Life Sciences 231 (2019) 116530

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

3,4-dimethoxybenzyl isothiocyanate enhances doxorubicin efficacy in LoVoDX doxorubicin-resistant colon cancer and attenuates its toxicity *in vivo*

Mateusz Psurski[a,b,*], Beata Filip-Psurska[a], Monika Cuprych[a], Joanna Wietrzyk[a], Józef Oleksyszyn[b]

[a] Department of Experimental Oncology, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland

[b] Department of Medicinal Chemistry and Microbiology, Wroclaw University of Science and Technology, Wybrzeże Wyspiańskiego 29, 50-370 Wrocław, Poland

ARTICLE INFO

Keywords:
Isothiocyanates
Doxorubicin
Combined therapy
Chemoresistance
Cardiotoxicity
Drugs interaction

ABSTRACT

Aims: The aim of the study was to evaluate the potential of naturally occurring isothiocyanates and doxorubicin in combined treatment of doxorubicin-resistant colon cancer. Doxorubicin is a cytostatic commonly used to treat many different types of cancer but its usage is often abrogated by severe side-effects and drug-induced resistance.
Main methods: The antiproliferative potential of the combined treatment was analyzed *in vitro* by the SRB method (sulforhodamine B) and further evaluated for the mechanisms that determine the treatment outcome using a series of assays which included oxidative stress, apoptosis and compounds accumulation assessment. Ultimately, a combined treatment potential was assessed *in vivo* utilizing doxorubicin-resistant colon cancer model.
Key finding: The results indicate that naturally occurring isothiocyanates, represented by 3,4-dimethoxybenzyl isothiocyanate (***d*MBITC**) increase doxorubicin the efficacy in doxorubicin-resistant human colon adenocarcinoma model by attenuated drug efflux, an increased reactive oxygen species production and an increased rate of apoptosis. In *in vitro* studies, over a 3-fold decrease in doxorubicin IC_{50} value was observed on the LoVoDX cell line when used in combination with suboptimal concentrations of ***d*MBITC**. The combined therapy exhibited a significantly higher efficacy than doxorubicin-alone treatment (*c.a.* 50% tumor growth inhibition in comparison to *c.a.* 25% for doxorubicin-alone treatment) *in vivo*. At the same time, the combined treatment attenuates doxorubicin toxicity as evidenced by improved animals body mass, main organs weight and biochemical markers of toxicity.
Significance: The adopted approach provides evidence that isothiocyanates can be successfully applied in the treatment of doxorubicin-resistant colon cancer, which warrants further studies.

1. Introduction

The brassicaceae family, which encompasses many frequently consumed vegetables (like cauliflower, horseradish, Brussels sprouts, broccoli *etc.*), is a rich source of chemically diverse glucosinolates, biologically inert precursors of isothiocyanates. These naturally occurring compounds for over 30 years receive sustained attention as potential anticancer agents [1]. The high reactivity of isothiocyanates arises from the electrophilic character of the central carbon atom in the isothiocyanato moiety and is responsible for its rapid addition to sulfhydryl groups of which the most abundant intracellular source is glutathione. The adduct and the products of its metabolism (including the *N*-acetylcysteine derivative) can act as an intracellular depot of isothiocyanates, which allows for the protein's sulfhydryl groups subsequent modifications *via* transthiocarbamoylation [2]. This unique feature enables rapid isothiocyanates intracellular accumulation and the modulation of multiple signaling and metabolic pathways, which is a result of the covalent modification of over 30 proteins, including cytochromes P450, Kelch-like ECH-associated protein 1 (Keap1), α- and β-tubulin, and many more [3]. In numerous studies isothiocyanates and their mercapturic derivatives exhibited a high anticancer activity at multiple diverse cancer models (both *in vitro* and *in vivo*) with but negligible toxicity [4].

Doxorubicin (**Doxo**) belongs to anthracyclines – antineoplastic antibiotics – and is one of the most potent and widely used chemotherapeutics, recognized by the World Health Organization (WHO) as the

* Corresponding author at: Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Rudolfa Weigla 12, 53-114 Wrocław, Poland.
E-mail address: mateusz.psurski@hirszfeld.pl (M. Psurski).

https://doi.org/10.1016/j.lfs.2019.06.005
Received 9 April 2019; Received in revised form 28 May 2019; Accepted 2 June 2019
Available online 04 June 2019
0024-3205/

essential medicine. Its usage covers, but is not limited to, several types of sarcomas, lymphomas, breast, colon and bladder cancer. Unfortunately, apart from the activity as an intercalating agent and topoisomerase II inhibitor, doxorubicin causes severe toxic effects because it affects negatively mitochondrial respiration and elevated reactive oxygen species (ROS) production [5]. The most dangerous side effect is cardiomyopathy whose rate strongly correlates with doxorubicin cumulative dose (600 mg/m^2 body surface is estimated as the highest dose that can be received in a lifetime). Prolonged anthracycline-based treatment is often associated with the occurrence of chemoresistance, mainly caused by the elevated expression and activity of ABC multidrug-resistance pumps, which further decreases the compound's usability [6].

Recently, isothiocyanates and their metabolites – mercapturic acids – have been sparking a keen interest as a common diet constituent whose regular consumption substantially reduces tumor incidence [7]. In several studies, isothiocyanates have been revealed to exhibit considerable capabilities as adjuvants for classical chemotherapeutics with markedly increased treatment outcomes [8]. Herein, we report that naturally occurring 3,4-dimetoksybenzyl isothiocyanate not only increases doxorubicin antitumor potential in the doxorubicin-resistant colon adenocarcinoma model, but also significantly reduces its toxicity.

2. Materials and methods

2.1. Reagents and chemicals

Unless stated otherwise, all chemicals, culture media supplements and solvents used in this study were provided by Sigma-Aldrich (Poznan, Poland) at the highest available purity and applied as supplied. 3,4-dimethoxybenzyl isothiocyanate (***d*MBITC**) as well as *N*-Acetyl-*S*-(*N*-(3,4-dimethoxy)benzylthiocarbamoyl)-L-cysteine (**NAC-*d*MBITC**) were synthesized from the corresponding primary amine (3,4-dimetoxybenzyl amine) *via* procedure described previously with minor modifications (for details see supplementary materials) [9]. Doxorubicin (**Doxo**; Urtica, Wroclaw, Poland) was applied as a *ready-to-use* stock 2 mg/mL solution and stored at 2–8 °C. ***d*MBITC** and **NAC-*d*MBITC** were dissolved in DMSO (dimethyl sulfoxide) and stored as 50 mM aliquots at −80 °C.

2.2. Cell culture

The LoVo (human grade IV colorectal adenocarcinoma) cell line was purchased from the American Type Culture Collection (ATCC; Rockville, USA); LoVoDX (doxorubicin-resistant LoVo subline) cell line was kindly provided by Professor E. Borowski (Gdansk University of Technology, Poland). All cell lines are maintained at the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET, PAS), Wroclaw, Poland. The LoVo and LoVo/DX cell lines were cultured in a 1/1 (v/v) mixture of RPMI-1640 and OPTI-MEM culture media (both Life Technologies, Warsaw, Poland), supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, and 1 mM sodium pyruvate with additional supplementation with doxorubicin (100 ng/mL) for LoVo/DX. All culture media contained antibiotics: 100 U/mL penicillin (Polfa Tarchomin, Warsaw, Poland) and 100 μg/mL streptomycin. All cell lines were cultured in a humidified atmosphere at 37 °C with 5% CO_2 and passaged twice a week using EDTA-Trypsin solution (pH 8; HIIET, Wroclaw, Poland) as a detachment agent.

2.3. Cell proliferation SRB assay

Cells were seeded on 96-well plates (Sarstedt, Warsaw, Poland) at 10^5 cells/well density and after overnight incubation, compounds or their combination at various concentrations were applied. After 72 h, the sulforhodamine B (SRB) assay was performed, as described by Skehan et al. [10] with minor modifications [11]. Based on raw absorbance readings at 540 nm, using Biotek Synergy H4 Hybrid Reader (Biotek Instruments, Bad Friedrichshall, Germany), mean cell proliferation inhibition and IC_{50} (compound concentration causing 50% growth reduction) were calculated as previously described [12].

2.4. Caspase-3 activity assay

Cells were seeded on 24-well plates at 5×10^4 cells/well density and after overnight incubation, compounds or their combination at various concentrations were applied. After another 24 h, the caspase-3 activity assay described previously was performed [11]. Results are presented as a fold caspase-3 activity increase over a vehicle-treated control samples.

2.5. Cell-free assessment of the permeability glycoprotein (P-gP) activity

These were performed according to the assay instruction provided by the manufacturer (Promega Corp., Wisconsin, USA; #V3601) with the application of various concentrations of doxorubicin and 3,4-dimethoxybenzyl isothiocyanate-derived mercapturic acid (**NAC-*d*MBITC**). The results are presented as a fold P-gp activity increase in comparison to a vehicle-treated control.

2.6. Doxorubicin intracellular accumulation

Cells were seeded on 24-well plates at 5×10^4 cells/well density and after overnight incubation, compounds or their combination at various concentrations were applied. After 4 h incubation samples were washed with PBS (phosphate-buffered saline), harvested by trypsinization and immediately analyzed for doxorubicin content by flow cytometry (BD LSRFortessa; BD Bioscience, San Jose, USA) using 488 nm laser for excitation and 610/20 nm filter for emission. The results are presented as fold change in doxorubicin intracellular content.

2.7. Reactive oxygen species production

Cells were seeded on 24-well plates 5×10^4 cells/well density and after an overnight incubation the medium was replaced with Hank's buffer (HBBS; IIET, Wroclaw, Poland) containing 10 μM 2′,7′-dichlorofluorescin diacetate (DCF-DA). An hour later, the buffer was replaced with a full culture medium containing compounds or their combinations at various concentrations. After a 4 h incubation, cells were harvested by trypsinization and immediately analyzed for reactive oxygen species level by flow cytometry (488 nm laser excitation, 530/30 nm emission filter). The results are presented as a fold ROS level increase over a vehicle-treated control.

2.8. Glutathione reduced (GSH) analysis

Cells were seeded on 96-well plates at 10^5 cells/well density and after an overnight incubation, compounds or their combination at various concentrations were applied. After a 4 h incubation, samples were analyzed for the glutathione reduced content by a *ready-to-use* assay (Promega Corp.; #V6912). The results are presented as glutathione reduced percentage in comparison to the vehicle-treated control.

2.9. In vivo experiments

6–8 weeks old female NOD/SCID mice were subcutaneously (*s.c.*) inoculated (right flank of the body, one inoculation per mouse) with LoVo or LoVo/DX cells (5×10^6 per animal) suspended in 100 μL of Hank's buffer (HIIET, Wroclaw, Poland) and matrigel (#354248; BD Bioscience, San Jose, USA) 1/1 (v/v) mixture. On the 8th day mice were randomly divided into experimental groups (10 animals/group) and

received the designed treatment schedule. Control groups received colza oil orally (*p.o.*) 5 times a week (QD × 5) in four series; ***d*MBITC**-treated groups received 300 μmol/kg b.w. (body weight) of the isothiocyanate dissolved in colza oil 5 times a week (QD × 5; *p.o.*) in four series; **Doxo-**treated groups received 1 mg/kg b.w. of doxorubicin intravenously (*i.v.*) once a week (QW), four injections per animal during the experiment. Tumor growth was monitored every other day by the measurement of the largest diameter (a) and perpendicular diameter (b), and tumor volume (TV) was calculated with the formula $TV\ [mm^3] = 0.5 \times a[mm] \times b^2[mm]$. Animals' welfare was monitored daily by body weight measurements and behavior observations. By the end of each experiment, a full autopsy was performed with main organs weighing and whole blood collection for morphological analysis (utilizing Mythic 18, C2 Diagnostics, Lomianki, Poland), followed by blood centrifuging (15′, 4 °C, 2000 ×g) and plasma analysis for various biochemical parameters (Cobas c111, Roche Diagnostics, Warsaw, Poland). The mice were maintained in specific pathogen free (SPF) conditions with water and fodder supplied *ad libitum*. All experiments were approved by the Local Committee for Experiments with the Use of Laboratory Animals (Wroclaw, Poland) and followed the highest ethical standards.

2.10. Statistical analysis

All datasets were evaluated for outliers by the MAD (median absolute deviation) method [13] and Gaussian distribution (Brown-Forsythe and Bartlett's tests), followed by main statistical analysis applying one-way ANOVA and appropriate multiple comparison tests (see, data description in Results section for details). Tumor growth inhibition (TGI) was calculated as $TGI\ [\%] = 100 - \left[\left(\frac{TV_X^D}{TV_{ctrl}^D}\right) \times 100\right]$, where $TV_X{}^D$ refers to mean tumor volume in group X at day D and $TV_{ctrl}{}^D$ refers to mean tumor volume in the control group at the same day. The hypothetical outcome from the combined treatment was calculated with the formula based on Peters' approach [14] $\%HYPO = 100 - [(100 - \%\,\textbf{\textit{Doxo}}) \times (100 - \%\,\textbf{\textit{NAC}} - \textbf{d}\textbf{\textit{MBITC}})]/100$, where %**Doxo** and %**NAC-*d*MBITC** refers to the biological effect of single compounds treatment (tumor growth inhibition). GraphPad Prism 7.03 (GraphPad Software, Inc., La Jolla, USA) was used in all statistical analyses; *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. 3,4-dimethoxybenzyl isothiocyanate increases doxorubicin activity on doxorubicin-resistant colon cancer cell line

Out of over 120 naturally occurring isothiocyanates [1], based on our comprehensive studies, we have selected 3,4-dimethoxybenzyl isothiocyanate (***d*MBITC**) for further studies as the most promising anticancer agent. LoVo and LoVoDX cells were treated *in vitro* with ***d*MBITC** at various concentrations for an hour, followed by medium replacement with a fresh one and cell growth assessment by SRB assay after next 72 h. Such an approach reflects isothiocyanates high metabolism and excretion *in vivo* [15]. Our studies revealed a significantly different dose-response curve than that for the continuous treatment in both cell lines (Suppl. Fig. 1). The 1 h pre-treatment with ***d*MBITC** efficiently inhibited LoVo and LoVoDX cell growth with IC_{50} 8.4 ± 1.7 and 9.0 ± 1.4 μM respectively. At the same time, continuous treatment with the isothiocyanate resulted in IC_{50} 4.2 ± 0.5 and 3.5 ± 0.7 μM in LoVo and LoVoDX, respectively. In all subsequent *in vitro* experiments cells were pretreated for 1 h with ***d*MBITC** at a sub-optimal, 5 μM concentration, which influenced cell growth in a limited manner: cell growth inhibition did not exceed 10%. This pretreatment with the isothiocyanate was followed by a treatment with **Doxo** for a time indicated in a description of each experiment.

The LoVoDX cells pretreated with 5 μM ***d*MBITC** exhibited a significantly higher sensitivity to doxorubicin. The doxorubicin IC_{50} value decreased over 3 times for pretreated cells (4.5 ± 1.1 μM) from 14.6 ± 2.4 μM assessed for non-pretreated LoVoDX cells and the related dose-response curves differed significantly (as evidenced by extra sum-of-squares F test; Fig. 2A). This increased activity observed in the antiproliferative assay was reflected in the pro-apoptotic assay where ***d*MBITC-**pretreated LoVoDX cells exhibited a significantly higher caspase-3 activity, regardless of **Doxo** concentrations used (Fig. 2B). The highest increase in caspase-3 activity was observed for samples treated with 10 μM **Doxo**. At the same time, no such activity increase was observed for drug-sensitive LoVo cell line and dose-response curves obtained on this cell line did not differ significantly (Fig. 2 **C**). The IC_{50} value for ***d*MBITC**-pretreated cells was even slightly higher than for **Doxo**-only treated cells (0.56 ± 0.12 μM and 0.41 ± 0.14 μM, respectively), and caspase-3 activity did not change significantly after ***d*MBITC** pretreatment (Fig. 2C–D).

3.2. *d*MBITC influence Doxo intracellular accumulation in LoVoDX cells

***d*MBITC** failed to increase **Doxo** activity in drug-sensitive LoVo cells, which suggests that its advantageous influence is associated with doxorubicin intracellular accumulation impaired in LoVoDX by an increased expression of multi-drug resistance proteins [6], including P-gp. In the cell-free assay for P-gp activity (Fig. 3A) both **Doxo** and **NAC-*d*MBITC** (the final product of ***d*MBITC** metabolism *via* mercapturic acids pathway) proved to be substrates for P-gp efflux pump, indicated by an increased P-gp activity in the presence of the compounds. Since ***d*MBITC**, like other isothiocyanates [16] can accumulate at very high, milimolar concentrations, it is highly plausible that it impairs **Doxo** efflux, and thus, increases cytostatic intracellular concentrations and the resulting antiproliferative activity. In order to evaluate this hypothesis, cells pretreated with culture medium, 5 μM ***d*MBITC** or 20 μM Verapamil (**Ver**, a known P-gp substrate) for 1 h, were incubated with **Doxo** at various concentrations for 3 h, and drug intracellular level was assessed by flow cytometry (Fig. 3B). Both ***d*MBITC** and **Ver** significantly increased the **Doxo** intracellular level regardless of the cytostatic concentration used. The mean fluorescence (MFU) observed in the case of ***d*MBITC**-pretreated samples was *c.a.* 2-times higher in comparison to samples pretreated with culture medium. In both cases **Ver** was slightly more potent as doxorubicin efflux inhibitor. It should be noted that (regardless of concentrations) **Doxo** intracellular levels, even after pretreatment with ***d*MBITC,** were at least 2-times lower than in LoVo cells treated with **Doxo**. Both ***d*MBITC** and **Ver** had limited influence on **Doxo** accumulation in the LoVo cell line (data not shown) since their activity in this matter arises mainly from direct interactions with MDR pumps, whose expression in the LoVo cell line is negligible [6].

Further studies indicated that increased **Doxo** intracellular retention resulted in significantly higher reactive oxygen species production (Fig. 3C) and a slightly reduced glutathione level (Fig. 3D). The results indicate that ***d*MBITC** acting as a **Doxo** efflux competitive inhibitor increases the cytostatic intracellular level and renders its molecular targets more prone to modulations.

3.3. *d*MBITC increases Doxo effectiveness in vivo on LoVoDX colon cancer model

The **dMBITC** beneficial influence on **Doxo** biological activity was evaluated on NOD/SCID mice bearing subcutaneously implanted LoVoDX cells. The LoVoDX tumors growth kinetics in each experimental group is presented in Fig. 4A. **Doxo** administered *i.v.* once a week (1 mg/kg b.w.) influenced tumor growth in a limited manner – the TGI did not exceed 25% at any day, whereas the combined treatment with ***d*MBITC** administered every day caused significantly higher tumor growth inhibition exceeding 50% during the entire experiment (Fig. 4B). Tumors were significantly smaller in the combined treatment

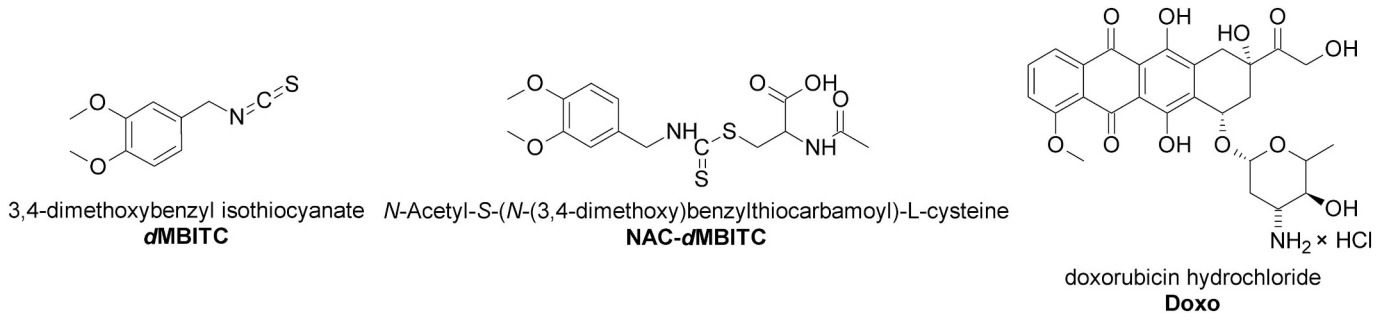

Fig. 1. Chemical structure of the compounds used in this study.

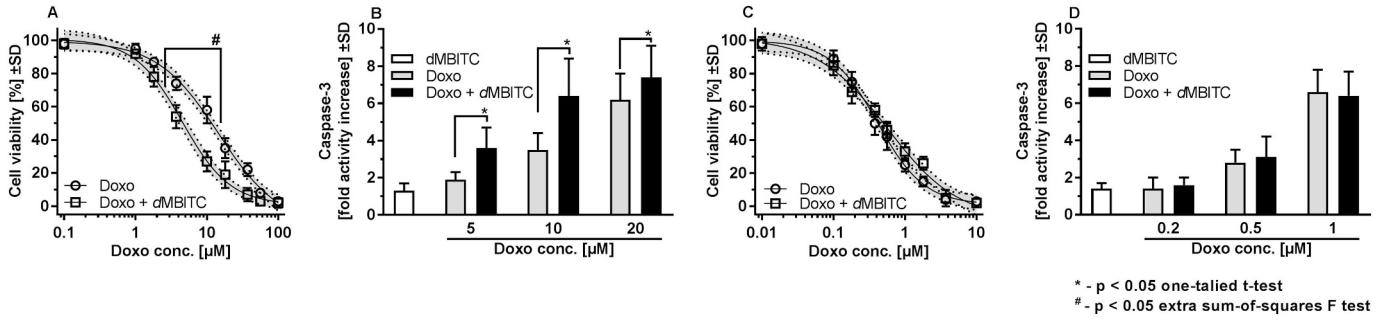

Fig. 2. Antiproliferative (A, C) and proapoptotic potential (B, D) of **Doxo**, ***d*MBITC** and the combined treatment on LoVoDX (A, B) and LoVo (C, D). In all experiments cells were pretreated with 5 μM ***d*MBITC** for 1 h and further incubated with the indicated concentrations of **Doxo** for 72 h and 24 h for antiproliferative and proapoptotic tests, respectively. Shaded areas on figs. A and C represent 99% confidence bands for dose-response curves calculated in GraphPad Prism 7.03 software using [inhibitor] *vs.* response equation (four parameters).

group since day 19 in comparison to the vehicle-treated control (**Ctrl**) and since day 26 in comparison to the **Doxo**-treated group ($p < 0.05$, one-way ANOVA with Dunnett's test for multiple comparisons). The hypothetical, additive outcome, calculated with Peter's method, ranged from 45 to 50% (dashed curve on Fig. 4B), thus, the observed effect of combined treatment can be interpreted as slightly synergistic. An analogous treatment regime (**Doxo** alone, 1 mg/kg b.w., *i.v.*, 4 doses) applied for LoVo bearing NOD/SCID mice resulted in 55 to 69% tumor growth inhibition (**Suppl. Fig. S2**), revealing that the combined treatment applied for the LoVoDX model almost completely abrogated cells resistance.

3.4. *dMBITC attenuates Doxo toxicity in vivo*

Mice body weight measurement conducted during the course of the experiment revealed significant **Doxo** toxicity, which was indicated by high body weight loss exceeding 10% of initial mice body weight and visible changes in animals behavior from day 22 (Fig. 4C). At the same time, mice treated with ***d*MBITC** or its combination with **Doxo** retained good health and normal behavior, despite the initial 3–7% body weight loss on days 13–15. This positive influence of ***d*MBITC** on **Doxo** toxicity was further supported by *post mortem* analysis of major organs weight, blood morphological and biochemical parameters (Fig. 5). The weight of all major organs was significantly reduced in the **Doxo**-treated group, whereas the weight in ***d*MBITC** and combination-treated mice remained similar to control and was significantly higher in comparison to **Doxo**. Subsequent plasma biochemical analyses confirmed doxorubicin cardiotoxicity (indicated by elevated levels of creatinine phosphokinase, especially its MB isoform) and hepatotoxicity (increased levels of alanine and aspartate transaminases). In both cases, combined treatment significantly reduced the level of toxicity markers to those observed in a vehicle treated control group, which supported our previous observations about the beneficial influence of ***d*MBITC** on **Doxo** toxicity. Kidneys function markers (creatinine and urine levels) were influenced in a smaller manner. However, combined treatment caused a significant decrease in the erythrocytes and hemoglobin level as well as an increased level of platelets indicating that in the case of the prolonged application of this regime, its periodic withdrawal for recovery might be required. It should also be noted that the combined treatment increased the overall leukocytes level but failed to reverse **Doxo**-induced increase in granulocytes fraction. In the tumor bearing control NOD/SCID mice lymphocytes percentage was about 60–68% with

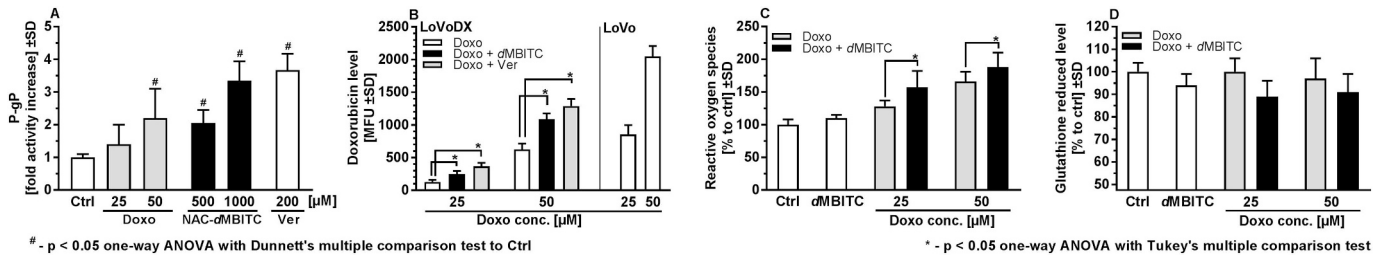

Fig. 3. Mechanism of the combined treatment arises from doxorubicin increased intracellular efflux associated with increased ROS production and reduced glutathione level. (A) Compounds influence P-gP pumps activity in a cell-free assay. **Doxo** intracellular accumulation (B) or its influence on ROS (c) and GSH (D) when used alone or in a combination with 5 μM ***d*MBITC** or 20 μM **Ver** (1 h pretreatment followed by 3 h of treatment with **Doxo**) in the LoVoDX cell line.

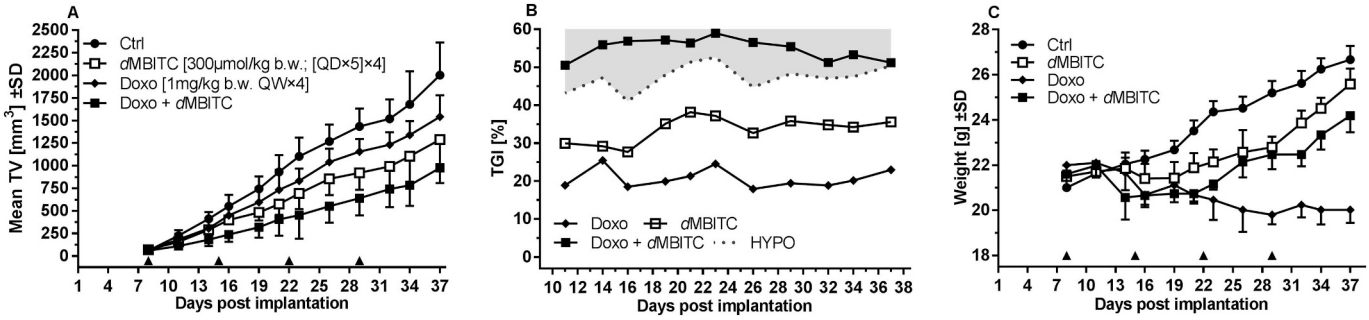

Fig. 4. *In vivo* evaluation of the combined treatment efficacy in LoVoDX tumor-bearing (*s.c.*) NOD/SCID female mice. (A) Mean tumor volume measured in the course of the experiment was used for tumor growth inhibition calculation (B). Mice body weight (C) was monitored through the entire experiment. ▲ indicates **Doxo** administration (1 mg/kg b.w., *i.v.*).

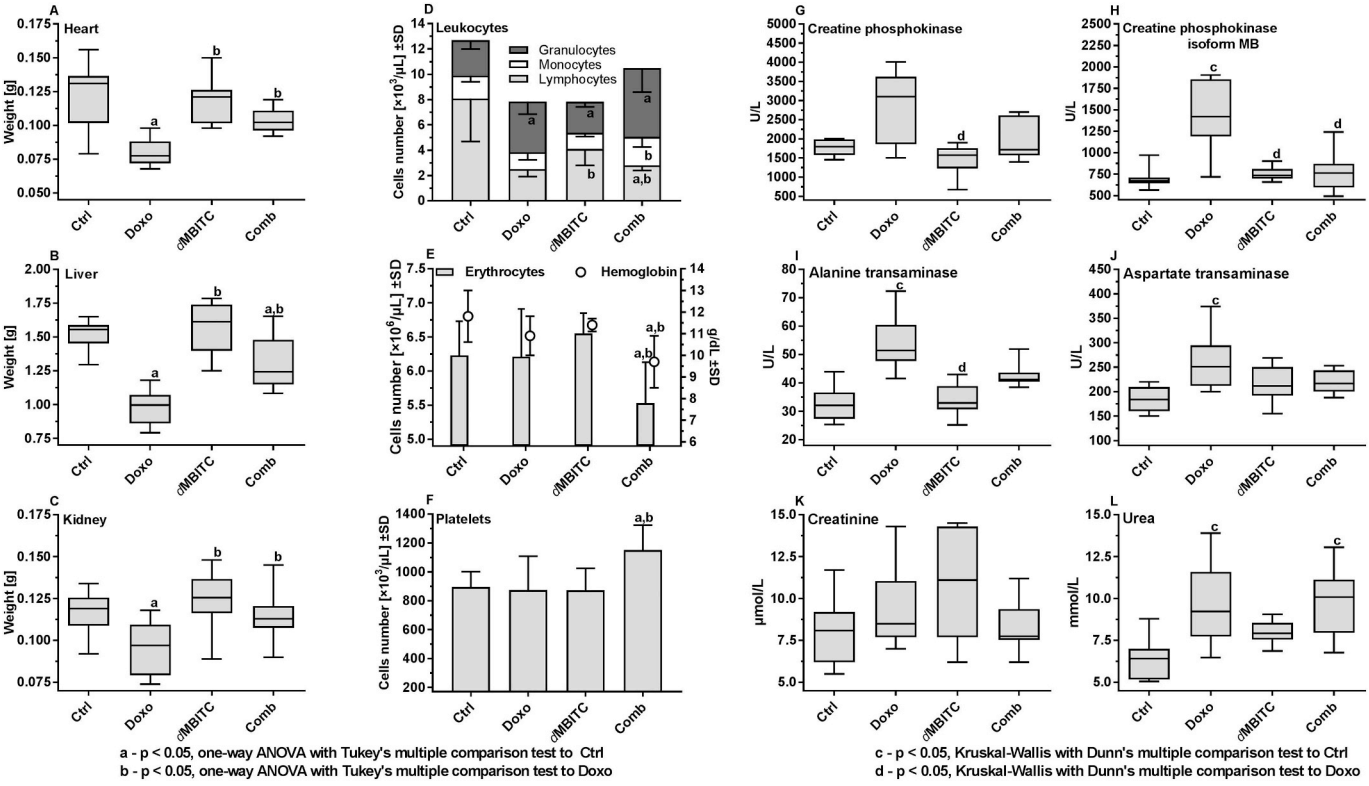

Fig. 5. *Post mortem* analysis of major organs weight (A-C, left column), morphological (D-F, middle column) and biochemical parameters (G-L, right columns) of blood derived from LoVoDX tumor-bearing (*s.c.*) NOD/SCID female mice. Whiskers in box and whiskers convention indicates minimal and maximal values observed within a group.

granulocytes percentage not exceeding 25%, whereas in both groups receiving **Doxo** lymphocytes percentage a significant decline to 27–32% was accompanied by a significantly increased percentage of granulocytes to 50–52%.

4. Discussion

Combined treatment regimens are widely used in oncology, since they provide an opportunity to target several different attributes of cancer at once, which results in an significantly enhanced treatment efficacy. Naturally occurring compounds, especially those abundant in human diet, are particularly interesting candidates for such approach, since their incorporation into the treatment scheme might be accomplished simply by diet optimization or supplementation (functional food). Compounds like curcumin, quercetin, resveratrol, betulinic acid and many others proved to possess valuable biological potential accompanied by relatively low toxicity (especially in comparison to some commonly used cytostatics) [17,18]. Many of these compounds were evaluated *in vitro* and/or *in vivo* as potential partners for classical chemotherapeutics readily enhancing their effectiveness [19,20] as well as potentially cardioprotective agents against doxorubicin associated cardiotoxicity [21,22].

A list of naturally occurring compounds with a high potential in combined therapy encompasses also naturally occurring isothiocyanates [8]. This class of relatively simple compounds includes several sub-types characterized by the presence of isothiocyano moiety associated with different chemical groups (from simple aliphatic, olefins to substituted aromatic ring-containing compounds) [1]. 3,4-dimetoksybenzyl isothiocyanate (***d*MBITC**) belongs to this last group and in our studies was identified as one of the most potent representative of the isothiocyanates tested so far, which is why it was chosen for studies on combined therapy. In the *in vivo* colon cancer model (LoVoDX), ***d*MBITC** synergistically increased **Doxo** efficacy from *c.a.* 25% to 50% of tumor growth inhibition. The observed effect is slightly less

pronounced than those observed in Eisa et al. studies on phenethyl isothiocyanate in Ehrlich ascites carcinoma (EAC, *s.c.*) [23] or Bose et al. studies on sulforaphane in the breast cancer model (13,762 MAT B III, *ort.*) [24]. We are the first to provide evidence that naturally occurring isothiocyanate can modulate doxorubicin efficacy in doxorubicin-resistant cells (LoVoDX) *in vivo*. The isothiocyanate co-treatment almost completely abrogated the resistant character of LoVoDX cells, as it is indicated by the comparable cytostatic output on LoVo (when used alone) and LoVoDX (when used in a combination) *in vivo*. Moreover, isothiocyanates used in the mentioned studies differ substantially. These results taken together provide evidence that the beneficial influence on doxorubicin treatment is a common feature of all isothiocyanates and as such can be obtained with the use of the mixture of them, preferentially through cruciferous vegetables-rich diet.

Such a conclusion is further supported by the cardioprotective feature observed for sulforaphane [24–26], phenethyl isothiocyanate [23] and 3,4-dimetoksybenzyl isothiocyanate in this study. This phenomenon was evident both *in vitro* and *in vivo* by cardiac functions preservation [24], reduced oxidative stress level (*e.g.* reduced serum malondialdehyde (MDA) level or 4-HNE adducts) [23,25], reduced level of troponin [25], improved mitochondrial function associated with the increased nuclear factor erythroid 2-related factor 2 (Nrf2) activation in H9c2 (rat cardiomyoblasts) cell line [25]. The Nfr2 activation is the most likely phenomenon responsible for isothiocyanates' cardio- and hepatoprotective properties. In our studies, doxorubicin toxicity was observed as significantly reduced mice body weight which corelates with a dramatic decrease in main organ weight and an increase in biochemical indicators of cardiotoxicity (creatinine phosphokinase and its heart-specific MB isoform) [27] and hepatotoxicity (alanine and aspartate transaminases). For all of these parameters, a significant improvement was observed when doxorubicin was used in combination with 3,4-dimetoksybenzyl isothiocyanate. The joint results from all these studies provide strong evidence for cardioprotective features of several different isothiocyanates available in cruciferous vegetables.

The multimodal character of isothiocyanate biological activity provides at least several different possible mechanisms that synergistically influence **Doxo** antiproliferative activity. Both antracyclins and isothiocyanates are potent apoptosis inducers and this mechanism was positively evaluated *via* TUNEL assay, active caspase-3 level and phosphatidylserine exposure in most of the previous reports [23,28,29] and further confirmed in our study. However, we observed that ***d*MBITC** significantly increases **Doxo** effectiveness only in adriamycin-resistant subline LoVoDX, but not in the parental LoVo cell line. Distinctive features of LoVoDX are an increased multidrug-resistance pumps (MDRP) expression and a slightly increased level in glutathione reduced intracellular pool [6] associated with over 30-fold increase in **Doxo** IC_{50} value [11]. Isothiocyanates can readily accumulate in cells' cytosol at milimolar concentrations [30] as a result of their fast conjugation with glutathione [31], being at the same time a substrate for MDRP [32]. Thus, they can attenuate other drugs efflux even when used at low concentrations. Hu K. and Morris ME. evaluated certain isothiocyanates (but not the related amines and isocyanates) as potent enhancers of daunomycin (doxorubicin analog) accumulation in MCF-7/ADR cells [33] and their influence on efflux pumps was postulated as a major mechanism. However, since doxorubicin and the related compounds are co-transported with GSH in a 1:1 stoichiometric ratio [34], the impact of isothiocyanates on glutathione pool is probably a secondary mechanism associated with this feature. We only observed in our studies an increased **Doxo** intracellular retention in the resistant subline, thus, the involvement of the above mentioned mechanisms is plausible.

5. Conclusions

Classical chemotherapeutics such as doxorubicin, despite its relatively high toxicity, remain widely used in oncology. The results of our study indicate that naturally occurring isothiocyanates, represented here by 3,4-dimetoxybenzyl isothiocyanate exhibit a capability of increasing doxorubicin efficacy in the doxorubicin-resistant human colon adenocarcinoma model by attenuated drug efflux, increased reactive oxygen species production and an increased rate of apoptosis. At the same time, the combined treatment exhibited a significantly lowered toxicity *in vivo* in comparison to doxorubicin administered alone. The evaluated approach provides evidence for isothiocyanates' usefulness as an element of doxorubicin-resistant colon cancer. Since isothiocyanates' clinically relevant doses can be potentially acquired by a diet switch towards cruciferous vegetables-based cuisine, the presented results warrant further studies on this matter.

Declaration of Competing Interest

All authors declare no conflicts of interest.

Acknowledgements

Funding: This work was supported by the Polish National Science Centre (grant no. UMO-2011/01/N/NZ4/03361). JO acknowledges financial support from Wroclaw University of Science and Technology (statute funds no. 0401/0195/17).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.06.005.

References

[1] J.W. Fahey, A.T. Zalcmann, P. Talalay, The chemical diversity and distribution of glucosinolates and isothiocyanates amoung plants, Phytochemistry 56 (2001) 5–51, https://doi.org/10.1016/S0031-9422(00)00316-2.
[2] T. Shibata, Y. Kimura, A. Mukai, H. Mori, S. Ito, Y. Asaka, S. Oe, H. Tanaka, T. Takahashi, K. Uchida, Transthiocarbamoylation of proteins by thiolated isothiocyanates, J. Biol. Chem. 286 (2011) 42150–42161, https://doi.org/10.1074/jbc.M111.308049.
[3] L. Mi, A.J. di Pasqua, F.L. Chung, Proteins as binding targets of isothiocyanates in cancer prevention, Carcinogenesis 32 (2011) 1405–1413, https://doi.org/10.1093/carcin/bgr111.
[4] B. Abbaoui, C.R. Lucas, K.M. Riedl, S.K. Clinton, A. Mortazavi, Cruciferous vegetables, isothiocyanates, and bladder cancer prevention, Mol. Nutr. Food Res. 62 (2018) 1800079, , https://doi.org/10.1002/mnfr.201800079.
[5] K. Renu, V.G. Abilash, P.B. Tirupathi Pichiah, S. Arunachalam, Molecular mechanism of doxorubicin-induced cardiomyopathy – an update, Eur. J. Pharmacol. 818 (2018) 241–253, https://doi.org/10.1016/j.ejphar.2017.10.043.
[6] O. Wesołowska, J. Wiśniewski, K. Środa, A. Krawczenko, A. Bielawska-Pohl, M. Paprocka, D. Duś, K. Michalak, 8-prenylnaringenin is an inhibitor of multidrug resistance-associated transporters, P-glycoprotein and MRP1, Eur. J. Pharmacol. 644 (2010) 32–40, https://doi.org/10.1016/j.ejphar.2010.06.069.
[7] F. Bianchini, H. Vainio, Isothiocyanates in cancer prevention, Drug Metab. Rev. 36 (2004) 655–667, https://doi.org/10.1081/DMR-200033468.
[8] L. Mielczarek, Z. Chilmonczyk, P. Suchocki, P. Wroczyński, K. Wiktorska, Combinations of isothiocyanates with drugs – a chance or threat to chemoprevention and cancer treatment? Acta Pol. Pharm. - Drug Res. 75 (2018) 829–841, https://doi.org/10.32383/appdr/78769.
[9] M. Vermeulen, B. Zwanenburg, G.J.F. Chittenden, H. Verhagen, Synthesis of isothiocyanate-derived mercapturic acids, Eur. J. Med. Chem. 38 (2003) 729–737, https://doi.org/10.1016/S0223-5234(03)00141-7.
[10] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, J.T. Warren, H. Bokesch, S. Kenney, M.R. Boyd, New colorimetric cytotoxicity assay for anticancer-drug screening, JNCI J. Natl. Cancer Inst. 82 (1990) 1107–1112, https://doi.org/10.1093/jnci/82.13.1107.
[11] M. Psurski, Ł. Janczewski, M. Świtalska, A. Gajda, T.M. Goszczyński, J. Oleksyszyn, J. Wietrzyk, T. Gajda, Novel phosphonate analogs of sulforaphane: synthesis, in vitro and in vivo anticancer activity, Eur. J. Med. Chem. 132 (2017) 63–80, https://doi.org/10.1016/j.ejmech.2017.03.028.
[12] D. Nevozhay, Cheburator software for automatically calculating drug inhibitory concentrations from in vitro screening assays, PLoS One 9 (2014) e106186, , https://doi.org/10.1371/journal.pone.0106186.
[13] C. Leys, C. Ley, O. Klein, P. Bernard, L. Licata, Detecting outliers: do not use standard deviation around the mean, use absolute deviation around the median, J. Exp. Soc. Psychol. 49 (2013) 764–766, https://doi.org/10.1016/j.jesp.2013.03.013.
[14] I.V. Bijnsdorp, E. Giovannetti, G.J. Peters, Analysis of drug interactions, Methods

Mol. Biol. 731 (2011) 421–434, https://doi.org/10.1007/978-1-61779-080-5_34.
[15] E. Lamy, C. Scholtes, C. Herz, V. Mersch-Sundermann, Pharmacokinetics and pharmacodynamics of isothiocyanates, Drug Metab. Rev. 43 (2011) 387–407, https://doi.org/10.3109/03602532.2011.569551.
[16] Y. Zhang, Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells, Carcinogenesis 21 (2000) 1175–1182, https://doi.org/10.1093/carcin/21.6.1175.
[17] S. Nobili, D. Lippi, E. Witort, M. Donnini, L. Bausi, E. Mini, S. Capaccioli, Natural compounds for cancer treatment and prevention, Pharmacol. Res. 59 (2009) 365–378, https://doi.org/10.1016/J.PHRS.2009.01.017.
[18] P. Kuppusamy, M.M. Yusoff, G.P. Maniam, S.J.A. Ichwan, I. Soundharrajan, N. Govindan, Nutraceuticals as potential therapeutic agents for colon cancer: a review, Acta Pharm. Sin. B 4 (2014) 173–181, https://doi.org/10.1016/J.APSB.2014.04.002.
[19] A. Lodi, A. Saha, X. Lu, B. Wang, E. Sentandreu, M. Collins, M.G. Kolonin, J. DiGiovanni, S. Tiziani, Combinatorial treatment with natural compounds in prostate cancer inhibits prostate tumor growth and leads to key modulations of cancer cell metabolism, Npj Precis. Oncol. 1 (2017) 18, , https://doi.org/10.1038/s41698-017-0024-z.
[20] A. Rejhová, A. Opattová, A. Čumová, D. Slíva, P. Vodička, Natural compounds and combination therapy in colorectal cancer treatment, Eur. J. Med. Chem. 144 (2018) 582–594, https://doi.org/10.1016/j.ejmech.2017.12.039.
[21] S. Ojha, H. Al Taee, S. Goyal, U.B. Mahajan, C.R. Patil, D.S. Arya, M. Rajesh, Cardioprotective potentials of plant-derived small molecules against doxorubicin associated cardiotoxicity, Oxidative Med. Cell. Longev. 2016 (2016) 5724973, , https://doi.org/10.1155/2016/5724973.
[22] M. Songbo, H. Lang, C. Xinyong, X. Bin, Z. Ping, S. Liang, Oxidative stress injury in doxorubicin-induced cardiotoxicity, Toxicol. Lett. 307 (2019) 41–48, https://doi.org/10.1016/J.TOXLET.2019.02.013.
[23] N.H. Eisa, N.M. El Sherbiny, A.M. Shebl, L.A. Eissa, M.M. El-Shishtawy, Phenethyl isothiocyanate potentiates anti-tumour effect of doxorubicin through Akt-dependent pathway, Cell Biochem. Funct. 33 (2015) 541–551, https://doi.org/10.1002/cbf.3153.
[24] C. Bose, S. Awasthi, R. Sharma, H. Beneš, M. Hauer-Jensen, M. Boerma, S.P. Singh, Sulforaphane potentiates anticancer effects of doxorubicin and attenuates its cardiotoxicity in a breast cancer model, PLoS One 13 (2018) e0193918, , https://doi.org/10.1371/journal.pone.0193918.
[25] P. Singh, R. Sharma, K. McElhanon, C.D. Allen, J.K. Megyesi, H. Beneš, S.P. Singh, Sulforaphane protects the heart from doxorubicin-induced toxicity, Free Radic. Biol. Med. 86 (2015) 90–101, https://doi.org/10.1016/j.freeradbiomed.2015.05.028.
[26] B. Li, D.S. Kim, R.K. Yadav, H.R. Kim, H.J. Chae, Sulforaphane prevents doxorubicin-induced oxidative stress and cell death in rat H9c2 cells, Int. J. Mol. Med. 36 (2015) 53–64, https://doi.org/10.3892/ijmm.2015.2199.
[27] S.H. Kim, K.-J. Kim, J.-H. Kim, J.-H. Kwak, H. Song, J.Y. Cho, D.Y. Hwang, K.S. Kim, Y.-S. Jung, Comparision of doxorubicin-induced cardiotoxicity in the ICR mice of different sources, Lab. Anim. Res. 33 (2017) 165–170, https://doi.org/10.5625/lar.2017.33.2.165.
[28] S. Mukherjee, S. Dey, R.K. Bhattacharya, M. Roy, Isothiocyanates sensitize the effect of chemotherapeutic drugs via modulation of protein kinase C and telomerase in cervical cancer cells, Mol. Cell. Biochem. 330 (2009) 9–22, https://doi.org/10.1007/s11010-009-0095-4.
[29] Q. Fan, X. Zhan, Z. Xiao, C. Liu, Phenethyl isothiocyanate enhances adriamycin-induced apoptosis in osteosarcoma cells, Mol. Med. Rep. 12 (2015) 5945–5950, https://doi.org/10.3892/mmr.2015.4187.
[30] Y. Zhang, The molecular basis that unifies the metabolism, cellular uptake and chemopreventive activities of dietary isothiocyanates, Carcinogenesis 33 (2012) 2–9, https://doi.org/10.1093/carcin/bgr255.
[31] A. Adesida, L.G. Edwards, P.J. Thornalley, Inhibition of human leukaemia 60 cell growth by mercapturic acid metabolites of phenylethyl isothiocyanate, Food Chem. Toxicol. 34 (1996) 385–392, https://doi.org/10.1016/0278-6915(96)00124-X.
[32] E.C. Callaway, Y. Zhang, W. Chew, H.-H.S. Chow, Cellular accumulation of dietary anticarcinogenic isothiocyanates is followed by transporter-mediated export as dithiocarbamates, Cancer Lett. 204 (2004) 23–31, https://doi.org/10.1016/J.CANLET.2003.09.021.
[33] K. Hu, M.E. Morris, Effects of benzyl-, phenethyl-, and α-naphthyl isothiocyanates on P-glycoprotein- and MRP1-mediated transport, J. Pharm. Sci. 93 (2004) 1901–1911, https://doi.org/10.1002/jps.20101.
[34] M. Salerno, A. Garnier-Suillerot, Kinetics of glutathione and daunorubicin efflux from multidrug resistance protein overexpressing small-cell lung cancer cells, Eur. J. Pharmacol. 421 (2001) 1–9, https://doi.org/10.1002/jps.20101.