ORIGINAL ARTICLE

Age-dependence of Poly(Adenosine Diphosphate-Ribos)yl Polymerase 1 Inhibition in Liver and Thymocyte Nuclei after the Treatment of Rats with Cisplatin

Anush L Asatryan*, Irina G Artsruni, Karine S Matinyan, Emil S Gevorgyan

Department of Biophysics, Faculty of Biology, Yerevan State University, Yerevan, Armenia

Abstract

Objective: Poly(ADP-ribos)yl polymerase 1 (PARP1) inhibitors enter into clinical trials for mono and combination cancer chemotherapy, improving curative potential of DNA-alkylating agents. It is documented that therapeutic outcomes after the treatment of patients with broad spectrum of drugs are age-dependent and reveal sexual dimorphism. We investigated age- and sex-dependent differences in PARP1 inhibition by benzamide (Bam) and adenosine triphosphate (ATP) in rat liver and thymocyte nuclei after the treatment of rats with cisplatin. **Materials and Methods:** The drug was injected intraperitoneal. Animals were treated according to the regulations of National Centre of Bioetics (Armenia). Cell nuclei were isolated according to the standard procedure. PARP 1 activity was evaluated by nicotinamide adenine dinucleotide (NAD)+ consumption. Data are expressed as mean ± S.D. Statistical differences in the results between groups were evaluated by the Student's *t*-test. A probability (*P*) value of <0.05 was considered significant. **Key results:** Basal PARP 1 activity in rat thymocyte and liver nuclei decreases in the period of animal growth from 6 to 10 weeks. The treatment of intact rat with cisplatin differentially affected PARP 1 activity in liver and thymocyte nuclei. It was shown that efficiency of PARP1 inhibition in thymocyte and liver nuclei by Bam and ATP is age-dependent phenomenon which is affected by *in vivo* treatment of intact rat with cisplatin. It is suggested that the design of personalized combination therapy regimen should consider cisplatin induced age-dependent changes in PARP 1 inhibition by competing/allosteric inhibitors.

Key words: Adenosine triphosphate, Age-dependence, Benzamide, Cell nuclei, Cisplatin, Poly(ADP-ribos)yl polymerase 1 inhibition

INTRODUCTION

Cisplatin [cis-diammine-1,1-cyclobutanedicarboxylate platinum (II)] is one of the most commonly used antitumor drugs and is recognized as highly effective cytotoxic agent, which forms DNA-adducts due to chemical modification of nuclear DNA hindering cell proliferation and tumor growth. Cells immediately respond to DNA damage with post-translational poly(ADP-ribos)ylation (PARylation) of proteins that are involved in DNA damage repair. Poly(ADP-ribos)yl polymerase 1 (PARP 1), PARP 2, and PARP 3 are DNA-damage dependent members of PARP family located in cell nuclei. Activity of these enzymes is promptly stimulated by DNA-damage or non-canonical DNA structures. However, a pivotal role in burst of PAR production is addressed to PARP 1. Activation of PARP 1 after genotoxic stress consumes most of the nicotinamide adenine dinucleotide (NAD)+ in a cell (more than 90%). PARP 1 performs sequential addition of adenosine diphosphate (ADP)-ribose units cleaved from substrate NAD+ to mainly itself and to adjacent protein molecules. PARP 1 modified proteins harbor long branched chains of poly(ADP-ribose) moieties, which substantially affect the net negative charge of acceptor molecule. PAR modification is reversible and it is proposed that once

Address for correspondence:
Anush Asatryan, Department of Biophysics, Faculty of Biology, Yerevan State University, Yerevan, Armenia.
Mobile: +37491180810. E-mail: anush.asatryan@ysu.am

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other repair proteins have localized to the damaged DNA, PAR needs to be removed before repair can take place.

PARylation of chromatin-associated proteins is engaged in regulation of gene expression, broadly changes cellular physiology, and directs metabolic processes toward cell survival or death, dictating responses to chemotherapy.[1] Tumors may harbor high numbers of non-proliferating cells, potentially making them less sensitive to agents that selectively target dividing cells. In general, mechanisms underlying tumor resistance are poorly understand and the higher doses of cisplatin are applied in clinical protocols to overcome this obstacle. However, elevating resistance of cancer cells to cisplatin treatment does not simply undermine the curative potential of the drug, but its accumulation in normal tissues and organs leads to development of undesirable side effects: Secondary leukemia, thymomas, and other pathologies of hematopoietic and immune systems considerable time after the drug treatment.[2]

Cellular consequences of the treatment with cisplatin are harmful for immune system breaking into process of T cell differentiation in the thymus gland.[3] One of cisplatin off- target effects is suppression of glucose metabolism in cells.[4,5] This off-target effect of cisplatin on energy metabolism common for all types of cells is potentially harmful regardless health status and can affect drug detoxifying potency of liver. To overcome complications arising from accumulation of cisplatin in host cells, combination therapy with PARP 1 inhibitors is employed in cancer patient therapy.[6]

The drug-drug interactions in the course of combination therapy of cancer patients are complex and entangled with individual physiologic context, maintained by age- and sex-dependent variables. Evaluation of effectiveness of PARPi in anti-cancer therapy should take into consideration possible changes of PARP 1 activity and availability for inhibition after cisplatin treatment.

Bam is PARP inhibitor of first generation, which interacts with the nicotinamide pocket of PARP 1 and acts as competitor of NAD+.[7] Adenosine triphosphate (ATP) selectively inhibits PARP 1 auto-poly(ADP-ribosilation), hinders enzyme dissociation from DNA-binding sites, and "trapped" the enzyme to the DNA.[8,9] PARP 1 "trapping" to the DNA could play prominent role in mechanisms that are responsible for killing of cells.[10]

In the present study, we focused on examination of PARP inhibition by Bam and ATP in rat liver and thymocyte nuclei and collected from cisplatin treated animals of different age and sex.

MATERIALS AND METHODS

Isolation of nuclei from rat liver

Albino inbred male and female rats (6 and 10 weeks old) were used throughout experiments. The animals were standardized by weight in either age group (to 100 g and 150 g in 6 and 10 weeks old correspondingly). All reagents were purchased from Sigma-Auldrich. Vehicle (saline) and cisplatin (10 mg/kg weight) were injected intraperitoneally. Animals were sacrificed under light ether anesthesia by decapitation after 48 h treatment with cisplatin. Liver and thymocyte nuclei were isolated according to.[11] Sucrose solutions utilized throughout the nuclei isolation procedure were buffered with 20 mMTris containing 15 mMNaCl, 60 mMKCl, 0.15 mM spermine, and 0.5 mM spermidine, pH 7.4.

PARP1 activity assay

The enzymatic assay for PARP 1 activity was performed according to the original method based on estimation of residual NAD^+ concentration in PARP assay mix,[12] adapted by us to quantify NAD^+ consumed by isolated nuclei. Briefly, nuclei were gently suspended in PARP assay buffer containing 20 mM Tris, 6 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.4. Density of nuclear suspension was normalized to 1 mg DNA/ml. PARP reaction was initiated by addition of NAD^+ stock solution to 1000 µl aliquot of nuclear suspension (0.5 mM NAD^+ final concentration). The reaction was carried out for 10 min at 37°C followed by centrifugation at 13,000g, 4°C for 2 min to discard nuclear pellet. 50 µl of supernatant was transferred to the Falcon ultraviolet visible transparent 96-well plate. NAD^+ quantitation was performed by sequential addition of 2M KOH, acetophenone (20% in EtOH), and 88% formic acid, in accordance with the original assay. Absorbance of PARP assay mix containing 0.5 mM NAD^+ was measured at 378 nm and set as a standard. The amount of NAD^+ was determined using NAD^+ calibration curve and PARP 1 activity was defined as NAD^+ consumed by nuclei in 10 min per mg of DNA.

Light microscopy and histopathological procedures

The thymus gland and liver were collected from rat, fixed in 10% formalin in saline, and dehydrated in ascending grades of alcohol. Thymus gland and liver were sliced transversely and paraffin embedded for light microscopic examination. Paraffin-embedded sections (5 mkm) were dewaxed with xylenes and stained with hematoxylin and eosin. Histopathological changes in organ were assessed in 30 randomly selected tissue sections from each group studied. Slides were observed under Olympus microscope (Model no. BX51).

DNA electrophoresis

Thymocyte nuclei DNA isolation and electrophoresis were performed according to the standard protocols.[13] Gels were stained with 1 mkg/ml ethidium bromide.

Statistical analysis

Data are expressed as mean ± S.D. Statistical differences in the results between groups were evaluated by the Student's *t*-test. A probability (p) value of >0.05 was considered significant.

RESULTS

In present study, we examined age- and sex-dependent differences in PARP1 inhibition by NAD^+ competing inhibitor benzamide (Bam) and ATP. To circumvent physiological complications and to segregate direct effect of Bam and ATP on PARP1 activity in cell nuclei from pharmacodynamics and pharmacokinetic effects of inhibitors in the case of their administration to hall animals, we introduced inhibitors directly into nuclei incubation media in 15 min before addition of constituents of PARP 1 reaction mixture. Nuclei were isolated from liver and intrathymic thymocyte collected from rats of control and cisplatin treated groups.

Toxicity

Changes in histopathological appearance were revealed in liver collected from 6-week-old male and female rat in 48 h after administration of cisplatin to animals [Figure 1]. Dissolution of liver lobules, degeneration of hepatic cords, and massive periportal fibrosis were observed. The cytoplasm of the hepatocytes contained transparent vacuole relevant to clustered lipids and pronounced steatosis emerged [Figure 1, low panel]. These changes evidenced hepatotoxic effect of cisplatin in young animal. Adult animals exhibit higher resistance to hepatotoxicity of cisplatin in the time interval examined in the present study.

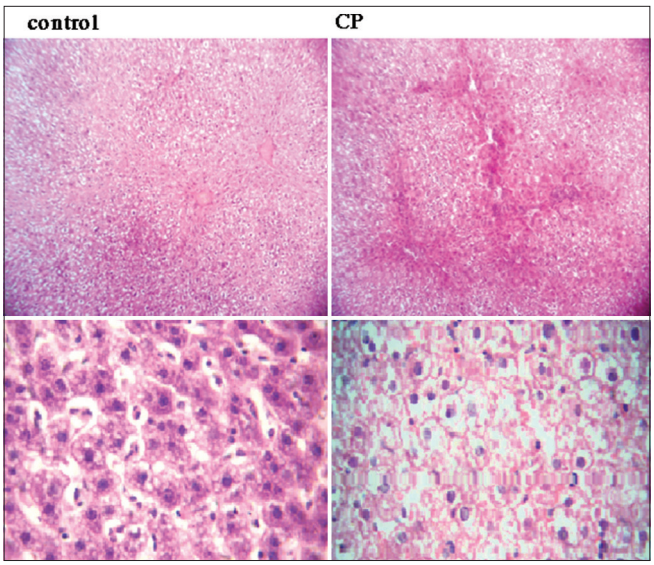

Figure 1: Histopathological changes in liver tissue of 6-week-old rats treated with cisplatin. Upper panel-magnification ×100; lower panel-magnification ×400

During 6–10 weeks of rat growth, thymus weight increased nearly by 70–75% in male and female, respectively. After cisplatin administration to rat, the weight of thymus dramatically decreased in 48 h after administration of the drug. The weight of 6-week-old male thymus decreased by 65%, whilst in females it diminished by 40%. Thymus glands of adults (10 weeks) were less vulnerable and after 48 h of cisplatin injection into intact rat; they demonstrated weight loss by 70% and by 50% in males and females correspondingly. The weight loss of thymus caused by cisplatin administration into rat emerged sex-bias. Reduced weight and size [Figure 2a,b and c] were accompanied by decrease in cortical lymphocytes and loss of corticomedullary demarcation. Thymus of 6-week-old rat treated with cisplatin for 48 h developed distinct dark-staining cortex with small lymphocytes, shows reduction in cortical thickness, and reduced distinction between cortex and medulla. There are patchy areas in cortex where small lymphocyte density reduced. Toxic atrophy of gland was accompanied by the same changes in histological aspect in sex-independent manner.

Although thymus is known to be apoptosis prone and thymocytes exhibited characteristic apoptotic DNA ladder after the *in vivo* treatment of 6-week-old rats with hydrocortisone, the toxic atrophy of the gland was not accompanied by characteristic feature of apoptosis, for

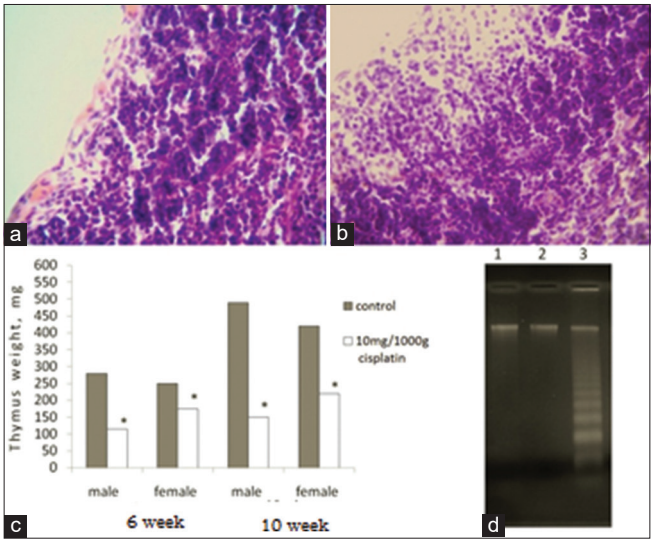

Figure 2: (a) A thymus of 6-week-old rat. (b) Thymus of 6-week-old rat treated with cisplatin for 48 h. (c) Thymus gland of 6-week- and 10-week-old rat shows different weight loss after *in vivo* treatment with cisplatin for 48 h. (d) DNA isolated from thymocyte nuclei of rats (1) Treated with vehicle; (2) With cisplatin 48 h; and (3) 24 h hydrocortizone (10 mkg/1000 g weight). Hydrocortizone was injected peritoneal

example, DNA olygonucleosomal fragmentation [Figure 2d].

Modulations of PARP 1 activity in liver nuclei after administration of cisplatin to intact rat

Sex-dependent differences in basal PARP 1 activity in liver nuclei of 6-week-old rat were not reliable and according to our data; they disappeared during growth. In general, PARP 1 activity decreased nearly 2, 5-fold during the growth from 6 to 10 weeks regardless sex [Figure 3].

Sex-dependent decrease in PARP 1 activity in liver nuclei of rat treated with cisplatin was determined only in young rat (3, 5 and 2, 5 fold in male and female nuclei, respectively). In contrast, administration of cisplatin to 10-week adult rat stimulated PARP 1 activity in liver nuclei more than 2-fold in sex-independent manner [Figure 3].

Effect of inhibitors on PARP 1 activity in liver nuclei

Inhibitory potency of Bam in 10-week-old rat liver nuclei increases nearly 2-fold [Figure 4a]. In contrast, the inhibitory potential of 1 mM ATP (representing low physiological concentration) in the course of rat growth disappeared.

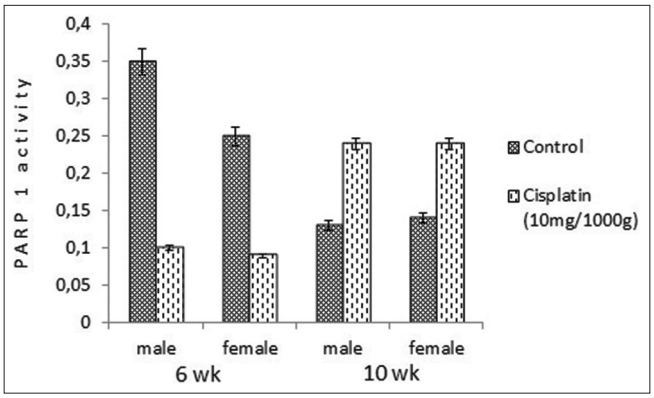

Figure 3: The effect of cisplatin on age-dependent modulationof Poly(ADP-ribos)yl polymerase 1 activity in rat liver nuclei

It seems that age-dependent decline of baseline PARP1 activity in liver nuclei is accompanied with elevated resistance to inhibition by ATP [Figure 4b]. High physiologic concentration of ATP (5 mM) in liver nuclei incubation medium completely suppressed PARP 1 activity in nuclei collected from all examined groups (data not shown). These data demonstrate that there are no sex dependent differences in PARP 1 inhibition in rat liver nuclei by Bam and ATP, whereas reliable age-dependent differences in PARP 1 inhibition were apparent.

In 48 h, after cisplatin administration to rat inhibitory efficacy of Bam did not change in [Figure 4a], whilst efficacy of ATP increased [Figure 4b].

Age- and sex-dependent modulations PARP 1 activity in thymocyte nuclei after *in vivo* treatment of rats with cisplatin

Sex-dependent differences in thymus weight, male, and female thymocytes display nearly the same PARP1 activity. Growth of thymus during 6–10 weeks paralleled with diminution of PARP1 activity nearly by 35% regardless in sex-independent manner.

In 48 h after administration of cisplatin to female rat, PARP 1 activity in thymocyte nuclei displays nearly 40% and 60% stimulation (6-week- and 10-week-old rat correspondingly) [Figure 5].

The results presented in Figure 6 demonstrate that Bam inhibited PARP 1 in thymocytes nuclei of 6-week-old male rat by 30–35%, whilst does not suppress the enzyme activity in thymocyte nuclei collected from female rat. PARP 1 inhibition in thymocyte nuclei of 10-week-old rat was more sensitive to Bam (50–55% inhibition). Thymocyte nuclei display sex- and age-dependent modulation of Bam inhibitory potency.

According to our data, 1 mM ATP (relevant to low physiological concentration), which was added into nuclei incubation media in 15 min before addition of NAD^+ inhibited PARP1 in 6-week-old male and female thymocyte nuclei nearly by 45%, whereas elicited negligible inhibitory

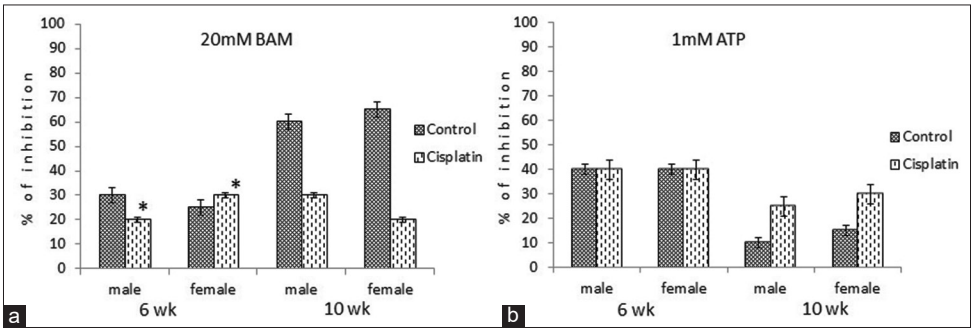

Figure 4: Inhibition of Poly(ADP-ribos)yl polymerase 1 (PARP 1) by BAM (a) and adenosine triphosphate (b) in liver nuclei of rats treated with cisplatin. *$P < 0.05$, PARP 1 activity in nuclei isolated from liver of rat treated with vehicle was set as 100% activity

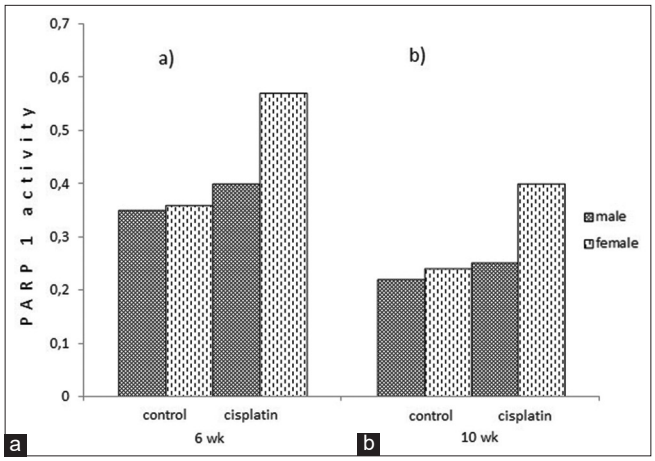

Figure 5: Sex-dependent activation of Poly(ADP-ribos) yl polymerase 1 in thymocytes of rat treated with cisplatin; (a) thymocytes were collected from 6-week-old rat; (b) from 10-week-old rat

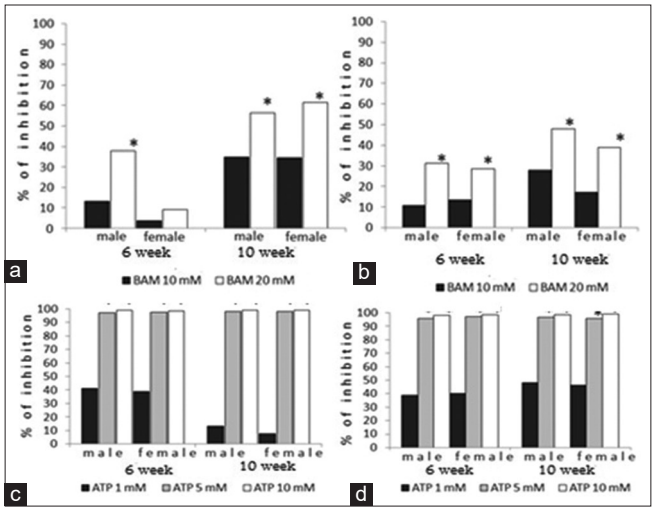

Figure 6: Poly(ADP-ribos)yl polymerase 1 (PARP1) inhibition by Bam and adenosine triphosphate in rat thymocyte nuclei; (a and c) PARP1 inhibition in nuclei of thymocytes collected from animals treated with vehicle; (b and d) nuclei were isolated from thymocytes of rats treated with cisplatin for 48 h

effect in thymocyte nuclei from 10-week-old rat (10% inhibition of PARP 1 activity). Administration of cisplatin to rat did not affect PARP 1 inhibition by ATP in young animal thymocyte nuclei, whilst increased inhibitory potency of ATP in thymus nuclei of 10-week-old rat up to 40% inhibition [Figure 6c and d]. These data demonstrate that inhibition of PARP 1 in thymocyte nuclei by ATP (allosteric inhibitor) is age-dependent and had no sex bias. The treatment with cisplatin caused increase of Bam efficacy by 15–20% in thymocyte nuclei of young female rat, but had no reliable effect on PARP 1 inhibition by Bam in 10-week-old rat thymocyte.

DISCUSSION

PARP 1 plays a prominent role virtually in all chromatin-associated nuclear functions and is the most abundant member of PARP enzyme family localized to nuclei.[14] Pharmacologic inhibition of the enzyme increases cytotoxic potential of cisplatin and benefits therapeutic responses in cancer patient treated with DNA-damaging agents. However, the entrance of poly(ADP-Ribose) polymerase (PARP) inhibitors into clinical trials must be revalidated from the viewpoint of the challenges; they present in integrating them with existing anticancer therapies and should be constrained in patient-tailored chemotherapy.[15]

Sex- and age-related difference in drug pharmacokinetics and pharmacodynamics is widely recognized phenomenon.[16,17,18] The vast majority of PARP 1 inhibitors applied in clinical trials share characteristics of competing inhibitors. It is widely recognized that PARP1 comprises two different activities: Auto- and trans- poly(ADP-ribos)ylation. ATP specifically inhibits auto-poly(ADP-ribos)ylating activity of PARP 1 and is recognized as allosteric inhibitor, whereas Bam is well established competing inhibitor.[19]

In the present study, we focused on age-and sex-dependent modulation of PARP 1 activity and efficacy of Bam and ATP in liver cell and thymocyte nuclei after the *in vivo* treatment of rats with cisplatin. The experimental system employed here (naked nuclei) and minimizes disparities between inhibition kinetics of purified and cellular PARP 1 forms.[20] From the other hand, cell-free system comprising isolated nuclei discriminates non-specific effect of PARP 1 inhibitors on cellular bioenergetic pathways.[21]

PARP1 inhibition is released through two different routes: Disturbance of mechanisms responsible for the enzyme protein binding to coenzymic DNA and second, suppression of catalytic domain function. Bam is well known competing PARP1 inhibitor of first generation, which represents a family of structural analogs of NAD+ employed in clinical trials. NAD+ analogs block the binding of NAD+ to PARP 1 catalytic domain, thereby inhibiting the enzyme.

In 2004, Kun *et al.* suggest bioenergetic model for PARP 1 regulation in cells by ATP.[22] This model emphasized the role of ATP as specific inhibitor of auto-ADP-polyribosylating capacity of PARP 1 in the *in vitro* system.

Our data come to show that PARP1 basal activity in rat thymocyte and liver nuclei declined during animal growth during 6–10 weeks. Age-dependent down-regulation of PARP 1 activity was more significant in liver nuclei (2, 5 fold in liver vs. 35% in thymocyte). We suppose that PARP 1 basal activity decreases due to decline in NAD+ synthesis in the process of aging detected by other investigators.[23] Our data come to show that down-regulation of PARP 1 activity can start in the phase of sexual maturation of young rat. Age-dependent variability bear not only PARP 1 activity, but also affects enzyme inhibition by Bam. Suppression of PARP 1 activity in liver nuclei of rat in

6–10 weeks of growth paralleled with elevation of inhibitory potency of Bam. We suppose that elevation of Bam efficacy is coupled with age-dependent drop in NAD+ content which serves as PARP 1 substrate.[24] It was detected earlier that less actively growing organs of elder animal exhibit low level of auto-poly(ADP-ribos)ylating activity of PARP 1. Our data which indicated on diminished efficacy of PARP 1 inhibition with ATP in liver and thymocyte nuclei of 10-week-old rat are complementary to this observation and come to support hypothesis about age-dependent tuning of PARP 1 auto- and trans-ribosylating activity in cell.

Inhibition of PARP 1 activity with Bam in thymocyte nuclei emerges sex bias only in 6-week-old rat. Sex-depending variation displayed by thymocyte of young rat disappears during sexual maturation in concert with elevation of inhibitory potency of Bam.

In 48 h after administration of cisplatin, we observed changes in liver and thymus morphology of 6-week-old male and female rat. Thymus and of cisplatin treated 6-week-old rat exhibit characteristic features of toxic atrophy, for example, weight loss and dramatic change in histopathologic appearance. Liver morphology also underwent alterations indicating on destruction of tissue architecture and hepatotoxicity in general. Apparently, the organism of adult rat is more resistant to toxic impact of cisplatin and no alterations in liver and thymus histology occurred.

Nevertheless, thymus toxic atrophy induced by cisplatin, PARP 1 activity in 6-week-old female rat thymocyte nuclei increased nearly by 40%. PARP 1 simulation was more significant in 10-week-old female rat thymocyte (nearly by 60%). We suppose that cisplatin-induced PARP 1 activation in female rat thymocyte could be determined by estrogen-induced changes in thymus metabolism documented earlier by Gui *et al.*[25]

Morphological changes in liver of 6-week-old male and female rat indicating on hepatotoxic impact of cisplatin coincided with suppression of PARP 1 activity, whereas the treatment with cisplatin had no appreciable effect on morphology of 10-week-old rat liver. The absence of histopathological changes in liver of adult rat coincided with significant stimulation of PARP 1 activity. We consider that higher detoxifying potential of adult rat liver may be responsible for postponing of tissue damage, which could emerge later than 48 h after cisplatin administration. Less pronounced effect of cisplatin on energy metabolism in liver of mature rat determines prevalence of survival programs coupled with PARP 1 activation.

Physiological status is extremely important and determines the role of PARP 1 as a trigger of cell survival or death programs.[26,27] Cisplatin-induced down-regulation of metabolic activity in 6-week-old rat liver which resulted glucose and NAD+ metabolism inhibition, eventually caused inhibition of PARP 1 activity determined in our study. The data reported here demonstrated that the treatment of rats with cisplatin affected PARP 1 activity in rat liver nuclei in age-dependent manner and in contrast to thymocyte nuclei had no sex bias.

PARP 1 inhibitors often are exploited as a constituent of multidrug therapy of patients. Mounting evidence demonstrates that drug-drug interactions can modify pharmacologic effects and attenuate therapeutic outcomes. We were interested to investigate whether the *in vivo* treatment of hall animal with cisplatin could influence PARP1 inhibition in liver and thymocyte nuclei by Bam and ATP.

Administration of cisplatin increased inhibitory potency of ATP in thymus and liver nuclei of 10-week-old rat regardless animal sex and had no appreciable impact on efficacy of inhibitor in young animal. The treatment with cisplatin caused negligible increase in Bam efficacy by 15–20% in thymocyte nuclei of young female rat. In liver, nuclei treatment with cisplatin did not affect inhibitory potential of Bam. These data demonstrate that cisplatin treatment elicits different impact on efficacy of PARP 1 competing (Bam) and inhibitor responsible for PARP 1 "trapping" (ATP).[28] The results of our investigation come to show that sex bias in curative potential of PARP 1 inhibitors demonstrated by Soldin *et al.* [Soldin *et al.*, 2006] can be determined rather by sex-dependent differences in pharmacokinetics and pharmacodynamics, than pharmocogenetic differences.[29]

CONCLUSION

The data of present investigation come to show that PARP1 basal activity in rat thymocyte and liver nuclei declined during animal growth during 6–10 weeks. It was shown that efficiency of PARP1 inhibition in liver nuclei by competing inhibitor Bam increased in age-dependent manner in contrast to inhibitory potency of allosteric inhibitor ATP. We demonstrated that efficacy of PARP inhibitors in liver and thymocytes nuclei of rats is modulated by treatment of rats with cisplatin. The data reported here demonstrated that the treatment of rats with cisplatin affected PARP 1 activity in rat liver nuclei in age-dependent manner and in contrast to thymocyte nuclei had no sex bias. Administration of cisplatin increased inhibitory potency of ATP in thymus and liver nuclei of 10-week-old rat regardless animal sex and had no appreciable impact on efficacy of inhibitor in young animal. In general our data come to show that age- and sex-dependent variables are extremely important and should be taken into account in design of anti-cancer multi-drug treatment.

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