (99%) of myocytes, while the supernatant was centrifugated to obtain a population of small size immature cells.

The progenitor populations obtained were resuspended in 10 ml of culture medium containing Iscove's Modified Dulbecco's Medium (IMDM, Sigma) supplemented with 1%

penicillin- streptomycin (P/S, SIGMA), 1% insulin-transferrin-selenite (I/T/S, SIGMA), 10 % fetal bovine serum (FBS , Sigma ) and 10 ng/ml basic- fibroblast growth factor (FGF -b , SIGMA ) and were seeded in Petri dishes (Corning , USA) incubated at 37 ° C and 5 % $CO_2$ for their amplification .

At the daily microscopic observation of progenitor populations, two different populations of adherent cells was seen: one fibroblast-like and one with monomorphic features, that consisted in cardiac progenitor cells characterized by clonogenic and multipotent growth . These cells were amplified with several steps and cryopreserved in aliquots with a medium composed of FBS supplemented with 1 % dimethylsulfoxide (DMSO, SIGMA).

Myocytes were indeed seeded at a density of 20000cell/cm2 in chambre slides previously treated with laminin (5ml/ml in Hank's Solution) in the culture medium and placed at 37 ° C - 5 % $CO_2$ and treated immediately after seeding.

The CPC were subsequently seeded in chambre slides at a concentration of 2500 cells/ml and treated after 24 hours of adherence.

Both cell cultures were exposed to 5 mM IM (IM5) or 50 mM IM ( IM50 ) for 6, 12 and 24h or DOXO 0.1 mM , 0.5 mM , and 1 mM for 12 , 24, 48 h .


**Data Analysis and Statistics.** Results are presented as mean±SD. Significance between two comparisons was determined by unpaired and paired Student's *t*-test and among multiple comparisons by Bonferroni test. Mortality was measured by log-rank test. All *P* values are two-sided and *P*<0.05 was considered to be significant.

# 4) RESULTS

**HUMAN MYOCARDIUM**

•       **to assess the number of CPCs and myocardial cell turnover in DOXO and IM-induced human cardiomyopathic heart**

**TISSUE ANALYSIS**

**Myocardial damage**

*DOXO.* The first step was the analysis of fibrotic tissue on Masson's Trichrome staining that could reflect the necrotic damage caused by the drug. An example of scattered acute and chronic injuries with marked myocardial fibrosis is documented in figure 2. Collagen accumulation was also present in the myocardium of all other cases, although with different magnitude. The volumetric fraction of fibrosis (Fig. 3) in patients treated with DOXO was 2 fold higher (15,17% ± 6.42) than in control group (8.45% ± 2.50 n.s.)

Apoptotic cell death and DNA oxidative damage were then analyzed by TUNEL assay and the detection of DNA Double Strand Breaks (dSBs) by $\gamma H_2 AX$, respectively (Fig. 5). Compared to controls, high levels of apoptotic cell death and DNAdSBs were present in cardiomyocytes and non-cardiomyocytes of the cardiotoxic heart. The incidence of apoptosis and of DNAdSBs were 6-fold higher in patients treated with chemotherapy. The

apoptotic death in DOXO group was 7,2 fold higher (0.36±0.06%) than ctrl group (0.05±0.035%, p<0.001).

*IM*. The fibrotic fraction in IM treated patient was low (3.57 %), indicating that fibrotic damage is not a major cause of IM related cardiotoxicity. Also genotoxic damage (Fig. 5) and apoptotic death (0.048±0.012) were no significantly increased.

**Myocardial Cell Proliferation**

*DOXO*. The ability of myocardial cells to respond to damage by entering cell cycle and divide was evaluated. This goal was obtained by the immunofluorescence analysis of mcm5 and phH3, respectively. Quantitatively, cardiomyocytes in mitotic phase in the heart of patients treated with DOXO were 4-fold than in control and cycling cells showed an increase of 70% in patient treated (p<0,05) (Fig. 6)

*IM*. Also in IM case the cell proliferation index was increased. Mitotic cardiomyocytes were 3-fold higher than in control and mcm5$^{pos}$ cells showed an increase of 80% (Fig. 6).

**Cardiac Progenitor Cells**

To support the hypothesis that DOXO and IM affect cell compartments responsible for cardiac homeostasis, the effect of cardiotoxic drugs on resident progenitor cells was evaluated by measuring the number of c-kit$^{pos}$ CPCs on tissue sample. The numerical density

of CPCs resulted in 31% lower in the heart of patients treated with DOXO, but without statistic significance, and 10% in the patient treated with IM.

The increased cell turnover documented characterized by high level of both cell death and cell proliferation likely reflects the attempt of the heart to repair the damage produced by chemotherapy. This finding, together with the evidence of reduced number of progenitor cells, suggests that in the toxic cardiomyopathic heart the endogenous repairing machinery is triggered.

Importantly, these observations also suggest that not only the number of CPCs, but above all functional properties of CPCs may be responsible for cardiac failure. This contention is clearly supported by the in vitro analysis on human CPCs later described in the present investigation.

Interestingly, one of the case described (case 1) prompted us to provide further evidence for the presence of myocardial regeneration by progenitor cells later in life when overt CHF developed. She had delivered a healthy boy, after being treated for NHL. We hypothesized that male cells could migrate during the course of pregnancy participating to the reconstitution of myocardial damage of the cardiomyopathic heart. Importantly, chimerism can be easily recognized in women transplanted with male bone marrow cells or male cardiac allograft by the detection of Y chromosome. Indeed, the identification of male cells in female hearts transplanted to male recipients has challenged the view of the heart as a post-mitotic organ [45.]

Thus, we have performed FISH analysis to detect Y chromosome in parenchymal cells of myocardial tissue of this female patient. We have been able to document that

cardiomyocytes and vascular structures carrying XY chromosomes took part on the composition of the female myocardium (Fig.8). Chimerism was present in the healthy portion of the heart and cells with Y chromosome were not distinguishable from the surrounding spared myocardium. Importantly, the time interval from pregnancy to the documentation of chimerism was 25 years. These findings demonstrate that primitive cells from the fetus were translocated to the heart of the mother and upon their activation and differentiation were able to generate functional myocardium. The long term survival of these cells and their ability to multilineage differentiation strongly suggest their stem cell nature.

## IN VITRO ANALYSIS

- **To determine whether DOXO and IM differently affect in vitro cell growth of hCPC, hBEC and hLEC.**

**Effect of treatment on cell growth of hCPC, hBEC and hLEC.**

Cardiac progenitor cells and endothelial cell lines were exposed to different doses of DOXO (0.1 μM, 0.5 μM, 1 μM) and IM  (1 μM, 5 μM, 25 μM, 50 μM) in order to analyse the in vitro effects of the drugs. The interest in analysing the effect on endothelial cells is due to the important structural rearrangement noticed in human myocardium and to the differences in the different compartment of the heart seen in the animal model described below.

Daily quantification of hCPC, BEC e LEC, after incubation with different doses of DOXO and IM for 6 days was performed.

*DOXO*. A reduction in cell growth was seen in all the cell lines analysed, in a dose and time dependent manner (Fig. 9). The prolonged exposure of hCPC to all tested doses of DOXO showed an early cell growth inhibition starting after 24 hours. During exposure no cytotoxic damage was observed, because after 4 days of exposition the effects were the same than after 1 day. Interestingly, with the dose of 0.1 μM, after 4 days of treatment, an increase in hCPC proliferation was seen. This effect was not seen with higher doses of DOXO. In BEC and LEC line, at the dose of 0,5-1μM an important cytotoxic damage was seen, with extensive cell death after 3 days of treatment. The lower dose of 0,1μM induced a cytostatic effect, because after an initial reduction at the second day the number of BEC and LEC remained at the steady state. Interestingly, the hCPC incubated with DOXO have a residual growing ability, and this phenomenon is likely due to stem carachteristics. They also have many efflux pump, ATP binding cassettes or protein multidrug resistance (MRD) that lead to a more efficient drug elimination.

*IM*. Higher doses of IM (25 and 50 μM) were cytoxic on all cell lines, in a dose and time dependent manner, while at lower conentration (1μM) a cell growth inhibition was seen in BEC and LEC but not on hCPC. In all cell lines, after 6 days of exposure to IM 1 μM the number of cell was similar to that of CTRL group (Fig. 10).


**Angiogenetic assay on Matrigel**

The in vitro angiogenic properties of BEC and LEC after treatment with different concentration of DOXO (0.1 μM e 0.5 μM) and IM  (1 μM e 5 μM) were tested.

*DOXO*. A reduced ability in forming tube was observed in BEC and LEC treated with 0,5 μM, while an increased ability was oserved with lower doses of the drug (0,1 μM) (Fig. 11A).

*IM*. An inhibition effect of IM was observed only on lymphatic endotelial cells, at both doses. No effect was seen on BEC ability in forming tube  (Fig. 11B)

**Wound-Healing assay.**

Monolayers of BEC and LEC, incubated with different doses of DOXO (0.1 μM e 0.5 μM) and IM (1 μM e 5 μM) at different time (T0, 24h and 48 h) were subjected to wound healing assay (Fig. 12 and 13)

*DOXO*. Doxorubicin inhibited the migration of both cell lines at both concentration of drug.

*IM*. The treatment with IM didn't affect the proliferation of BEC, while it reduced the ability in proliferation of LEC at the dose of 1 and 5 μM.

• **To determine whether cell proliferation and death of CPC and tumor cell exposed to therapeutic and toxic doses of DOXO and IM are differently affected in vitro**

*DOXO*. The effect of DOXO on hCPC was compared with that on MCF-7, the breast cancer cell line. This analysis was performed in order to understand whether cellular and molecular mechanism may be differentially involved in neoplastic and cardiac cells.

DOXO was added to MCF7 and hCPC cultures at different concentration of 0.1, 0.5, 1 and 10 µM and the exposure was prolonged for 4,12, 24 and 48 hours.

By MTT test, the cell viability was tested. At 12 hours hCPCs sensibility to DOXO-induced metabolic damage was higher than in MCF-7 suggesting that progenitor cells are less resistant to the toxic insult in vitro. At 24 and 48 hours the two cell lines equally responded to different DOXO concentrations. The genotoxic damage is dose and time dependent.

*IM*. hCPCs proliferation was not affected by IM 5µM after 24 and 48h of treatement, while a singnificant inhibition was observed with higher dose of IM (50 µM), also after 3 hours of exposure. The effect of IM was than compared with that on K562. In these cells, after 24 and 48 hours of exposure, an inhibition of viability at both doses (5 and 50 µM) was seen. After 24 hours in K562 a reduction in genotoxicity was observed, maybe due to DNA repairing or to a selection of resistant population.

Oxidative stress response in DOXO treated hCPC was analyzed by the quantification of MnSOD and CuZnSOD. No significant changes were observed in hCPC, whereas in MCF7 cells a dose and time dependent increase in these SOD enzymes was present in response to DOXO.

The phosphorilated form of p53 (usually activated by DNA damage) has been evaluated in our cultured cells and a time and dose dependent increase was shown. The increase in p53

stimulated by DNA damage activates on one hand cell cycle arrest, in order to let the cell to repair DNA and restart cycling, or on the other hand apoptosis, a mechanism triggered to remove pathologic cells.

Doxorubicin resulted in an average 30% shortening of telomeres in CPCs. Dysfunctional telomeres trigger DNA damage response in which the major determinant is the transcription factor p53.

p53 resulted activated by DOXO in both CPC and MCF7. However, higher values were observed of p53 expression in CPC. This phenomenon toghether with oxidative stress inhibit the growth and survival of CPCs.

A dose and time dependent reduction in detection of BrdU was noted in both cell lines. However, as expected, a higher fraction of BrdU positive MCF-7 cells was present at 48 hours supporting the notion that a selection of a resistant population maintain the growth capacity of cancer cells compared to normal cardiac progenitors

In addition to BrdU, the analysis of the cell cycle was measured by the CyclD/cdk4 complex. The synthesis of cyclin D is initiated during G1 and drives the G1/S phase transition. Its interaction with cdk4 is of great importance for cell cycle progression. CyclD/cdk4 complex was reduced in both CPC and MCF-7 cultures in a time and dose dependent manner. With respect to control conditions there was a 50% reduction of CycD1 and cdk4 in both cell lines. The analysis of phosphorilated Rb confirmed the effects of DOXO on cell cycle progression. A time and dose dependent reduction of p-Rb was more apparent in hCPC. By this mechanism promoting G1 arrest, DOXO significantly lowered cycling CPC in a time and dose dependent manner.

Evaluation of apoptosis was made by measuring at biochemical level bax/bcl2 ratio. In both cell coltures bax/bcl2 increased in a dose but not time dependent manner. Thus, alteration of

molecular pathwways involved in cell death and survival is present in both hCPCs and neoplastic cells exposed to DOXO.

## IN VIVO ANALYSIS

## Sperimental model – rat myocardium

Mortality rate with IM50 and IM100 was of 25%. A significant decrease in body weight was observed.

## Cardiac morphology and function

*DOXO*. At the end of the treatment an increase in diameter (11%) and of volume of left vetricular camera (17%) was observed. The thickness of left ventricular wall resulted significantly reduced after treatment with DOXO in rispect to that of control (p<0.05). One week after the end of treatment a reduction in LVS function was seen, with reduction in Ejection fraction and in dP/dt and an increase in telediastolic pressure.

*IM*. After 3 weeks treatment a dose dependent increase of telediastolic pressure was observed with an impairement in +dP/dt e - dP/dt and a small reduction in sistolic pressure (Fig. 14)

The morphometrc analysis showed an increased thickness of left ventricular wall with a reduction in volume of the camera leading to an increased ratio thickness of LV wall/radius of camera (Fig. 16 and 17). Interestingly, when the ratio of cardiac wet to dry weight was measured, compared to control values, a dose dependent increase of theoretical water

content in treated hearts was observed (Fig. 15).

In conclusion, the remodelling of the heart in DOXO group is very different from the one post IM treatment. In particular the volume of the camera is increased in hearts treated with DOXO and the thickness of the wall reduced, according to a dilatative type cardiomyopathy. After treatment with IM the volume of the camera is reduced and the thickness of the wall normal or little increased, suggesting a restrictive type cardyomypathy.

**Fibrosis**

*DOXO*. As in human sample, an important deposition of collagen was observed in rat treated with DOXO 12% at 3 weeks, 18% at 6 weeks, $p < 0.05$ vs CTRL (fig. 18).

*IM*. No changes in fraction of fibrosis were seen (fig. 19).

Fibrosis is a characteristic of DOXO-induced damage: the loss of myocytes leads to a reduction in wall thickness and dilation of heart camera, with reduced contractility strength and reduced ejection fraction. Instead, the TKI related cardiomyopathy couldn't be explained with fibrotic damage.

**Effects on number of cardiomyocytes**

*DOXO*. The number of cardiomyocytes was reduced of 50% and 68% after 3 and 6 weeks of treatment, respectively. The loss of myocytes was not replenished by new formation of cells and this phenomenon explains the reduction of parenchymal cells in LV.

*IM*. Differently, an increased number of cardiomyocytes was detected in rat treated with IM 50 (11%) and in IM 100 (19%) versus ctrl.

**Proliferation**

The effect of the drugs on proliferation was evaluated by detection of Ki-67$^{pos}$ and PH-H3$^{pos}$ The formation of new myocytes has been measured by evaluation of the number of cells that coexpress Ki67 and sarcomeric a-actin.

*DOXO*. A reduction of 95% and 65% in the proliferation of smaller cardiomyocytes was observed after 3 and 6 weeks of treatment with DOXO (p<0,05) vs ctrl, respectively.

*IM*. The mitotic index was differently influenced by IM doses, where an increasing in cellular division was seen with lower doses of drug. Indeed, IM50 showed an increase of 1,68 fold in the number of cardiomyocytes PH-H3$^{pos}$, while at higher doses a reduction in mitotic index of 62% was observed.

**Evaluation of DNA damage**

IM. Quantitatively, with doses of IM50 an increase of 30% of DNA damage of cardiomyocytes compared with controls was observed, while the same phenomenon at higher doses was not documented. The analysis of DNA damage in all population of myocardial cells did not show a significant effect of IM.

**Apoptosis**

*DOXO*. In the hearts treated with DOXO, the apoptotic death was 7-times at 3 weeks and 4-times at 6 weeks compared to control hearts. An increase in the fraction of cardiomyocytes

expressing p16INK4a, a marker of senescence, was also observed, that was 2-fold after 3 weeks and 3-fold after 6 weeks. More than 70% of the LV myocytes were positive for p16INK4a to 6 weeks.

*IM*. The treatment with IM50 has led to an increase of 3-times of apoptosis in cardiomyocytes compared to controls, after 3 weeks of treatment. The fraction of myocardial cells in toto that were undergoing apoptosis was increased in a dose dependent manner.

**Cardiac Progenitor Cells**

*DOXO*. Compared to control hearts, in DOXO treated heart an increased apoptosis in the CPC was seen of 5 times and 8 times at 3 and 6 weeks, respectively. Also the fraction of CPC positive for p16INK4a resulted increased, suggesting an irreversible growth arrest. In contrast, the percentage of CPC positive for Ki67 was seriously reduced by the treatment. These results are consistent with the increased oxidative stress and DNA damage promoted by DOXO, as documented by the generation of 8-OHdG in the nuclei of the CPC. Acting on apoptosis and senescence, the treatment with DOXO was able to reduce the compartment of functionally competent CPC in LV of 79% and 94% at 3 and 6 weeks, respectively (fig. 20A).

*IM*. Even treatment with IM resulted in a significant reduction of 25 % in the number of CPC kit[pos] in left ventricular myocardium (fig. 20B).

**Vascular structure**

With the intention to get information on the different potential remodelling in the vascular structures caused by DOXO and IM, morphometric measurements of the arteries, blood and lymphatic vessels was performed, using antibodies that specifically recognize smooth muscle cells (a smooth muscle actin), blood (Factor vW) and lymphatic (LYVE1) endothelial cells.

*DOXO*. Treatment with DOXO did not cause a substantial change in the arteriolar density compared to control hearts, while the density of lymphatic vessels resulted 1.7 times increased (fig. 21A).

*IM*. Doses of 50 mg/kg induced a reduction of both arterioles and lymphatic vessels, of 30% and 70% respectively (fig. 21B and C). The capillary density was reduced of 1.8-fold with lower doses and of 2.7-fold with higher doses (p <0.05 vs. CTRL).

## IN VITRO STUDIES ON RAT CELLS

### Rat cardiomyocytes

*DOXO*. We have not performed the same analysis after exposure of cardiomyocytes to DOXO because repeatedly published by several groups who have described the direct damage caused by the drug in vitro.

*IM*. The cardiomyocytes was separated enzymatically from the rat heart and exposed for 24 hours at doses of 5 and 50 μM of IM. Morphological alterations were observed only with

higher doses of the drug. The evaluation of adult cardiomyocytes could be performed only in the short term, for this reason their number provides only limited information on the effect of IM on these cells.

TUNEL test was performed on cardiomyocytes to detect the presence of apoptosis, after in vitro treatment with IM. A significant dose-dependent increase in the percentage of apoptotic cardiomyocytes after 24 hours of exposure to IM was found (1.17 times IM5μM and 3.39 IM50μM vs CTRL).

**In vitro effects of DOXO and IM on CPC**

To verify the in cico observations, which showed a reduced number of progenitors in the hearts of animlas, the in vitro effects of DOXO and IM on CPC isolated from rat was tested.

*DOXO*. c - kit$^{pos}$ CPC isolated from rat heart were treated with concentrations of 0.1 μM, 0.5 μM and 1 μM of DOXO for 12 , 24 and 48 hours , and cell viability by MTS test was assessed.

Any effect on survival has been shown at 0.1 μM concentration.

A reduction in the vitality of 24% and 33% at 24h and 66% and 90% at 48 hours was seen at the concentrations of 0.5 μM and 1 μM, respectively.

The impact of DOXO on proliferation of CPC was determined by immunofluorescence for BrdU and PH-H3 . The reduction of number of BrdU$^{pos}$ and PhH3$^{pos}$ cells correlate with increasing dose of DOXO, demonstrating a dose and time dependent response.

*IM*. CPC were exposed for 24 hours to drug concentrations of 5 μM and 50 μM. IM concentration of 5 μM does not significantly influence the number of CPC, while IM 50 μM

reduces of 4.5 times the number of cells in culture. So, only toxic doses of IM are able to change in the short term the number of CPC in vitro.

**Effects of DOXO on apoptosis of CPC.**

Apoptosis has been evaluated by TUNEL test, DNA laddering and activity of caspase 3. All these indicators documented induction of apoptosis in a time and dose dependent with a peak after 48 hours of treatment with DOXO 1 μM. The TUNEL assay was limited by the presence of adherent cells after a 48-hour treatment with DOXO 1 m M. The number of adherent CPC  has been reduced by 90%, indicating that the drug has promoted apoptosis of nearly all cells .

**Effects of DOXO on oxidative stress of CPC.**

The oxidative stress on the CPC has been evaluated by detection of 8-OHdG in nuclear level with immunohistochemistry and confocal microscopy. Treatment with DOXO has resulted in a considerable increase in the number of CPC positive for 8-OHdG. The espression of antioxidant enzymes Mn SOD, Cu/Zn SOD and catalase were not modified, while the activity of these enzymes decreased significantly to 48 hours, resulting in a deficiency of the systems able to repair the DNA damage caused by ROS.

**Effects of DOXO on telomerase length of CPC**

DOXO lead to a shortening of telomeres average of 30% and a left shift in the distribution curve of telomere length. The percentage of CPC with telomere length <8 kilobase pairs

(kbp) was increased by 4 times with the administration of DOXO. This shortening occurred despite the presence of telomerase activity in the CPC.

**Effects of DOXO con DNA rapair and senescence of CPC.**

The dysfunctional telomeres trigger a DNA damage response in which the decisive factor is the transcription of p53. The protein kinase ATM is required for the phosphorylation of p53 on serine 15 : the ATM protein and p53 phosphorylated are upregolated in the CPCs treated with DOXO .

The expression of ATM kinase has reached a peak at 12 hours, while p53 phosphorylated increased especially at 12 and 24 hours, and remained elevated at 48 . The phosphorylation of p53 activates a cascade of post-transcriptional modifications that activate transcription of target genes , followed by the activation of apoptosis or cellular senescence. In our study, phosphorylation of p53 was associated with an increased but transient expression of p21Cip1 , which could represent an attempt to promote DNA repair. In addition, an increase of the apoptotic proteins Bax and Bad was observed in CPC treated with DOXO. The sustained upregulation of p16INK4a in CPCs is consistent with the role of this protein in the modulation of the irreversible growth arrest and cell senescence .P16INK4a is rarely associated with double-strand breaks in DNA and represent a delayed response , which follows the induction of p53 and p21Cip1 .

So, the anthracyclines promote oxidative stress and the activation of p53 with the inhibition of growth and survival of the CPC .

**Effects of IM on autophagy**

Cell turnover in the myocardium of animals treated with IM, measured by the rate of mitotic

and apoptotic index, can not fully explain the severe impairment of ventricular function following the systemic administration of the TKI .

To investigate alternative ways of response of myocardial cells to IM, the expression of genes involved in autophagy (beclin -1, LC3 , mTOR) and its relationship with apoptosis ( Bcl -2) and DNA repair (ATM and ATR) have been studied (fig. 22 and 23). For this reason, RNA extracted from human CPC and from K562 cells exposed for 12 hours to IM was studied by RT-PCR .
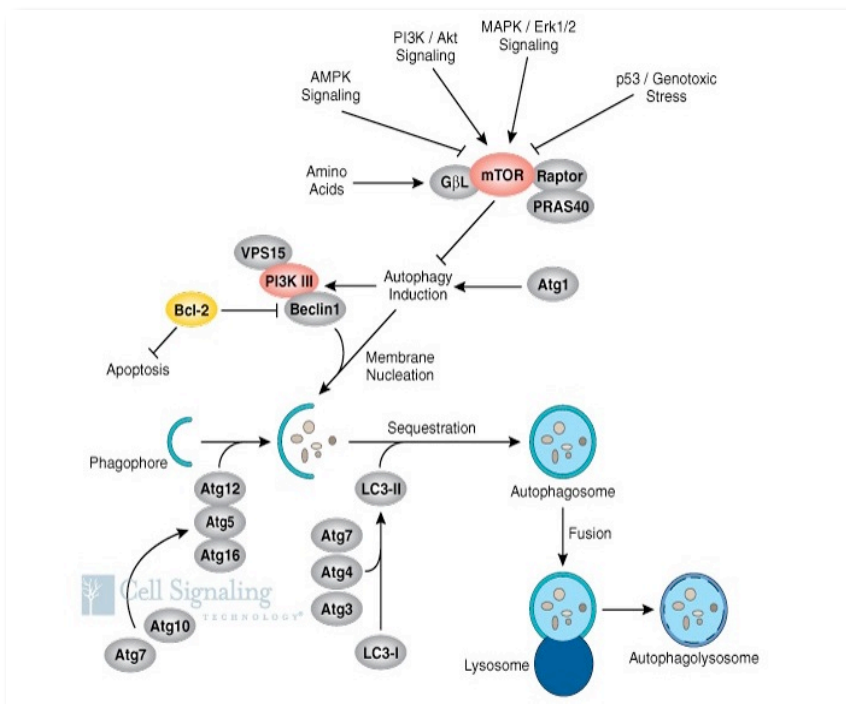


*Image 4. Pathway implicated in autophagic process.*

We documented that the CPC, in response to therapeutic doses of IM (5 μM) , activate transcription of Beclin -1, LC3 and mTOR pathways involved in autophagy. In the same

conditions the CPC does not seem to trigger the response mediated by the anti-apoptotic Bcl -2.

In the leukemia cell line K562 that possess molecular target of IM, the treatment with the drug induces a significant transcription of genes implicated in the autophagy at low doses (1 μM) in the absence of overexpression of Bcl -2 .

We don't have at the moment definitive data on the involvement of autophagy in response to DOXO CPC (fig. 24). However, we tested the drug-sensitive breast cancer line (MCF7). After 12 and 24 hours of treatment in vitro we have seen an extensive toxicity, for this reason we have reduced the time of exposure to 3 hours. We thus documented that autophagy is not involved in the cytotoxicity of anthracyclines. Any concentration of DOXO lead to transcription of Beclin - 1 , LC3 and mTOR, while doses of 0.5 uM determined a marked activation of Bcl -2 .

The analysis of the genes involved in response to DNA damage have shown that the two main protagonists of this cellular function were differently modulated in CPC and K562 exposed to IM . In fact, while ATR was almost exclusively overexpressed in CPC with values  30 - times higher than in the control , in the K562 cells the transcription of ATR and ATM increased of 12 -fold and 16 - fold, respectively.

For the comparison with DOXO, we have only data relating the line MCF7. It was especially interesting to note that intermediate doses of DOXO (0.5 μM) induced in tumor cells high transcription of ATM , coinciding with the documented increase of Bcl -2 involved in the response to apoptosis.

# 5)    PREVENTIVE APPROACHES IN DRUG RELATED CARDIOTOXICITY

**CONVENTIONAL APPROACHES**

In order to find therapeutic strategies to treat or better prevent drug-related cardiotoxicity several approaches and drugs have been tested, like different rate of administration, medication with known positive effects on heart remodelling, or drugs with antioxidant effects.

Concerning the rate of administration, a prolonged infusion therapy has been tested for DOXO to reduce the peak serum concentrations that seems to be responsible of toxicity. Even if this practice has become standard, a randomized trial showed that a continuous doxorubicin infusion over 48 hours did not offer a cardioprotective advantage over the bolus infusion, increasing, on the other hand, hospitalization and costs, and creating additional stress. Moreover, both ways of administration caused cardiac abnormalities.

Certain cardioprotective medications have demonstrated benefit in ameliorating the cardiotoxic effects of anthracyclines [46]. A pharmacological approach has been made with early treatment with enalapril, an angiotensin converting enzyme inhibitor (ACE-I), in patients with evidence of myocardial cell injury after chemotherapy. However, the development of cardiotoxicity and associated adverse clinical events [47,48] noted in childhood cancer survivors treated with anthracycline, there is no evidence for long-term therapeutic benefits of ACE-I [49, 50, 51, 52,53.] The improvement was transient and primarily related to a decreased diastolic blood pressure, which was identified as the only effective benefit of ACE-I. There was no prevention in the lack of progression of LV dysfunction and wall

thinning.

A significant interest has been rised by dexrazoxane for cardioprotection during anthracycline chemotherapy [54]. Dexrazoxane is a *bis*-ketopiperazine, which diffuses in cardiomyocytes and hydrolyses leading to the formation of a diacid-diamide with strong iron-chelating properties. As it is known that iron potentiates the toxicity of anthracycline-derived reactive oxygen species (ROS) and that secondary alcohol metabolites further deregulate iron movements in the cell, an iron chelator like dexrazoxane would be the perfect candidate to mitigate anthracycline cardiotoxicity. Although the cardioprotective effect of dexrazoxane has been documented [55] neither a consensus agreement nor high level of recommendation has been reached. Moreover, one clinical trial has suggested its possible interference with objective tumor response [56] pinpointing to an important issue related to the difficult goal to preserve normal cells from the desired toxic action of antineoplastic therapy on tumor cells. No data are available about the long-term effects of dexrazoxane so that there is still no evidence of improvement in rate of survival for childhood cancer.

In light of the growing number of cancer survivors, there is a great need of identifying effective interventions after anthracycline treatment that would result in evidence-based recommendations [57]. To mitigate the cardiotoxic risks associated with anthracycline, studies are in progress to examine the efficacy of nonanthracycline-containing regimens in the adjuvant setting or better the efficacy of alternative composition of DOXO, like pegylated or liposomal forms. For example, liposomal anthracyclines have been developed to reduce DOXO cardiotoxicity while preserving its antitumor efficacy. The changes in tissue distribution of liposomal anthracyclines lead to less drug exposure in sensitive organs. Also, the release of the drug is slow, which may avoid high peak plasma concentrations [58]. Pegylated liposomal doxorubicin can improve cardiac safety. In one

randomized control trial, 509 patients with metastatic breast cancer received cumulative doses of at least 500–550 mg/m2. The overall risk of cardiotoxicity was significantly higher with DOX than with the pegylated liposomal form in the presence of similar overall survival [59]. Finally, clinical trials are undergoing to determine whether a molecular modification of DOX, consisting in an albumin-binding prodrug, effectively improve anti-tumor efficacy and reduce toxicity [60].


**POTENTIAL PREVENTIVE AND THERAPEUTIC APPROACHES**

Due to the absence of conventional drugs able to treat or prevent cardiotoxicity, it appears to be necessary to find new preventive strategies. Innovative strategies based on novel mechanisms to prevent or protect patients from developing cardiomyopathy are proposed. Starting from the idea that chemoterapic drug affect stem cells, new strategies based on mobilization of progenitor cell pool of the Bone Marrow and stimulation of the cross talk between BM and heart and the engraftment of circulating progenitor cells in damaged organ could be proposed.


The first step in this modelled treatment is based on the possibility to increase the engraftment of progenitor cells in targeted tissue. This approach has to be assembled to increase the number of progenitor cells in damaged tissues without reducing number of circulating progenitor cells in the presence of cytotoxic drugs. A potential advantage in cardiac repair may be tested in patients because all the compounds indicated in the scheme are clinically tested.

This proposed model and the possible mechanism of action of compounds used are briefly described.

After 3 days from chemotherapy, Progenitor Cells mobilizers, as G-CSF or AMD3100, are given to stimulate progenitor cells to circulate and reach the sites damaged by chemotherapy.

Growth factors able to expand the progenitor cell pool like Growth Hormone (GH) or Stem Cell Factor (SCF) could be also used in an attempt to increase the number of stem cells present in both the bone marrow and target organs.

This hypothetic model is build to protect progenitor cells from the cytotoxic insult of drugs and allow tissue repair.

The use of G-CSF and AMD3100 is based on the role of Stromal Derived Factor-1 (SDF-1) and Tissue Committed Stem Cells system. The chemokine SDF-1/CXCR4 receptor system plays a critical role in the homing of Tissue Committed Stem Cells (TCSCs), as previously demonstrated for Hematopoietic Stem Cells (HSCs). The expression of the CXCR4 receptor on HSCs and TCSCs shows a crucial role in their homing. Thus, mutant mice in CXCR4 and SDF-1 have defects in heart muscle and large vessel development, indicating the relevance of the SDF-1/CXCR4 axis in organogenesis and stem-cell trafficking. SDF-1 is secreted in the heart representing the most potent chemoattracting gradient for circulating CXCR4+ TCSCs. The secretion of SDF-1 increases during tissue damage, such as during ischemia, playing an important role in chemoattracting TCSCs, that is necessary for tissue regeneration. Since SDF-1 may attract CXCR4+ stem cells, the diseased heart may contain sites of homing of CXCR4+ CSCs but also empty spaces to accept circulating CXCR4+ SCs. Interestingly CXCR4+ TCSCs, similarly to CXCR4+ HSCs, circulate in the peripheral blood and compete for SDF-1-positive niches in various organs. Thus, circulating TCSCs can be triggered by myocardial injury. However, complete identification of the factors secreted by the damaged organs to favor the engraftment and function of regenerating cells

requires more detailed experimental observations. Whether the SDF-1/CXCR4 system is altered in drug induced cardiotoxicity is an important unanswered question. Primitive changes in the chemoattractive properties of the heart by drug-induced cardiomyopathy and/or in the sensitive pool of BM and circulating cells may be operative in the onset and development of cancer treatment cardiotoxicity. The SDF-1/CXCR4 is an important interaction for movement into, retention within and survival of HSC and HPCs in the bone marrow microenvironment. Modulating this interaction has allowed for enhanced mobilitation or homing/engrafment.

*Sitagliptin*

CD26 is a cell-surface protein which is a dipeptidylpeptidase IV (DPPIV). DPPIV has the capacity to truncate SDF-1/CXCL12. The truncated SDF-1/CXCL12 is both inactive as a chemotactic molecule, and also blocks the chemotaxis of full-length SDF-1/CXCL12. Inhibition of CD26, by specific small peptide inhibitors, enhances chemotaxis of HSCs/HPCs to SDF-1/CXCL12. This treatment also enhances homing of HSCs in vivo in lethally irradiated mice in competitive and noncompetitive HSC assays. That means that this drug could increase the chemoattraction of circulating progenitor cells to the different organ, increasing the secretion of SDF1.

*AMD3100 (Plerixafor)*

AMD3100 is a specific antagonist of CXCR4, that interfere with the SDF1/CXCR4 axis specific, leading to an increased cell mobilization. Commercially available Plerixafor

injection in combination with granulocyte-colony stimulating factor (G-CSF) increase the efficacy in mobilizing hematopoietic stem cells to the bloodstream, where they can be collected. Autologous transplantation requires at least 2 million stem cells per kilogram of body weight, which may take 3 or 4 hours during multiple days in some patients, and other patients are not able to reach this goal. Pivotal phase 3 trials showed that the number of stem cells mobilized for collection was significantly increased in patients receiving plerixafor vs current standard of care, and that the time needed for patients to mobilize sufficient numbers of stem cells for autologous transplantation was decreased. The target number of at least 5 million stem cells/kg body weight in 4 or fewer apheresis sessions was achieved in 59% percent of patients with NHL who received plerixafor and G-CSF vs 20% of patients who received placebo. The median number of days to reach this target stem cell count was 3 days for patients receiving plerixafor and was not evaluable in the placebo group.

*Dipyridamole*

Dipyridamole is an old drug, with an antiaggregant action, and used, for this specificity, in thromboembolism and ischemic ictus. Its cardioprotective effect is mediated by action on vasodilation and it also has an have an antiarrhythmic efficacy in treatment of reperfusion arrhythmias[61]. Finally, it's also acts as adenosine deaminase (ADA) inhibitor, acquiring pro-mobilizing action. It could have a double action in preventing or attenuating the cardiotoxicity drug related: one mediated by the action on ADA system and then on the crasstalk betwen organs and the other mediated by the antioxidant

*G-CSF*

Granulocyte colony-stimulating factors (CSF) are largely used in supporting chemoterapic treatment, to prevent febrile neutropenia, reducing the collateral effect of cytotoxic therapy, like bacterial or micotic infections. They stimulate the proliferation of white blod cell progenitors in Bone Marrow, in order to reduce the leukopenia post chemoterapic treatment.

*Statins*

Statins are inhibitors of the HMG-CoA enzyme provided by a lipidic lowering action. Because of the recent knowledge that these drugs activate mitochondrial-located antioxidative and antiapoptotic mechanisms improving LV function Raid et al[62] have investigated their ability to reduce cardiac toxicity of doxorubicin. They showed that pretreatment with statin improved LV function independently of direct effects on loading conditions, because statins influence neither preload nor after load, and they do not exert any inotropic effect. These data suggest that attenuation of cardiac nitrotyrosine expression, mitochondrial protection, and cardiac inflammatory response due to statin treatment may well contribute to improve heart function. Thus, the action of these drugs on the heart is also independent from their lipidic lowering action.

*Beta blockers*

Beta blockers are the most used drugs in the cardiac disease, above all when LV dysfunction is present, and, for this reason, they have been the first class of drugs implicated in the prevention of chemotherapeutic-related cardiotoxicity.

Regarding the pathogenetic mechanism of DOXO, there are several hypotheses including free oxygen radicals, apoptosis, mitochondrial dysfunction, and activation of matrix metalloproteinases. Free radical formation is generally accepted as the main mechanism. Cardiomyocytes have poor antioxidant defense systems, and free oxygen radicals can damage various targets in the cell. This may result in impairment of cardiac contractility and the development of cardiomyopathy. Betablockers, and in particular carvedilol, that blocks beta1, beta2-, and a$_1$-adrenoceptors, have been already indicated in treatment of DOXO-induced cardiomyopathy, to cease further deterioration in left ventricular function and to improve symptoms. Recently, it has been shown, how beta blockers could be used in the progression of cardiotoxicity at the early stage[63]. This ability is probably due to their potent antioxidant and antiapoptotic properties. Recent animal studies and experimental observations showed that carvedilol prevented the development of cardiac dysfunction, by antioxidant mechanism and inhibiting apoptosis in cardiomyocytes due to chemotherapy [64,65,66]. The action of this drug on the heart, documented by several reports, make imperative to test its protective effects in chemotherapy-related cardiotoxicity.


*GH*

The use of GH has been already proposed to prevent cardiotoxicity. GH exerts its effect on the heart through Insulin-like Growth Factor 1 (IGF-1), to mantain an adequate LV mass. GH deficiency is a common problem in cancer survivor children. Lipshultz et al. conducted a study where [67] GH-treated children (cancer survivors with a developed GH-deficiency) were compared to a control group (untreated cancer survivors). The LV wall was thinner before GH therapy and increased significantly during 3-years of treatment. GH therapy was

also able to increase LV mass, but did not decrease afterload. This beneficial effect was rapidly lost after discontinuation of therapy and, 4 years after, no significant difference was observed between those who received GH and the control group. This study concluded that no evidence of an effective persistent benefit of GH therapy was found. However, the study did not assess whether GH therapy may reduce the global cardiovascular risk in children treated with anthracycline. Indeed, a wide variability of the effects of GH therapy was present: there were patients who did not tolerate GH and developed cardiac failure leading to heart transplantation and other who actually showed a less dramatic cardiac deterioration, no deterioration, or even improvement in cardiac function. Though, many issues remain to be resolved before this approach will be totally dismissed.

It has been reported that GH therapy induces only myocyte hypertrophy and that LV wall thickness and mass are not restored because the number of cardiac myocytes is reduced. However, studies suggested that GH treatment results in myocardial growth by increasing capillary density, reducing the rate of apoptosis, and increasing myocardial cell number and size [68]. Additional studies have shown that an increase in cardiac myocyte number was possible only in young rats, while myocardial cell hypertrophy was the predominant mechanism of GH-induced LV growth in older animals[69].

GH also acts on resident cardiac stem/progenitor cells through IGF-1 receptor present on this population stimulating them to proliferate, differentiate and replace lost cardiomyocytes. This hypothesis is strongly supported by the observation that systemic or local IGF-1 activation promotes myocardial regeneration. Intriguingly, cardiac stem cells also express c-met the receptor for Hepatocyte Growth Factor (HGF) and it has also been shown that locally delivered HGF may have beneficial effect on DOX-induced cardiomyopathy.

Another potential mechanism implicates a GH mediated proliferation of BM progenitors

that, upon peripheral blood mobilization, home to the injured heart where by angiogenic and differentiating properties may repair anthracycline-induced cardiac damage. The demonstration that GH is able to expand BM stem cells in animals and humans and that circulating progenitors, by sensing cardiac injury, are recruited at the site of damage strongly favours the possible involvement of BM cells in DOX-induced cardiotoxicity. Thus, according to these working hypotheses, an increase in LV mass by GH therapy may be explained by the generation of new individual cardiomyocytes derived from BM or resident cardiac stem cell and their reparative properties.

*Stem Cell Factor and Cardiac Stem Cells*

There is increasing support for the presence of an endogenous population of $Lin^{neg}$ $c\text{-}kit^{pos}$ cardiac stem cells that proliferate in response to injury and that may be targets for novel approaches of activation. There is also evidence that cardiac stem cells, depleted after myocardial insults, are replenished by both endogenous proliferation and mobilization of bone marrow–derived stem cells. Administration of Stem Cell Factor (SCF) and G-CSF mobilizes pluripotent $Lin^{neg}$ $c\text{-}kit^{pos}$ cells from the bone marrow (BM) to the peripheral blood determining a 250-fold increase in the number of circulating $Lin^{neg}$ $c\text{-}kit^{pos}$ cells. Donor BM cells injected intravenously home to injured organs including heart, liver and skeletal muscle . Because of previous positive results of the direct intramyocardial injection of $Lin^{neg}$ $c\text{-}kit^{pos}$ cells, in infarcted mice treated with SCF and G-CSF it was demonstrated that $Lin^{neg}$ $c\text{-}kit^{pos}$ cells are responsible for cardiac repair. However, these results do not provide unequivocal demonstration of the origin of the cells reconstituting the myocardium. It could be likely that cytokine treatment mobilized bone marrow stem cells and resident

cardiac stem cells, which together participated in tissue regeneration. Thus, studies are required in an effort to document the plasticity of mobilized progenitor cells in a non ischemic cardiac insult such as after antineoplastic drugs.

The efficacy of cytokine treatment starting 5 days before myocardial infarction raises the question of the most effective therapeutic window. Additionally, it is not clear whether tissue repair is a result of the activation of resident CSCs or whether BM cells, once mobilized, randomly reach target organs and only when homed to the damaged myocardium rapidly proliferate and transdifferentiate. The former possibility is more attractive and supported by the rapid induction of SCF in a number of tissues, including the myocardium, in response to injury.

Thus, the hypothesis advanced is that SCF, by increasing the number of resident c-kit$^{pos}$ cells, may protect the heart form the cardiotoxic effect of antineoplastic drugs.

Sperimental data that support this hypothesis have been shown by Deangelis et al.

More knowledge is needed in order to confirm this new fascinating mechanism potentially exploitable to prevent or attenuate cardiotoxicity by regenerative approaches. All patients who received chemotherapy at any age, should be educated about the secondary prevention for cardiovascular diseases, since a healthy life-style may be helpful to prevent or decrease cardiotoxicity. Although the influence of a modified healthy diet on cardiotoxicity is limited ideal body weight should be maintained, since obesity is a major preventable coronary risk factor and is related to increased cardiovascular mortality and morbidity. Moreover, additional risk factors, such as diabetes, endocrinopathies, hypertension, or alcohol consumption, drug abuse and cigarettes smoking should be minimized.

Aerobic exercise may also be helpful, although patients should be tested to ensure a stable

cardiovascular function before starting a physical activity. A 12 weeks, hospital based physical activity program reduces body fat and improves general clinical conditions. Importantly, physical activity may be beneficial in some cancer survivors but harmful in others. Beside these studies, it is our opinion that aerobic exercise should be more critically evaluated for the early prevention of cardiotoxic complications. Indeed, a moderate pre-treatment training program may physiologically induce a better myocardial perfusion and a reduction in afterload, thus positively conditioning the heart, which has to support the subsequent adaptations to repeated drug-mediated cardiac insults. Interestingly, it has also been demonstrated that a long- and short-term voluntary physical exercise up-regulates cardiac telomere-stabilizing proteins and thereby induces anti-senescence and protective effects. These beneficial cardiac effects, mediated by TERT, eNOS, and IGF-1, may be exploited to prevent doxorubicin-induced cardiomyopathy.

## FUTURE PRECLINICAL DIRECTION

As underlied previously in the discussion, the first step in protection of cardiotoxicity could be the development of precocious detection of cardiotoxicity. Thus, the eventual goal could be the individuation of biomarkers able to determine which patients who could easily develop a chemotherapic drug related cardiomyopathy.

So far, there are no guidelines for oncologists (or cardiologists) that address how to approach patients who will be treated with cancer therapeutics. Strategies for follow-up do

not exist, and approaches involving treatment of patients who develop left ventricular dysfunction generally rely on guidelines written for patients with more traditional forms of heart failure. There is, however, a significant amount of interest in this issue, and position papers are beginning to appear [77]. Second, although recovery of left ventricular function has been seen in patients treated with trastuzumab and sunitinib, it is crucial to follow patients who have developed cardiotoxicity for longer periods of time. This lesson was learned from the studies on adult survivors of childhood cancer, in whom early recovery of left ventricular function was followed by significant and persistent declines in function [39]. Although it appears that the cardiotoxicity of kinase inhibitors may be intrinsically different to the cardiotoxicity resulting from anthracyclines or radiation, that conclusion can only be drawn after longer-term follow-up of patients.

*Imaging studies.* EF is a very insensitive marker of cardiotoxicity because of the multiple compensatory mechanisms that can be recruited to maintain contractile function. Thus, EF can be maintained even when the intrinsic contractile function is depressed. Therefore, determinations of EF may only detect those patients whose cardiovascular compensatory mechanisms are already compromised by co-morbidities (for example, CAD or uncontrolled hypertension). Furthermore, lveF determinations do not detect heart failure with normal systolic function (previously termed diastolic dysfunction), which is a major cause of admissions to hospital for heart failure. There is some evidence that tissue Doppler imaging/strain rate may be more sensitive than EF, but the true utility of that approach remains to be determined 78.

*Biomarkers.* A few cardiovascular safety biomarker studies have been conducted, with troponin I emerging as a potential candidate. elevations in troponin I levels are the major biomarker for cardiomyocyte necrosis in acute coronary syndromes**.** New technology now allows us to detect serum levels of troponin I in the picomolar range, but the significance of small, transient drug-related changes in troponin I is not clear [79,80]. That said, elevations appear to be predictive of cardiotoxicity with anthracyclines[81]. In one study involving patients receiving high-dose chemotherapy, an early elevation in troponin I predicted future left ventricular dysfunction, and treatment of troponin I-positive patients with an angiotensin-converting enzyme inhibitor led to better preserved left ventricular function82.

Although this is encouraging, it is not clear that using troponin I as a biomarker of cardiotoxicity to follow patients receiving cancer therapies that are less cardiotoxic (including kinase inhibitors) will be as predictive. For example, in the study of sunitinib in gastrointestina stromal tumours, troponin I elevations did not predict which patients would go on to develop CHF or significant declines in lveF6. by contrast, Cardinale *et al.* found that a troponin I elevation was the best predictor of trastuzumab-induced cardiotoxicity (the hazard ratio was 22.9) and also predicted those patients in whom ejection fraction (eF) would not improve after stopping drug treatment (the hazard ratio was 2.88) [83]. It is our view that in the long-term monitoring of patients taking kinase inhibitors, using biomarkers with increased sensitivity will increase the false positive rate. Although the above studies are encouraging, the value of using troponin I as a biomarker in the monitoring of patients taking kinase inhibitors chronically is unclear.

Biomarkers of heart failure (for example, the various measurements of B-type natriuretic peptide) are also being explored, but it is currently unclear whether these biomarkers will be prognostic or whether they will be able to identify patients significantly earlier than clinical

examination. That said, given the shared symptom complexes of dyspnea, fatigue and peripheral oedema in both cancer and heart failure, it is likely that b-type natriuretic peptide will be of clinical value in sorting out the individuals who have heart failure from those who do not. However, it is unclear whether such biomarkers will be of value in preclinical studies and/or have sufficient credibility for regulators in preapproval trials to identify agents that have the potential to cause cardiotoxiticy.

## MENAGEMENT OF CHEMOTHERAPY INDUCED CARDIOMYOPATHY

There is a notable absence of large scale randomized controlled trial data in treating cardiomyopathy and HF in patients who have received chemotherapy—not least because of their exclusion from HF trials. Certainly the longer the gap between the end of chemotherapy and the initiation of HF treatment in patients identified as having developed anthracycline induced cardiomyopathy, the less likelihood there is of recovery of LV systolic function, reinforcing the need for prompt cardiac assessment and management. If current guidelines are applied for the general management of HF as recommended, asymptomatic patients receiving chemotherapy without LV dysfunction (as an at-risk group) should have optimal blood pressure control, as well as control of other HF risk factors such as obesity, diabetes mellitus or insulin resistance, and smoking. Asymptomatic patients with reduced LVEF have been shown to have higher rates of cardiac events and should receive ARB/ACE inhibitors and β-blockers to prevent the development ofsymptomatic HF. The current European Society of Medical Oncology (ESMO) guidelines go further in recommending enalapril therapy for patients who are have a raised troponin I during

anthracycline chemotherapy. In the absence of troponin measurement the authors have suggested 3-monthly surveillance echocardiography for the first 12 months and annually thereafter. In addition to the ESMO guidance it would be suggested that patients at increased risk—such as those who have previously received high cumulative anthracycline doses (eg, in excess of 550 mg/m2 of body area), previous thoracic irradiation involving cardiac exposure or those who were treated with anthracyclines in early childhood—should be monitored closely, even if troponin titres are not raised. This should include 3-monthly evaluation to elicit signs or symptoms of HF, as well as echocardiographic assessment of LV systolic function. When the LVEF is still ≥40% there is less consensus; it has been suggested that appropriate HF medication should be considered, chemotherapy withheld if anthracycline based, and a further assessment of LV systolic function should be performed in 3 weeks. Reduction in LVEF to <40% with either anthracycline or HER2 based treatments necessitates appropriate HF treatment and cessation of chemotherapy. Patient with signs and symptoms of chemotherapy induced cardiomyopathy should be treated as per HF guidelines and receive ARB/ACE inhibitors, β-blockers, and both loop diuretics and aldosterone antagonists as required.

Referral to a specialist HF team can also improve patient education, which has been shown to improve hospital stay, time to hospitalisation, costs, and mortality. There is a strong argument that such co-care should be considered in all patients with chemotherapy induced cardiomyopathy [70].

# 6) CONCLUSION

Understanding the different pathogenetic mechanisms that underlie the cardiac abnormalities related to chemoterapic drugs is essential to understand what may be the targets of toxicity and of preventive and therapeutic strategies.

Anthracyclines act with different mechanisms: oxidative stress, DNA damage, modification of adrenergic system and of regolation in intracellular calcium and iron. DOXO could affect the cells in proliferation, without specificity or target molecule. It could affect cardiomyocytes, that because of their contractile activity continuously need a large amounts of ATP and a vascular supply, that can be compromised by therapy. The direct effect of anthracyclines on cardiomyocytes has been amply demonstrated. The morphometric assessments, in human and rat, showed, as expected, the formation of fibrotic foci in response to the necrotic damage determined by the drug on cardiomyocytes. Another hypothesis is that these foci are the result of vascular ischemic events induced by the drug. The damage of anthracyclines is also expressed on non-myocyte compartments of the heart: in vivo and in vitro studies have shown how the repairing ability of human endothelial and lymphatic cell lines is profoundly affected by the drug. The loss of cardiomyocytes, the presence of fibrosis that is formed in response to the necrotic damage and reduction of cardiac function lead to an impairment of LV with a reduction in ejection fraction. Particularly interesting is the observation that these changes worse over time. Heart failure could also arise years after discontinuation of therapy, and this fact is probably related to an irreversible damage.

On the other side we have the TKI that are target drugs, with a mechanism of action independent of the cell cycle phase and with a selectivity of action that allows precise molecule to interfere with the signal. Because of its specificity of action, this class of drug theoretically have a reduced cardiac toxicity. Although the incidence is much lower compared to anthracyclines, there are evidences about toxicity on heart related to TKI.

Studies in vivo and in vitro performed in our laboratory have shown that also imatinib have a toxic effect on cardiomyocytes, on endothelial and lymphatic compartment and that lead to a restrictive type remodeling of the heart. If fibrosis plays an important role in doxorubicine related cardiomyopathy, it doesn't seem to play a determinant role in IM cardiotoxicity. The toxic effect of IM on cardiomyocytes is explained based on the fact that the drug induces alterations at mitochondrial level, with consequent deficiency of ATP in cells with high energy consumption. This alteration can lead to cell death.

The quantification of apoptotic and proliferative index has documented an increased turnover in animal heart treated with low doses of IM , while higher doses prevent myocardial cellular response to the drug. This finding lead to the hypothesis that low doses of IM may induce repetitive injury (due to the daily administration of the drug) that is reversible, whereas higher doses don't let the heart to repair the damage.

In general, the documented increased cell turnover (human tissue) characterized by high level of cell death and proliferation likely reflects the attempt of the heart to repair the damage produced by chemotherapy .

Our hypothesis is that chemotherapic drugs have a toxic effect on the stem cell compartment and its ability to repair heart damage. This hypothesis is supported by the data reported.

About DOXO we mentioned the timing of administration of the drug. It might just be the mode of administration to explain in part the mechanism of damage on the CPC . It's

conceivable that their action is expressed at different times, resulting in damage of the stem cell compartment, expecially when the CPC activate the reparative processes for the damage done by the previous cycle. This effect on CPC can therefore affect their ability to regenerate the loss of contractile tissue, but can also determine an alteration of the physiological turnover of cardiomyocytes which is highlighted in the time. Unfortunately, the timing of onset and latency make it difficult to study the cardiotoxicity of anthracycline - mediated cardiomyopathy in humans.

Even if mechanism of toxicity of IM are still unknown, in vivo and in vitro studies show that this drug also has an action on CPC and that the ability of them to regenerate myocardium is affected by the drug . IM also has a toxic effect on endothelial and lymphatic vascular compartment, and on CPC.

Test performed in vitro and in vivo on CPCs document a reduction in myocardial cells positive for c-kit, which could be caused by a toxic effect related to the inhibition of IM on ABL or c-Kit, the receptor for stem cell factor (SCF).

From a structural point of view, all these modifications lead to changes in hemodynamic parameters and in cardiac anatomy.

Cardiac remodelling in IM is characterized by decreased LV volume and thickening of LV wall and appears to be dose-dependent. The reduction of chamber volume is associated with impaired mechanical function, demonstrated by an increase in left ventricular end-diastolic pressure and a decrease of the ratio dP/dT . The macroscopic structural composition of the myocardium does not change after treatment with IM, there is no evidence of fibrosis and the percentage of the heart muscle does not change. The absence of fibrosis in IM related cardiomyopathy could be explained by the fact that the action of PDGF on myofibroblasts, implicated in the mechanism of fibrosis, is inhibited by IM . Another possible explanation is

that IM does not lead to an inflammatory and necrotic damage, then there is not scar formation.

For this reasons we have investigated other mechanisms of cardiac toxicity induced by IM. Because of the action of the drug on PDGFR, we evaluated the action of IM on arterioles, capillaries and lymphatic vessels. After treatment with IM they resulted strongly reduced. This fact could be explained by inhibition of vascular progenitors expressing PDGFR. The reduction of the lymphatic vessels observed can be explained by the involvement of IM inhibition of PDGFR, a key effector of lymphangiogenesis .

These data are supported by in vitro studies, which show an inhibitory effect against growth of IM , angiogenic capacity and migration capability . The decrease of the arteries and capillaries may reduce the arrival of substrates , tissue oxygenation and elimination of waste substances. It is conceivable that a decrease of lymphatic vessels can lead to a condition of ineffective removal of liquids (with an increase, as documented, of water content) and toxic substances from the myocardium, including the drug itself.

Another mechanism designed for IM cardiomyopathy is the autophagy, a biological mechanism that seems to be involved also in other types of cardiomyopathies. Autophagy is a physiological mechanism, that can be induced under stress conditions. It seems to be an adaptive survival strategy that allows the cell to eliminate senescent and damaged organelles for recycling. However, an overload of this pathway can lead to exhaustion of lysosomal enzymes or their release into the cytoplasm. The research by RT-PCR of mRNA coding for protein involved in this process has shown a high activation of this pathway after treatment with IM, both in hCPC and CML cell line ( K562 ), used as a control. So from a structural point of view cardiotoxic effects of IM are explicable as alterations of homeostasis of the CPC, of the blood- lymphatic angiogenic mechanisms and activation of autophagy .

In conclusion, DOXO and IM are two drugs wth completely different mechanisms of action, pharmacological kinetics and bioavailability, and mode of administration. Their cardiotoxic action is expressed in a different way and with different mechanisms. But it seems that toxic effect that they have on progenitor cells and their damaged capacity in cardiac repairing is common to both drugs.

The role of the CPC in DOXO related cardiomyopatic heart has been highlighted by DeAngelis et al, who have shown how the infusion of isolated human cardiac stem cells in an experimental heart disease induced by DOXO could repair the damage and cardiac function.

Understanding the pathogenic mechanisms is essential in order to identify patients likely to develop cardiotoxicity, leading to the evaluation of variables such as the patient's medical history, which may have resulted in changes of the CPC.

Our findings on human samples, associated to animal models studies that demonstrate the therapeutic effect of cell therapy, open the possibility of isolate and expand autologous CPC to use for therapeutic purposes. In addition to cell therapy may other therapeutic strategies such as the administration of G -CSF and AMD3100 can be taken into consideration , stimulation of progenitor cells, mobilizing them into the bloodstream and promoting the engraftment at sites affected by chemotherapy. In addition, certain growth factors such as growth hormone ( GH) , Stem Cell Factor (SCF) and Hepatocyte Growth Factor ( HGF) are able to expand the pool of progenitor cells and therefore potentially usable for therapeutic purposes.

Even substances that are already used clinically in cardiovascular disease may have an important role in cardioprotection by anti-tumor therapy . For example , the involvement of ROS in the mechanism of toxicity of DOXO does suggest that it is possible groped to

counteract the damage by administering antioxidant agents . Among them, resveratrol seems to be a good candidate for therapeutic use in cardiac toxicity because it has a protective effect on the heart and is involved in the maintenance of functional stem cells.

The use of beta blockers, drugs used in the very heart disease , may be an additional therapeutic trial in counterstand cardiotoxicity . Within this class of drugs has been paid particular attention to carvedilol, also has antioxidant and protective properties against mitochondria. Also statins may protect cardiomyocytes through the activation of NO synthase and the channel of the mitochondrial ATP - sensitive potassium .

These last two classes of drugs may be effective in the heart induced by IM since they protect the mitochondria, which are among the most important targets of the action of tyrosine kinases.

However , our study aims to review the cardiotoxicity from a systemic point of view , focusing on the principle that organ homeostasis is maintained by a pool of stem cells. The anti-cancer strategies can affect the function of the cellular compartments deputies to repair the damage and then therapeutic approaches aimed at preventing this negative phenomenon are able to deal with a problem in an innovative and emerging clinical booming.

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