Doxorubicin *vs.* ladirubicin: methods for improving osteosarcoma treatment

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Abstract: Osteosarcoma is the most common primary bone tumor in children and adolescents, with a 5-year disease free survival rate of 70%. Current chemotherapy regimens comprise a group of chemotherapeutic agents in which doxorubicin is included. However, tumor resistance to anthracyclines and cardiotoxicity are limiting factors for its usage. Liposomal formulations of doxorubicin improve its anti-cancer effects but are still insufficient. The research in this area has lead to the production of anthracyclines analogues, such as ladirubicin, the leading compound of alkylcyclines. This new anticancer agent has shown promising results *in vivo* and *in vitro*, being effective against osteosarcoma cell lines, including those with a multidrug resistant phenotype. In phase I clinical trials, this molecule caused mild side effects and did not induce significant cardiotoxicity at doses ranging from 1 to 16 mg/m^2, resulting in a peak plasma concentration (C_{max}) ranging from 0.5 to 1.5 μM. The recommended doses for phase II studies were 12 and 14 mg/m^2 in heavily and minimally pretreated/non-pretreated patients, respectively. Phase II clinical trials in ovary, breast, colorectal cancer, NSCLC and malignant melanoma are underway. Given the improved molecular targeting efficacy of these new compounds, ongoing approaches have sought to improve drug delivery systems, to improve treatment efficacy while reducing systemic toxicity. The combination of these two approaches may be a good start for the discovery of new treatment for osteosarcoma.

Keywords: Anthracyclines family, doxorubicin, laudorubicin, osteosarcoma treatment.

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor and has a high incidence in children and adolescents, since it accounts for approximately 60% of primary malignant bone tumors diagnosed in the first two decades of life. It is characterized by an extremely aggressive clinical route with rapid development of metastases in 40-50% of patients, occurring mainly in lung [1, 2]. Conventional therapies for osteosarcoma include surgery (frequently amputation), chemotherapy and radiotherapy [3]. Until 1970, osteosarcoma treatment was based on amputation or radiotherapy, and death occurred in a short period of time due to lung metastasis; the 5-year disease free survival rate was about 12%. In 1978 neoadjuvant chemotherapy was introduced with the combination of doxorubicin (DOX), methotrexate (MTX), cisplatin and ifosfamide, significantly improving the clinical results in osteosarcoma treatment [4]. Current neoadjuvant chemotherapy protocols for high-grade osteosarcoma are based on DOX, high-dose MTX, and cis-dichloro-diammine-platinum (CDDP), with the addition of ifosfamide in the post-operative phase, increasing the 5-year disease free survival rate to 70%, in patients without metastasis [2]. However, current treatments for osteosarcoma have not resulted in improved prognosis during the last decade providing incentive for the development of new treatment options [5].

Osteosarcoma is one of the first solid tumors for which adjuvant chemotherapy proved to be beneficial [6]. The currently used drugs include cyclophosphamide, vincristine, melphalan, adriamycin (DOX), MTX, cisplatin, decarbazine, bleomycin, dactinomycin, actinomycin, and leucovorin rescue. The current standard treatment for osteosarcoma includes preoperative chemotherapy followed by surgery and postoperative chemotherapy. Preoperative chemotherapy aims to induce tumor necrosis in primary tumor, facilitating surgical resection and the eradication of micro-metastases. Postoperative chemotherapy, to manage metastases is chosen based on: the initial therapy; the site and the number of metastases or recurrent tumors; the length of the disease-free interval and the type of chemotherapy previously applied to the patients [7]. Although a multidrug regimen is used to treat osteosarcoma, the need for high doses of chemotherapeutic drugs to enhance prognosis remains a problem. Additionally some patients can be treated with radiation but only for radiosensitive tumors.

The major cause of failure of chemotherapeutic regimens is multidrug resistance (MDR). MDR has been correlated

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with multi-factorial processes such as: enhanced detoxification of the drugs through increased metabolism, decreased drug uptake, a reaction with increased levels of intracellular nucleophiles, enhanced repair of the drug-induced damage to DNA, or through overexpression of membrane-bound drug transporter proteins, such as P-glycoprotein (Pgp, ABCB1), multidrug resistance-associated proteins (MRP1, ABCC1 and MRP2, ABCC2) and the breast cancer resistance protein (BCRP, ABCG2) [1, 8].

Solid tumors, including osteosarcoma, consist of a heterogeneous population of cells that differ in their relative states of differentiation [9]. During the last years, the cancer stem cells (CSC) theory emerged as a model to account for the heterogeneity and renewal capacity of tumor cells. The CSC theory postulates that the greater part of a tumor mass contains more differentiated cells that are susceptible to radiation and chemotherapy because of their close vicinity to non- tumorigenic tissues and sufficient blood flow due to induced angiogenesis, or blood vessel growth [9]. In contrast, a small subset of cells with stem-like properties that is responsible for initiating and sustaining tumor growth were termed cancer stem cells because of the properties they share with normal stem cells, including their ability to self-renew and undergo differentiation [10]. Similar to the normal tissue stem cells, in some tumors, the CSCs are believed to reside in less oxygenated areas in a quiescent state. In fact, CSCs have several features that make them naturally resistant to conventional therapies. Most of the drugs used in cancer treatment target DNA and induce irreversible damage leading to cell death. CSCs seem to have enhanced DNA repair mechanisms allowing them to resist do damage induced by conventional therapies [11]. The multidrug resistance trait of CSCs is associated with an overexpression of proteins from the BCL-2 family, which protects CSCs from apoptosis and leads to an increase in expression of membrane proteins responsible for drug resistance [12]. In addition, an increased expression of transporting proteins such as MDR1 and ABC transporters is an important factor in chemotherapy resistance [13].

Recent studies have successfully identified the presence of CSCs in osteosarcoma. Gibbs *et al.* [14] have successfully isolated the CSCs subpopulation from nine established cultures from untreated osteosarcoma biopsies and a osteosarcoma cell line (MG 63) through sphere formation assay. Sarcospheres-derived cells expressed the MSC surface markers Stro-1, CD105 and CD44 and over-expressed embryonic stem cells pluripotency markers (OCT4 and Nanog). Wang *et al*. [15] observed similar results in four more human osteosarcoma cell lines. Murase *et al*. [16] also reported the existence of a subset of CSCs in human osteosarcoma cell lines identified through the extrusion of Hoechst 33324. These cells revealed higher tumorigenic potential *in vivo* and *in vitro*. These findings strongly suggest that osteosarcoma is enriched in cells with stem-like properties and that these cells may be responsible for drug resistance.

The high incidence of MDR in osteosarcoma and the difficulties in its treatment suggest new treatment options. This work firstly reviews a family of chemotherapeutic agents – *anthracyclines* – commonly used in osteosarcoma treatment, and one of its members, *DOX*. Secondly, it reviews a new chemotherapeutic drug, *ladirubicin*, the prototype drug of *alkylcyclines*, used to evade tumor resistance and to improve chemotherapy results.

ANTHRACYCLINES FAMILY

Anthracyclines belong to the group of the most effective anticancer drugs ever developed [17]. The first members of this family were originally isolated from the pigment-producing *Streptomyces peucetius* in the 1960s and were named doxorubicin (DOX) and daunorubicin (DNR) [18].

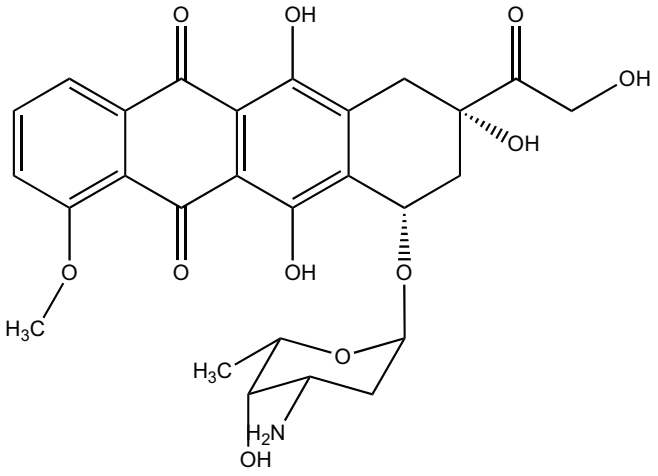

Fig. (1). Chemical structure of DOX.

According to (Figs. **1** and **2**), the only difference between DOX and DNR is the termination of side chain. DOX terminates with a primary alcohol whereas DNR terminates with a methyl group. Consequently, DOX is active against breast cancer, childhood solid tumors (like osteosarcoma), soft tissue sarcomas, and aggressive lymphomas while DNR is more active against acute lymphoblastic or myeloblastic leukemia [17].

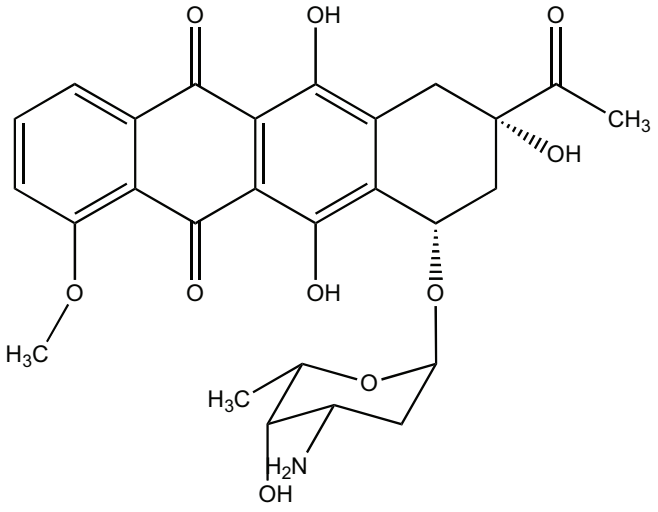

Fig. (2). Chemical structure of DNR.

The major drawbacks of these compounds are their cardiotoxicity leading to congestive heart failure and the development of spontaneous and acquired resistance. Intensive research to find analogues that circumvent these problems lead to the development of more than 300 new compounds, whereas more than 2000 analogues were issued from structural modifications of natural compounds or from synthesis [12]. However, from those only few members of the anthracycline family have reached the stage of clinical development and approval, including (Table **1**): DOX, DNR, epirubicin (EPI, 4'-*epi*-doxorubicin or Farmorubicin®), idarubicin (IDA, 4-demethoxy-daunorubicin or Zavedos®), pirarubicin (4'-tetrahydropyranyl-doxorubicin), aclacinomycin A (aclarubicin) and mitoxantrone [17, 19]. EPI is a semi-synthetic derivate of DOX obtained by an axial-to-equatorial epimerization of the hydroxyl group at C-4' in daunosamine (Fig. **3**). This structural change has little effect on its mode of action and spectrum of activity compared to DOX but it introduces significant pharmacokinetic and metabolic changes, like increased volume of distribution (V_d), 4-*O*-glucuronidation, and consequent enhanced total body clearance (CL) or shorter terminal half-time [20]. Moreover, EPI may be used at higher doses than DOX, without increased cardiotoxicity [17].

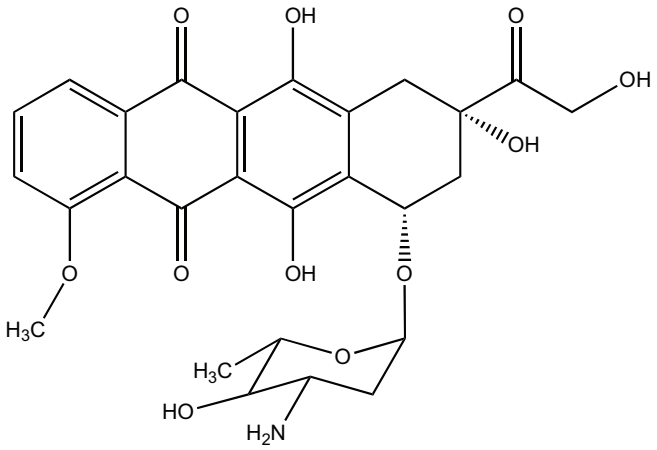

Fig. (3). Chemical structure of EPI.

IDA is an analogue of DNR obtained after removal of the 4-methoxy group in ring D (Fig. **4**) and is active in acute myelogenous leukemia, multiple myeloma, non-Hodgkin's lymphoma, and breast cancer [21]. IDA presents a higher spectrum of activity when compared to DNR, probably due to its increased lipophilicity and cellular uptake as well as improved stabilization of a ternary drug-topoisomerase II-DNA complex [17].

Pirarubicin (Fig. **5**) was synthesized in Japan [22] and subsequently marketed in Europe with the designation of Theprubicin®. This compound has discrete improvements over DOX in terms of drug resistance, and induces much less cardiotoxicity [17, 19].

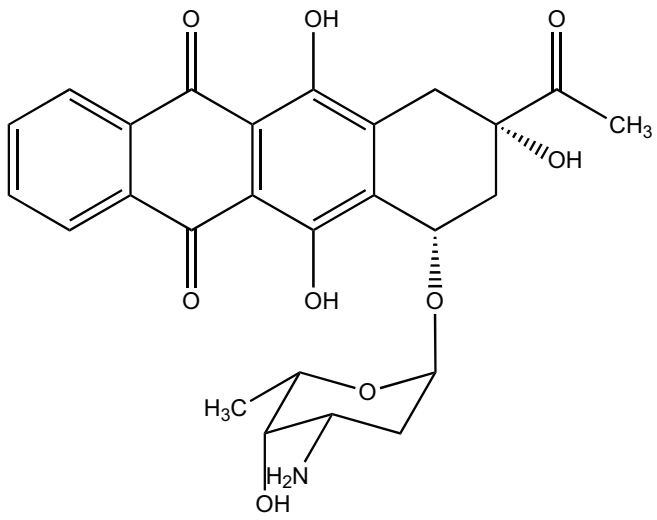

Fig. (4). Chemical structure of IDA.

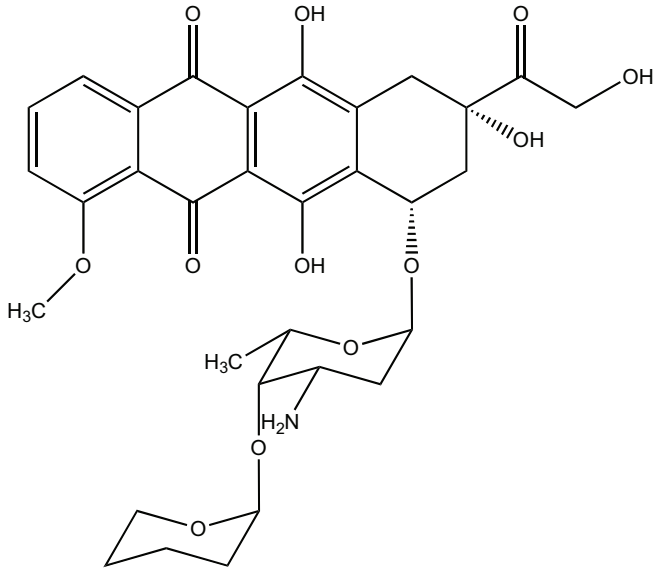

Fig. (5). Chemical structure of Pirarubicin.

Aclarubicin (Fig. **6**) is a trissaccharide anthracycline that also demonstrated little improvement over DNR in terms of drug resistance but was shown to be active and cardiac tolerable in adult patients with acute myeloblastic leukemia [17].

Mitoxantrone (Fig. **7**) is a substituted aglyconic anthraquinone that is active in breast cancer, acute promyelocytic or myelogenous leukemia, and androgen-independent prostate cancer. Its advantage in terms of cardiotoxicity compared to other family members is still questionable [17].

Table 1. Members of the Anthracyclines Family that Reached the Stage of Clinical Development and Approval; Pirarubicin and Aclarubicin are Not Currently Marketed. Information About Commercial Designation, Formulation, Therapeutic Indications and First Approval Date are Provided for the Remaining Members of the Anthracyclines Family

Name	Commercial Designation	Formulation	Therapeutic Indications	First Approval	Ref.
Doxorubicin	Caelyx	Liposomal formulation	Metastatic breast cancer, advanced ovarian cancer, progressive multiple myeloma in combination with bortezomib, AIDS-related Kaposi's sarcoma (KS).	21 June 1996	[23]
	Myocet	Liposomal formulation	Treatment of metastatic breast cancer in combination with cyclophosphamide.	13 July 2000	[24]
Daunorubicin	Daunorubicin hydrochloride	Intravenous formulation	Acute nonlymphocytic leukemia (myelogenous, monocytic, erythroid) and acute lymphocytic leukemia.	30 January 1998	[25]
	DaunoXome	Liposomal formulation	Advanced HIV-associated Kaposi's	15 January 1996	[26]
Epirubicin	Epirubicin hydrochloride	Intravenous formulation	Adjuvant therapy in patients with evidence of axillary node tumor involvement following resection of primary breast cancer.	9 August 2007	[27]
Idarubicin	Idamycin (USA)	Intravenous formulation	Acute myeloid leukemia (AML).	16 March 2006	[28]
	Zavedos (Europe)	Intravenous formulation	Acute nonlymphocytic leukemia.	5 November 1990	[29]
		Oral formulation	Acute nonlymphocytic leukemia, breast cancer in advanced stages.	1 August 1995	[30]
Pirarubicin	Not current marketed				
Aclarubicin	Not current marketed				
Mitoxantrone	Mitoxantrone hydrochloride (USA)	Intravenous formulation	Secondary (chronic) progressive, progressive relapsing, or worsening relapsing-remitting multiple sclerosis; advanced hormone-refractory prostate cancer, acute nonlymphocytic leukemia (ANLL), including myelogenous, promyelocytic, monocytic, and erythroid acute leukemias.	28 December 2009	[31]
	Mitoxantrone (Europe)	Intravenous formulation	Metastatic breast cancer, non-Hodgkin's lymphoma, acute nonlymphocytic leukemia, palliative treatment of primary hepatocellular carcinoma; advanced prostate cancer hormone-resistant.	29 December 1993	[32]

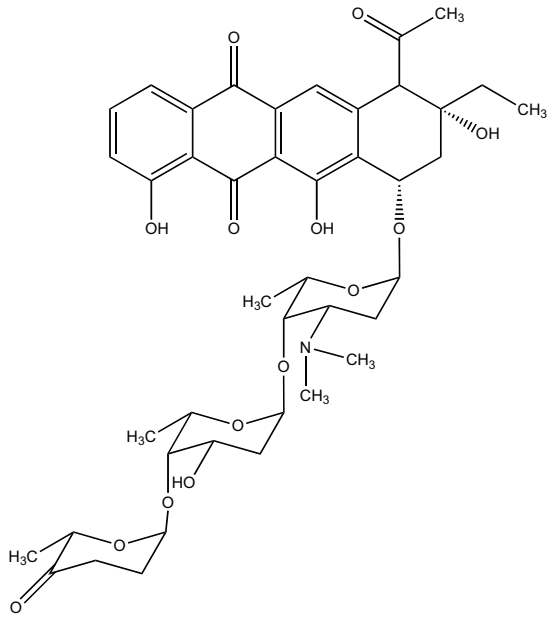

Fig. (6). Chemical structure of Aclarubicin.

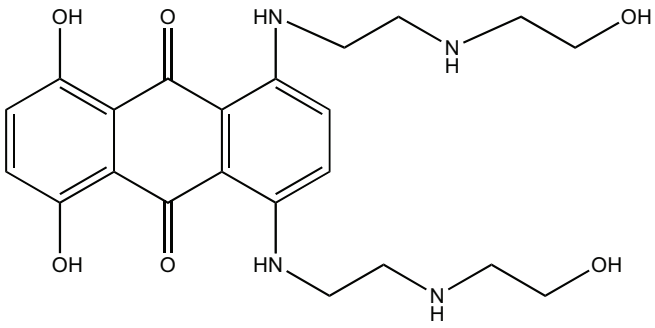

Fig. (7). Chemical structure of Mitoxantrone.

Mechanism of Action

The mechanism by which anthracyclines inhibit cancer growth is still not completely clear and multiple pathways are thought to be involved in the cytotoxicity of this class of anticancer drugs. According to Gewirtz [33] anthracyclines act by eight different mechanisms: 1) intercalation into DNA, leading to inhibited synthesis of macromolecules; 2)

generation of free radicals, leading to DNA damage or lipid peroxidation; 3) DNA binding and alkylation; 4) DNA cross-linking; 5) interface with DNA unwinding or DNA strand separation and helicase activity; 6) direct membrane effects; 7) initiation of DNA damage via inhibition of topoisomerase II; and 8) induction of apoptosis in response to topoisomerase II inhibition. However, these mechanisms did not occur all at the same dosage; more so, some of them were observed at concentrations considered too high to be administrated to patients.

Minotti *et al.* [17] considers that, at clinically relevant concentrations, anthracyclines may act as topoisomerase inhibitors or may induce apoptosis through DNA damage and p53. In the first case, anthracyclines act by stabilizing a reaction intermediate in which DNA strands are cut and covalently linked to tyrosine residues of topoisomerase II, eventually impeding DNA resealing. The formation and stability of an anthracycline-DNA-topoisomerase II ternary complex is crucial for anthracyclines activity; moreover, the external (non-intercalating) moieties of the anthracycline molecule (i.e., the sugar residue and the cyclohexane ring) seem to play an important role in formation and stability of this ternary complex. Topoisomerase II mediated DNA damage is followed by growth arrest in G1 and G2 and programmed cell death [34]. It follows that tumor cells may become resistant to anthracyclines because of altered topoisomerase II gene expression or activity [35].

As for the second mechanism of action of anthracyclines, it is known that DOX activates p53-DNA binding. However, the role of p53 in anthracycline-induced apoptosis is not certain, with contradictory reports [36, 37]. These uncertainties may be attributed to various factors such as the heterogeneity of the tumors examined or the methods used for assessing p53 status and tumor response. As for the role of p53 in regulating cell cycle transition, it is established that DOX-dependent p53 activation contributes to the induction of the WAF1/CIP1 p21 gene product, a strong inhibitor of cyclin-dependent kinases involved in G1 to S transition. Although these mechanisms may contribute to G1 arrest of p53 proficient cells, WAF1 expression might protect cells from DOX because the G1 block facilitates DNA repair before the cells undergo replication. On the other hand, the ability of p53-deficient cells to progress through the S phase may be a favorable event since the expression of the α-isoform of topoisomerase II is increased during DNA synthesis. Furthermore, Dunken *et al*. [38] shown that p53 might be important not only in connecting DNA damage to downstream execution of apoptosis but also in determining the levels of DNA strand breaks induced by DOX.

Uncertainties about the complex interplay between p53 and anthracycline-induced apoptosis and the contradictory reports about related mechanisms may also be justified by the presence of alternative networks that are not bound to an inhibition of topoisomerase II nor do they always require functional p53 [17].

Cardiotoxicity

One of the major problems of anthracyclines usage is related to cumulative dose-dependent cardiotoxicity, responsible for developing late-onset heart failure. Anthracycline-induced secondary cardiotoxicity is seen in 5-23% of patients [39, 40]. The mechanism behind the anthracyclines cardiac toxicity and their specificity to myocyte cells remains controversial and not completely understood. Sawyer *et al.* [39] propose several mechanisms to explain the cardiotoxicity of anthracyclines, including (Fig. **8**): 1) generation of oxidative stress through formation of hydroxyl radical, leading to myocyte cell dead; 2) inducing apoptosis via a mitochondrial pathway involving Bax, cytochrome c and caspase-3 activation; 3) inducing apoptosis through intercalation between base pairs in DNA, originating DNA damage; 4) DNA damage by suppression of expression and/or activity of transcription factors that modulate sarcomere synthesis, as well as cell survival; 5) suppression of sarcomere protein synthesis. These mechanisms may not occur all at once, they are probably induced by different doses of the chemotherapeutic agent.

Since anthracyclines are interesting therapeutic agents being its cardiotoxicity a major limitation, efforts to circumvent this problem include: limiting dose exposure; encapsulated anthracyclines in liposomes to reduce myocardial uptake; administering concurrently with the iron chelator dextrazone to reduce free iron-catalyzed reactive oxygen species formation; and modify anthracyclines structure in an effort to reduce myocardial toxicity [40].

Alternative Formulations of Current Anthracyclines

Doxorubicin plays an important role in osteosarcoma chemotherapeutic treatment, so its clinical unresponsiveness is a major concern. Liposomal doxorubicin (Caelyx® in Europe, Doxil® in USA) is currently approved for cancer treatment and its formulation has the advantage of enhancing the antitumor effect, reducing toxicity, and improving pharmacokinetics, when compared to free DOX. These improvements are due to several factors: the polyethylene glycol (pegylated) coating reduces the uptake of the liposomes by cells of the reticuloendothelial system (RES), thus prolonging the time of circulation; because normal blood vessels are not as fenestrated as tumor vessels, liposomes are confined to the intravascular space, reducing toxicity in normal tissues [41]. Besides Caelyx®, another two liposomal formulations of anthracyclines have showed promising results: an uncoated formulation in which citrate is included for increasing DOX encapsulation above the levels predicted by the maintenance of a transmembranar pH gradient; and a liposomal DNR (DaunoXome) [17]. The first formulation is not as advantageous as the pegylated liposomal DOX, but still is better than free DOX and its main indication is treatment of metastatic breast cancer [24]. Liposomal DNR also showed improved results when compared to free DNR and it is used as a first-line therapy of AIDS-related Kaposi's sarcoma, although it also has activity against refractory or relapsed acute myeloblastic leukemia, recently diagnosed or recurrent/refractory multiple myeloma and non-Hodgkin's lymphoma [26].

Tan *et al.* [42] produced an alternative drug delivery system composed by chitosan-dextran sulphate using a combinational coacervation method. DOX was successfully encapsulated into these microparticles and the *in vitro*

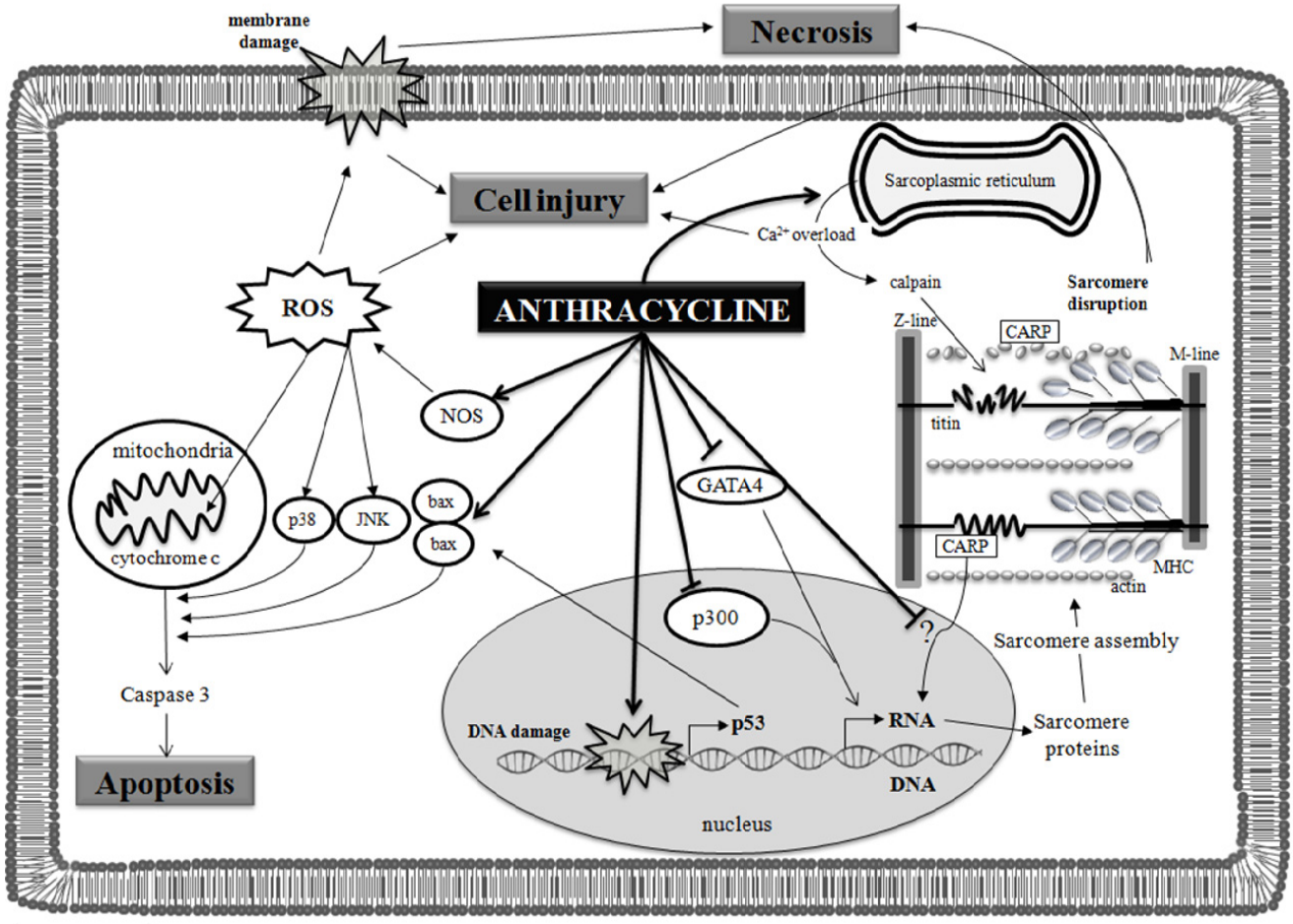

Fig. (8). Possible mechanisms by which anthracyclines causes cardiac toxicity. The formation of reactive species is induced by the quinone moiety of anthracyclines and by induction of nitric oxide synthase, leading to nitric oxide and peroxynitrite formation. Another method of anthracyclines cardiotoxicity is to intercalate into nucleic acids, causing suppression of DNA, RNA, and protein syntheses, as well as damaging some transcriptional regulatory proteins that seem important for regulation of cardiac-specific genes. Anthracyclines also accelerate myofilament degradation, leading to a net negative balance of sarcomeric proteins ("cardiac sarcopenia") and induce changes in adrenergic function and adenylate cyclase as well as abnormalities in Ca^{2+} handling, functions that are critical for cardiac function. By last, anthracyclines also induce necrosis and apoptosis of myocyte cells. ROS – reactive oxygen species; JNK – c-Jun N-terminal kinases; bax – Bcl-2-associated X protein; NOS – nitric oxide synthase; GATA4 – gene name, member of GATA family of zinc-finger transcription factors; MHC – myosin heavy chain; CARP – cardiac ankyrin repeat protein and cardiac adriamycin-responsive protein [39].

studies demonstrated a reduction in SaOS-2 cell viability through various cell death mechanisms such as necrosis, apoptosis and mitosis. Treatment of mice bearing orthotopic osteosarcoma with DOX microparticles decreased tumor volume, bone lysis, and reduced secondary metastasis to the lungs. Also the treated mice maintained their weight and did not appear to suffer from any visible side effects such as heart failure or dry skin.

Another approach to decrease anthracyclines cardiotoxicity is by its encapsulation into nanoparticles. Betancour *et al.* [43] reported acid-copped poly(lactic-co-glycolic acid) nanoparticles as a carrier for DOX that deliver the drug into MDA-MB-21 breast cancer cells quickly and in higher quantity than free DOX. Janes *et al.* [44] showed similar results using chitosan nanoparticles in human melanoma A375 cells. Bisht *et al.* [45] reviewed the usage of dextran-DOX conjugates encapsulated in chitosan nanoparticles in solid tumors therapy and showed that nanoparticles in the range of 10-100 nm diameter are able to deliver chemotherapeutic drugs to solid tumor. Susa *et al.* [1] incorporated DOX into a lipid-modified dextran based polymeric nano-system and demonstrated improved anti-proliferative effects against osteosarcoma cell lines compared to free DOX.

Wang *et al.* [46] demonstrated a way to overcome multidrug resistance in MCF-7/ADR cancer cells using a drug delivery system that tethers DOX onto the surface of gold nanoparticles with a poly(ethylene glycol) spacer *via* an acid-labile linkage (DOX-Hyd@AuNPs). These nanoparticles release DOX in response to pH of acidic

organelles after endocitosis, inducing elevated apoptosis in cancer cells. This process may be monitored by the fluorescence of DOX from quenching due to the nanosurface energy transfer between the doxorubicinyl groups and the gold nanoparticles.

New Anthracyclines Family Member

Another approach to increase anthracyclines anti-tumor activity and to decrease its cardiotoxicity is by chemical modification. It was shown that substitution in the sugar position at C-3' is critical for the ability of drugs to interfere with DNA topo II [47] and that the configuration of C-3'-NH_2 is fundamental for the ability of drugs to overcome MDR [48].

A new class of anthracyclines derivatives – **alkylcyclines** – was obtained by alkylation of position C-3' of the aminosugar of anthracyclines such as idarubicin, which increases lipophilicity and reduces the chemical reactivity of these molecules. This new class of compounds showed high cytotoxicity in cell lines resistant to doxorubicin and idarubicin [49, 50].

Ladirubicin (PNU-159548, 4-demethoxy-3'-deamino-3'-aziridinyl-4'-methylsulphonyl-daunorubicin) (Fig. **9**), the leading compound of this new class is characterized by the presence of an aziridinyl moiety at C-3' and esterification of –OH at C-4' with a methylsulfonic group, which is responsible for its high lipophilicity and for an increased stability of the alkylating moiety. This new class of compounds causes DNA damage, not by interaction with topoisomerase II but via the anthracyclines backbone binding covalently to guanines at the N7 position and adenines at N3 position via the reactive alkylating group in the sugar [17].

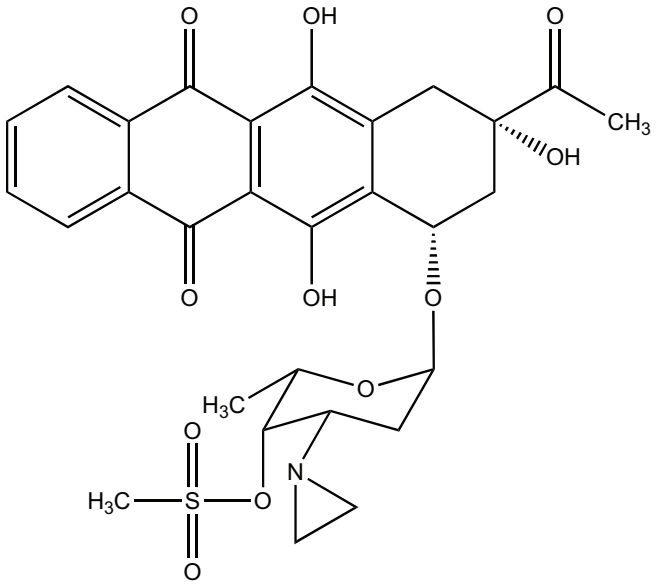

Fig. (9). Chemical structure of ladirubicin.

Gerani *et al.* [50] evaluated the antitumor activity of ladirubicin by an *in vitro* and *in vivo* cancer cell line panel and investigated its mode of action; also, they performed toxicity and pharmacokinetic studies. Ladirubicin was found to be active against both murine and human tumor cell lines *in vitro*, using a concentration 33 times smaller than the required for DOX, probably due to its higher lipophilicity. Moreover, the presence of bulky substituents at C-3' position of the amino sugar prevents drug stimulation of DNA topoisomerase II cleavage, making ladirubicin effective on cells presenting the topoisomerase II-related MDR. The *in vivo* studies indicated a wide spectrum antitumor activity against rapidly proliferating murine leukemias and on slowly growing transplantable human tumor xenografts. The toxicological profile of ladirubicin was pre-clinically defined in mice, rats, and dogs, and target organs were identified after single and repeated-cyclic-dose administration. The collateral toxic effects are dose-related and reversible and consisted in myelosuppression, lymphoid organ cell depletion, and intestinal toxicity. Conversely, in animals ladirubicin showed a cardiotoxicity remarkably lower then DOX at equimyelotoxic doses.

Marchini *et al.* [49] performed *in vitro* and *in vivo* studies to evaluate ladirubicin antitumor activity. Their results demonstrated that ladirubicin is active against cells expressing the MDR phenotype associated to *MDR-1* gene overexpression or to an alteration in the *topoisomerase II* gene (altered MDR). Ladirubicin was also active against cells showing resistance to several alkylating agents (cisplatin, cyclophosphamide, melphalan) and topoisomerase I-inhibitors.

Pasello *et al.* [2] evaluated ladirubicin to antitumor activity against 32 human osteosarcoma cell lines, including cell lines resistant to DOX, methotrexate or cisplatin. In their results ladirubicin maintained its activity in DOX-resistant osteosarcoma cell lines, which present a MDR phenotype as a consequence of *MDR-1* gene amplification/overexpression and increased levels of P-glycoprotein. The intracellular uptake of ladirubicin was not influenced by the presence of high levels of P-glycoprotein. When the authors used osteosarcoma cell lines resistant to methotrexate or cisplatin, ladirubicin exhibited similar efficacy to that found in drug-sensitive cell lines, indicating absence of cross-resistance mechanism between ladirubicin and methotrexate or cisplatin. They investigated as well the possibility of effectively combining ladirubicin with conventional anticancer drugs. The results revealed additive or synergistic interactions with DOX and cisplatin and antagonist effects with methotrexate, concluding that probably ladirubicin has effects on cell cycle, considering that methotrexate efficacy is strictly related to the presence of actively growing cells.

The preclinical studies demonstrated that ladirubicin is active against several human tumor xenografs: MX1 mammary carcinoma, DU 145 prostatic carcinoma, M14 melanoma, A431 epidermoid carcinoma, A2780, H207 and IGROV1 ovarian carcinomas, N592 SCL carcinoma, H460 NSCL carcinoma, HCT-116 colon carcinoma. Also, borderline activity was observed on A549 lung adenocarcinoma and HT29 colon carcinoma [50]. In addition, due to its high lipophilicity, ladirubicin is able to cross the blood-brain barrier, being effective against intracranial tumors, and the dose-limiting toxicity is myelosuppression whereas lack of cardiotoxicity may be

explained, at least partially, by high plasma clearance [19]. The *in vivo* studies demonstrated activity on MDR (P-glycoprotein and topoisomerase II related MDR) tumor cells. It does not inhibit topoisomerase II and it reaches similar intracellular levels in sensitive and MDR tumor cells. Furthermore, *in vivo* studies demonstrated an antitumor efficacy clearly superior and minimal cardiotoxicity compared to DOX. With this pre-clinical evidences, ladirubicin has been selected for Phase I clinical trials on patients with a variety of solid tumors, with the purpose of testing the feasibility and describe the clinical toxicity and pharmacokinetics of ladirubicin when administrated I.V. over a 10 min infusion [51]. The dose-limiting toxicity (DLT) of ladirubicin was thrombocytopenia and mild neutropenia, and the most prominent non-hematological side effects were nausea and vomiting, which were rare when in combination with 5-hydroxytryptamine-3 receptor antagonists. A less frequent but prominent side-effect was a complex of symptoms consisting of fever with chills, facial erythema and edema, and dyspnea, which was interpreted as an hypersensitivity reaction that developed during or shortly after the infusion and ceased, either spontaneously at interruption of the drug administration or following antihistamine therapy. The evaluation of the cardiac function demonstrated that ladirubicin did not induce significant cardiotoxicity at doses ranging from 1 to 16 mg/m^2, resulting in a peak plasma concentration (C_{max}) between 0.5 to 1.5 μM. Based on this study, the recommended dose for phase II studies was 12 and 14 mg/m^2 in heavily and minimally pretreated/non-pretreated patients, respectively. Phase II clinical trials of ladirubicin in ovary, breast, colorectal cancer, NSCLC and malignant melanoma are underway [51, 52].

DOXORUBICIN *VS.* LADIRUBICIN

Despite the lack of literature directly comparing DOX to ladirubicin, some aspects are clear and can be compared.

In terms of chemical structure, DOX is one of the firsts anthracyclines obtained and to exhibit an aglycolic and sugar moieties. The aglycone consists of a tetracyclic ring with adjacent quinone-hydroquinone groups in rings C-B, a methoxy substituent at C-4 in ring D, and a short side chain at C-9 with a carbonyl at C-13. The sugar (daunosamine) is attached by a glycosidic bond at the C-7 of ring A and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl moiety. Furthermore, DOX side chain terminates with a primary alcohol. Instead, ladirubicin is characterized by the presence of an aziridinyl moiety at C-3' and esterification of –OH at C-4' with a methylsulfonic group. In addition, the side chain of ladirubicin terminates with a methyl group, rather than a primary alcohol [17].

These structural differences lead to differences in the lipophilicity and cytotoxic mechanisms of both drugs. Ladirubicin is much more lipophilic than DOX and consequently has a higher volume of distribution [51], crosses the blood-brain-barrier and is able to delay the growth of intracranially implanted tumors [50].

The mechanisms of antitumoral activity of both drugs are different, which may justify the anti-tumoral activity of ladirubicin against DOX-resistant cell lines. DOX acts by interacting with topoisomerase II, stabilizing the topoisomerase II-DNA complexes, resulting in double-strands breaks in the DNA and cell arrest in the cell cycle at the G2 stage. In addition, as discussed earlier, DOX may also be involved in p53 pathways and inductions of programmed cell death. On the other hand, ladirubicin causes cell damage through DNA intercalation via the anthracycline backbone and bind covalently to guanines (N7 position) and adenines (N3 position) via the alkylating group in the sugar. As such, even in tumor cells that exhibit altered topoisomerase II gene expression or activity, ladirubicin is active [17].

Another aspect that distinguishes DOX from ladirubicin is the extent of cardiotoxicity as a result from treatment. In the rat, ladirubicin induces chronic cardiotoxicity which is less than one-20th of that caused by an equimyelotoxic dose of DOX [53]. Further, in phase I clinical trials, no cardiac toxicity could be discerned [51].

According to the *in vitro* studies performed by Geroni *et al*. [50], ladirubicin is more potent than DOX: the IC_{50} values of ladirubicin where in a range of 1.2 to 81.1 ng/ml, while for DOX those values are in a range of 72-1365 ng/ml, probably due to ladirubicin high lipophilicity, leading to faster accumulation in tumor cells.

As stated earlier, standard osteosarcoma treatment is based on pre-operative chemotherapy, surgery and post-operative chemotherapy. In pre-operative chemotherapy, the current used drugs are cyclophosphamide, vincristine, melphalan, DOX, MTX, cisplatin, decarbazine, bleomycin, dactinomycin, actinomycin, and leucovorin rescue. Post-operative chemotherapy includes DOX, high-dose MTX, and CDDP [2, 7]. According to Pasello *et al*. [2], the first lineuse of ladirubicin followed by DOX, MTX or CDDP results in synergetic or additive effects. On the other hand, using ladirubicin after administration of DOX, MTX of CDDP has antagonistic effects in the majority of the cell lines used. In addition, simultaneous exposure to ladirubicin and DOX or CDDP has additive or synergetic effects, while simultaneous exposure to ladirubicin and MTX result in antagonistic effects. The finding may be critical for ladirubicin usage, because its antitumoral effectiveness may not be superior to DOX if in a combinatory regimen with MTX. Additionally, based on these findings, ladirubicin would be more beneficial in pre-operative chemotherapy regimens.

Phase II clinical trials are ongoing and the results will certainly clarify these *in vitro* finding. Furthermore, combinatory studies are needed to evaluate the more beneficial combination with ladirubicin.

FUTURE DIRECTIONS

Cancer cells employ a host of different mechanisms to become resistant to one or more chemotherapeutic agents. To overcome drug resistance and reduce the side effects during chemotherapy, as well as to improve drug delivery and availability, nanotechnology holds a promising potential utilizing targeted drug delivery. A number of nanoparticles types are available: polymeric nanoparticles, dendrimers,

inorganic/metal nanoparticles, quantum dots, liposomes, micelles, and several other types of nanoassemblies [54].

Cardiotoxicity, a well-known adverse effect of anthracyclines, may become a manifest as late as 20 years after chemotherapy. Despite the intense research in this subject, there is no consensus over the standard of care for cardiac monitoring, prophylactic cardiac treatment, or selective therapies to reverse anthracyclines-induced cardiotoxicity [40].

Doxorubicin, one of the most important chemotherapeutic agents used in osteosarcoma treatment, has a high incidence in anthracyclines-induced cardiotoxicity. The liposomal formulation of doxorubicin, Caelyx®, has demonstrated to reduce the side effects and improve the anti-cancer effects of doxorubicin. However, cardiotoxicity is still observed in patients treated with Caelyx® [23] and its safety has constantly been under scrutiny due to the adverse side-effects still experienced by patients. For instance, incidences of dermatological toxic reaction – palmar-plantar erythrodysesthesia have been reported in up to 50% of all patients [55], while another 50% of patients suffered from various hematologic adverse reactions such as anemia, leucopenia, and neutropenia [56]. Alternative formulations have been studied like polymeric nanoparticles based delivery systems which offer a significant advantage over other nanocarrier platforms as there is a tremendous versatility in choice of polymeric matrices that can be used, allowing for the tailoring of nanoparticles properties to meet the specific needs they are intended to meet. Other advantages of these formulations are: easy surface modification; greater encapsulation efficiency of the payload; payload protection; large surface area-to-volume ratio; and slow or fast polymer erosion for temporal control over the release of drugs.

Furthermore, the development of nanostructured delivery systems that combine carriers with cancer-targeting molecules can potentially overcome the drawbacks presented by conventional approaches [54]. Salerno *et al.* [57] developed biodegradable, biocompatible nanoparticles made of a conjugate between poly (D, L lactide-co-glycolic) acid and alendronate, suitable for systemic administration and directly targeting the site of tumor induced osteolysis. These nanoparticles were loaded with doxorubicin and the *in vitro* and *in vivo* activity of the drug encapsulated in the carrier system was analyzed in a panel of human cell lines, representative for primary or metastatic bone tumors, and in an orthotopic mouse model for breast cancer bone metastasis. Their results showed a significant dose-dependent growth inhibition of all cell lines.

The use of polymeric nanocarriers allows the combination of various anti-cancer strategies. For example, some studies use magnetic nanoparticles loaded with chemotherapeutic agents [58-62]. This combination is suitable to use intracellular hyperthermia, a technique in which the particles are concentrated at the tumor site and are remotely heated using an applied magnetic field to the required hyperthermic temperatures (42-45ºC), thus killing the cancer cells with the heat generated [63, 64].

Ladirubicin, the prototype of alkylcyclines, has shown promising results *in vitro* and *in vivo* and in phase I clinical trials. It has anti-cancer activity against cell lines expressing the MDR phenotype and cancer cells resistant to alkylating agents and topoisomerase I-inhibitors. Also, in rats ladirubicin induces chronic cardiotoxicity which is less than one-20th of that caused by an equimyelotoxic dose of DOX and significantly milder than the predicted based upon the equivalent dose of IDA present in the drug´s backbone [17]. On the other hand, in drug resistant variants of osteosarcoma cells, there was evidence that a first exposure to the drug against which these cells are resistant, negatively affects and limits their subsequent sensibility to ladirubicin through mechanisms that still remain to be identified [2]. However, phase I clinical trials revealed some side effects such as thrombocytopenia, mild neutropenia, nausea, vomiting and hypersensitivity.

An alternative way to overcome anthracyclines problems profiting of ladirubicin advantages may be the encapsulation of ladirubicin into nanoparticles functionalized with tumor-targeted agents. This technique would provide specificity to the chemotherapy treatment, increase the anti-tumor activity, mainly in resistant variants of the tumor cells, decrease the side effects of ladirubicin and the cardiotoxicity would be almost absent. This technique has already been experimented for other cancer treatments. For example, Huh *et al.* [65] used magnetic nanocrystals conjugated to Herceptin, a cancer-targeting monoclonal antibody used for breast cancer treatment, and successfully monitored *in vivo* selective targeting events of human cancer cells implanted in live mice.

Wagner *et al.* [66] used a monoclonal antibody, DI17E6, covalently coupled to human albumin nanoparticles. DI17E6 is a monoclonal antibody directed against αv integrins that inhibit growth of melanomas *in vitro* and *in vivo* and also angiogenesis due to interference with αvβ3 integrins. Moreover, DOX was loaded in DI17E6 nanoparticles showing increased cytotoxic activity in αvβ3-positive melanoma cells compared to free drug.

CONCLUSION

Osteosarcoma is a relatively uncommon malignancy, with an overall incidence of 5 cases per million persons per year. However, among childhood malignancies, osteosarcoma is the eighth most common. Only leukemias, lymphomas, and neurological malignancies are more common. Osteosarcoma accounts for 8-9% of cancer-related deaths in children and carries an overall 5-year survival rate of 60%–70% [67]. Doxorubicin is one of the most important chemotherapeutic agents in osteosarcoma treatment. However, its cardiotoxicity and increase resistance are a limitation in its usage. So, there is a need for new treatment options. Also, ladirubicin is an analogue of idarubicin with promising results in phase I clinical trials, as such, liposomal formulations and anthracyclines analogues have been exhaustively explored in the last years, leading to the discovery of nanoparticles system able to deliver the drug to a specific site, maintaining the anti-cancer activity of the drug and reducing its side effects.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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