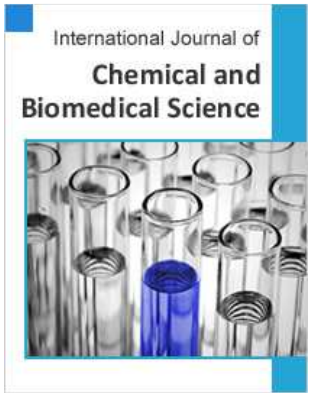
International Journal of Chemical and Biomedical Science
2015; 1(5): 103-108
Published online October 30, 2015 (http://www.aascit.org/journal/ijcbs)

American Association for Science and Technology



Keywords
PARP 1 Inhibition,
Thymocyte Nuclei,
Cisplatin,
Benzamide,
ATP

Received: August 31, 2015
Revised: September 17, 2015
Accepted: September 19, 2015

Age- and Sex-Dependent Modification of the Activity and Inhibition of Poly (ADP-ribose) Polymerase 1 by Cisplatin in the Rat Thymocytes

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Citation

Irina Artsruni, Anush Asatryan, Karine Matinyan, Emil Gevorgyan. Age- and Sex-Dependent Modification of the Activity and Inhibition of Poly (ADP-ribose) Polymerase 1 by Cisplatin in the Rat Thymocytes. *International Journal of Chemical and Biomedical Science*. Vol. 1, No. 5, 2015, pp. 103-108.

Abstract

Poly (ADP-ribose) polymerase 1 (PARP 1) inhibitors improve efficacy of DNA-damaging agents in cancer therapy and currently are tested in various clinical trials. However, the mechanisms responsible for the differences in their curative potential, especially in combination with other drugs are poorly understood. Present study examined age- and sex-dependent differences in PARP 1 inhibition by NAD^+competing inhibitor benzamide (Bam) and allosteric inhibitor ATP in rat thymocyte nuclei. Enzyme inhibition was analyzed after the *in vivo* treatment of pubertal age (6 week old) and young adult (10 week) rats with cisplatin. Cisplatin treatment was found to cause toxic atrophy of thymus manifested by the weight loss and morphological changes. Thymus glands of female animals treated with cisplatin undergo less severe atrophy which coincides with the PARP 1 activation. Efficiency of PARP 1 inhibition in thymocyte nuclei by Bam and ATP was found to be age-dependent and could be modified by the *in vivo* treatment with cisplatin. It is suggested that the design of personalized combination therapy regimen should consider cisplatin induced age-specific changes in PARP 1 inhibition by competing/allosteric inhibitors.

1. Introduction

Poly (ADP-ribose) polymerase 1 (PARP 1) inhibitors (PARPi) are recognized as promising agents which are currently entering clinical trials in combination with different DNA-damaging treatments to potentiate their cytotoxic effect [3, 10, 28]. Pharmacological endpoint of PARPi mediated down-regulation of enzyme activity in DNA damage bearing cells is the reduced DNA-repair and activation of different cell death pathways. The major challenge in chemotherapy is development of adverse drug reactions due to the off-target effects of cytotoxic drugs on healthy cells and from this viewpoint application of PARPi in combination with cytotoxic drugs adds potential risks arising from drug combination.

Currently, PARPi are entering clinical investigations in combination with cisplatin [21]. It was shown that cisplatin directly influences T cell differentiation within thymus [24] and one of the most undesirable adverse effects of this drug is leucopenia. Generation of immunocompetent thymocytes in thymus gland is a multistep process

comprising complex combination of cell death, survival and proliferation programs. It is widely accepted that PARP 1 plays a prominent role in all of these programs [17]. Considering that failure of thymocyte maturation can disturb proper leukocytogenesis, we suggest that examination of cisplatin effects on PARP inhibition in the *ex vivo* cell model system, such as isolated rat thymocyte nuclei will help to understand potential risks connected with the drug combination in cancer therapy.

PARP 1 possesses trans- and auto-polyADPribosylating activities which are tightly regulated by endogenous NAD^+ content [1, 2]. Benzamide (Bam) inhibits both auto-PARylating and trans-PARylating activities of the enzyme and represents a broad spectrum of NAD^+-competing inhibitors designed as benzamide derivates that are currently used in clinical trials [13]. In contrast to Bam, ATP, also a potent PARP 1 inhibitor acts via specific suppression of enzyme auto-PARylation as shown in the *in vitro* experimental settings [22, 23]. To circumvent complications stemming from the involvement of such a versatile molecule as ATP in the broad spectrum of intracellular metabolic pathways and to evaluate its role in PARP 1 regulation *in vivo*, we have studied ATP effects on PARP 1 in thymocyte nuclei, considering that such experimental model is rather realistic reflection of the *in vivo* situation.

Growing evidence demonstrates positive correlation between PARP 1 inactivation and aging [20]. However, modulation of PARP 1 activity in the young growing animals has not been properly studied thus far. To bridge this gap and to avoid the influence of the age-dependent thymic involution [8, 29], we have examined the baseline PARP 1 activity in the thymocytes collected from 6 and 10 week old (pubertal and young adult age) rats of both sexes.

2. Materials and Methods

2.1. Isolation of Nuclei from Rat Thymocytes

Albino inbred male and female rats (6 week and 10week old) were used throughout all experiments. The animals were standardized by weight in either age group (to 100g and 150g in 6 week and 10 week old correspondingly). Vehicle (saline), cisplatin (10mg/kg weight) and hydrocortisone (5 and 10 μg/kg) were purchased from Sigma-Auldrich (as well as all other reagents used in this study) were injected intra-peritoneally. Animals were sacrificed under light ether anesthesia by decapitation after 48 h treatment with cisplatin and 24 h after treatment with hydrocortisone. Thymocyte nuclei were isolated according to Hewish and Burgoyne [9]. Sucrose solutions utilized throughout the nuclei isolation procedure were buffered with 20 mMTris containing 15 mMNaCl, 60 mMKCl, 0, 15mM spermine, and 0. 5mM spermidine, pH 7, 4.

2.2. PARP 1 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 [25], adapted by us to quantify NAD+ consumed by isolated nuclei. Briefly, nuclei were gently suspended in PARP assay buffer containing 20mM Tris, 6mM $MgCl_2$, 1 mM $CaCl_2$, pH 7. 4. Density of nuclear suspension was normalized to 1mg DNA/ml. PARP reaction was initiated by addition of NAD^+ stock solution to 1000 μl aliquot of nuclear suspension (0. 5 mM final concentration). The reaction was carried out for 10 min at 37^0C followed by centrifugation at 13 000g, 4^oC for 2 min to discard the nuclei. 50μl of supernatant was transferred to the Falcon UV-Vis transparent 96-well plate. NAD^+ quantitation was performed by sequential addition of 2M KOH and 20% acetophenone (in EtOH), in accordance with the original assay [25]. The absorbance of PARP assay mix containing 0, 5mM NAD^+ was determined at 378 nm. The amount of NAD^+ was determined using NAD^+ calibration curve. PARP 1 activity was defined as NAD^+consumed by nuclei in 10 min per mg of DNA.

2.3. Light Microscopy

Thymus glands were collected from all groups, fixed in 10% formalin in saline, dehydrated in ascending grades of alcohol and embedded in paraffin. Paraffin-embedded 5μm sections were dewaxed with xylenes, stained with hematoxylin and eosin and examined by the light microscope.

2.4. DNA Electrophoresis

Thymocyte nuclei DNA isolation and electrophoresis were performed according to the standard protocols [27]. Gels were stained with 1μg/ml ethidium bromide.

2.5. Statistical Analysis

Data are expressed as mean ± s. d. Statistical differences in the results between groups were evaluated by the two-tailed Student's t-test. A probability (p) value of <0. 05 was considered significant.

3. Results

3.1. Cisplatin Affects the Weight and Histological Appearance of the Thymus

The data presented on Fig. 1A show that the average weight of thymus of 6 week old male rats is by 12% greater than the weight of their female counterparts. As expected, between 6^{th} and10^{th}weeks thymus weight was increased approximately by 60% in males and nearly 70% in females. 48 hours after cisplatin administration to the rats the weight of 6 week old male thymus decreased by 60%, whereas in females it diminished only by 30%. Thymus glands of young adults (10 weeks) treated with cisplatin demonstrated 70% weight loss in males and 50% in females.

Cisplatin triggered toxic atrophy of the gland was accompanied by morphological changes presented on Fig. 1B.

Reduced weight and size coincided with the decrease in the number of cortical lymphocytes and loss of corticomedullary demarcation. Though thymocytes are known to be apoptosis prone and they indeed demonstrated characteristic apoptotic DNA fragmentation after the *in vivo* treatment of rats with hydrocortisone (Fig 1C, lanes 4 and 5), the toxic atrophy of the gland after cisplatin treatment did not produce such characteristic apoptotic DNA olygonucleosomal fragmentation (Fig 1C, lane 3).

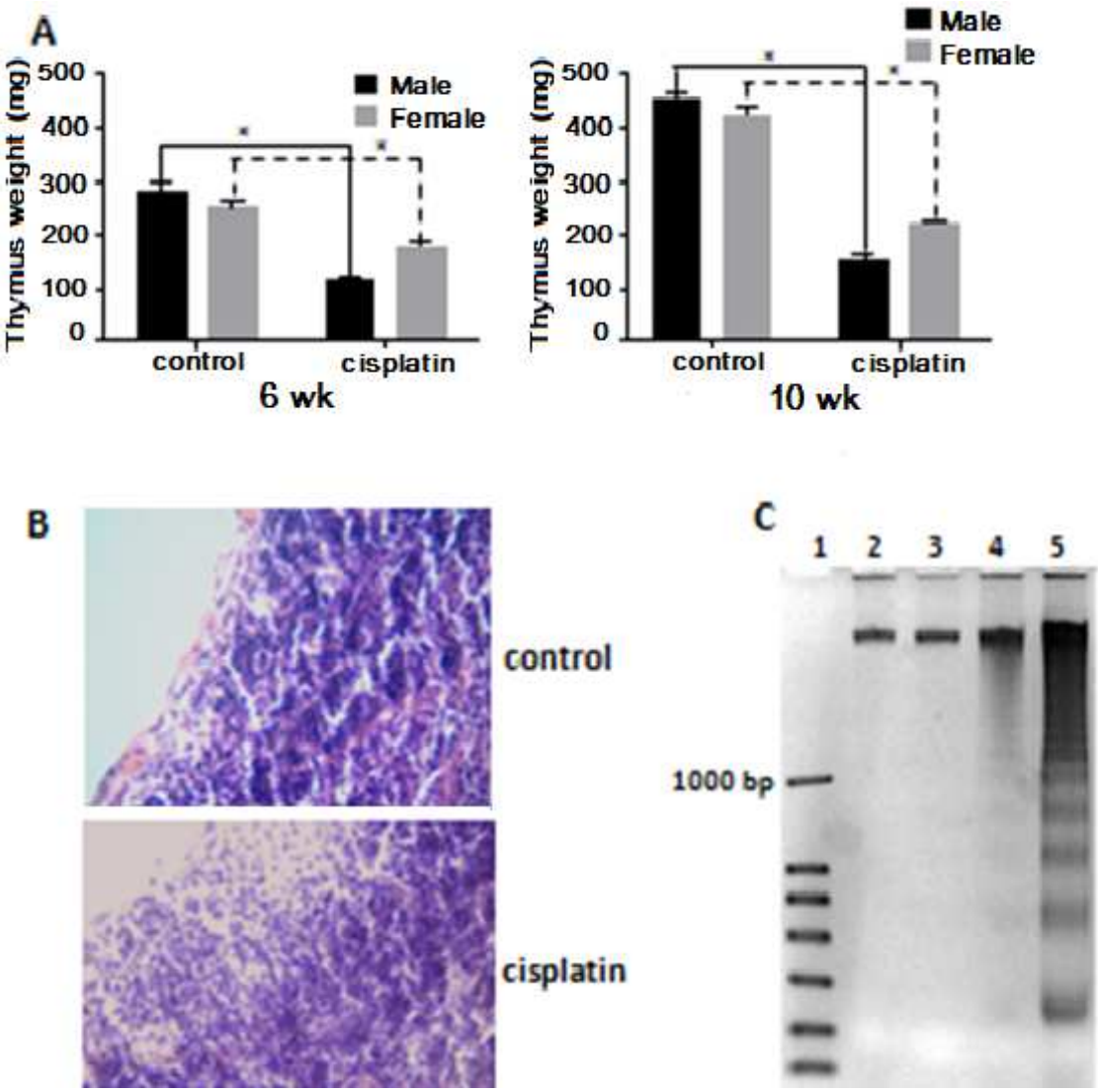

Figure 1. *Cisplatin impact on the weight and morphology of the rat thymus gland.*

A. 6 week (wk) and 10 wk old rats thymus show different weight loss after the *in vivo* treatment with cisplatin for 48 h. B. Hematoxilin-eosin staining of the 6 wk old rat thymus shows distinct dark-staining cortex with small lymphocytes (control, upper panel) and cisplatin induced reduction in cortical thickness and reduced distinction between cortex and medulla with patchy areas in cortex where small lymphocyte density is reduced (cisplatin, lower panel). Each histological section is representative of one group of animals (n=6). C. DNA isolated from the thymocyte nuclei of 6 wk old rats treated with vehicle (lane 2), cisplatin (lane 3), hydrocortisone, 5 µg/kg (lane 4) and hydrocortisone, 10µg/kg (lane 5) was separated on the agarose gel. Lane 1, DNA ladder.

3.2. Age, Sex and Cisplatin Dependent Modulation of Thymocyte PARP 1

As it is evident from the data presented on Fig. 2, PARP 1 activity measured in the nuclei isolated from the rat thymocytes has a tendency to decrease in course of rat maturation from 6 to 10 weeks. However, male and female thymocytes display nearly identical PARP 1 activity within the each age group.

Treatment with cisplatin had no appreciable effect on PARP 1 activity in the thymocytes from the male rats of pubertal age (6weeks; Fig. 2, left panel), whereas the activity of the enzyme increased by nearly 40% in the thymocytes of cisplatin treated female animals. Similar pattern was observed in the elder animals (10weeks) where the cisplatin

effect on the PARP 1 activity in female thymocytes was even stronger (increase by 60%; Fig. 2, right panel).

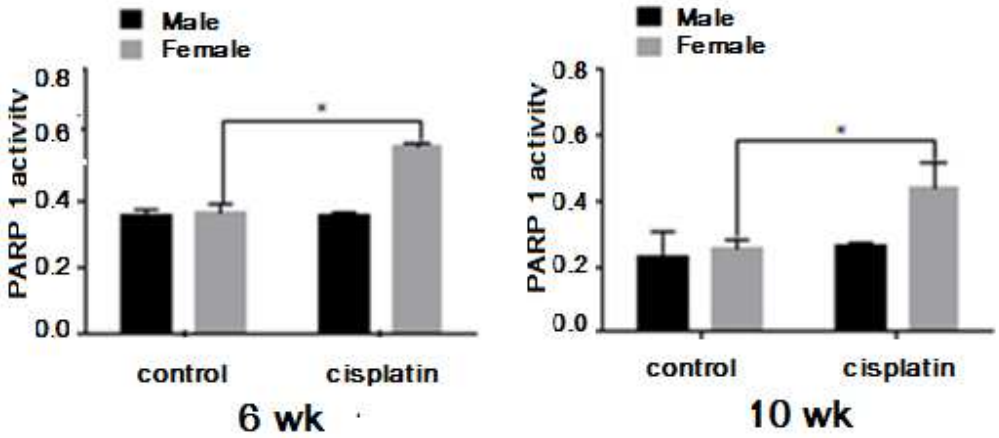

Figure 2. *Sex-biased cisplatin mediated activation of PARP 1 in the rat thymocytes.*

Thymocytes were collected from 6 wk (left panel) and 10wk (right panel) old rats. PARP 1 activity is expressed in mmoles of NAD^+consumed in 10 min per mg of nuclei DNA plus minus SEM *, p<0. 05.

Next we sought to reveal the effects of PARP 1 inhibition under our experimental conditions. To eliminate the nonspecific effects of benzamide and ATP onthe glucose metabolism [17, 2] and membrane-associated effects of cisplatin in thymocytes [4, 6], we have examined the impact of Bam and ATP on naked nuclei. Nuclei were isolated from thymocytes collected from rats before and after cisplatin administration.

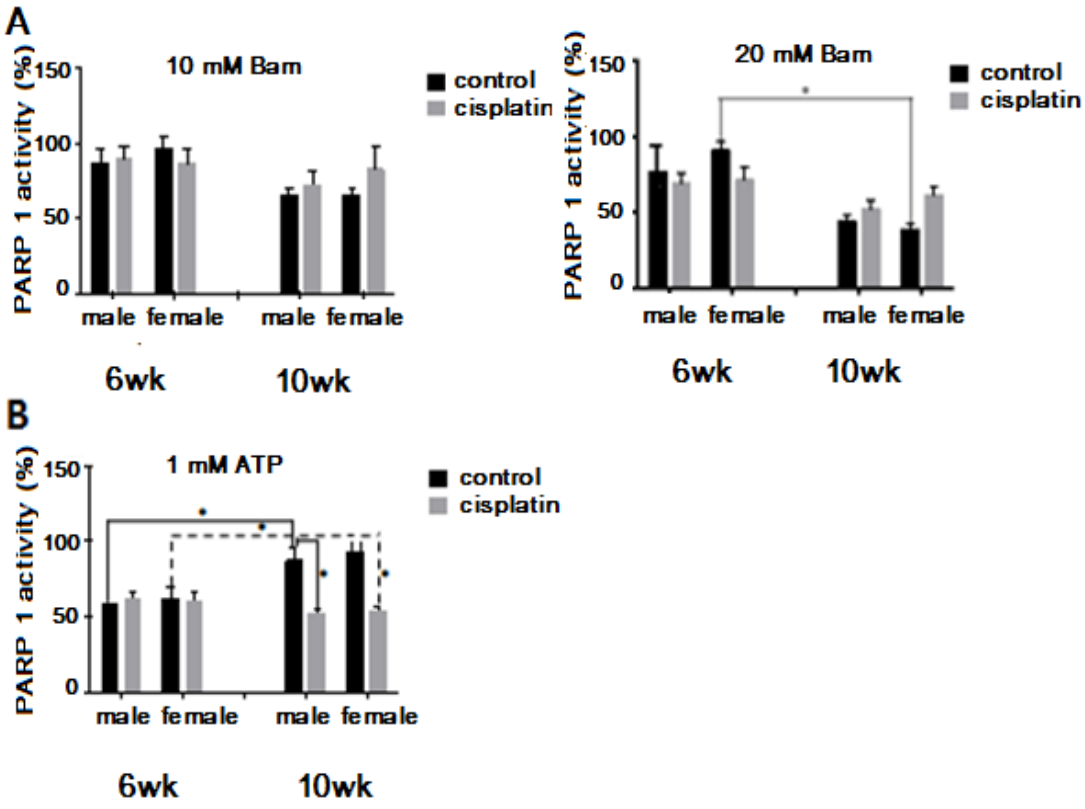

Figure 3. *PARP 1 inhibition by Bam and ATP in rat thymocyte nuclei modulated by cisplatin.*

A. PARP 1 inhibition by Bam; B, ATP mediated inhibition. Thymocytes were collected from 6 wk and 10wk old rats. PARP 1 activity is expressed in mmoles of NAD^+ consumed in 10 min per mg of nuclei DNA plus minus SEM*, p<0. 05.

The results presented on Fig. 3A, left panel demonstrate, that PARP 1 inhibition by Bam (10mM) in thymocytes nuclei of 6 week old rats of both sexes was negligible, whereas 10 week old rats exhibited higher sensitivity to inhibition. Cisplatin administration could not change that pattern in both sexes and age groups. As expected, inhibition by 20 mM

Bam (Fig. 3A, right panel) appeared to be stronger in either age group and here again, age-dependent increase in Bam inhibitory potency was apparent in 10 week old rats. Cisplatin administration to rats had no appreciable effect on the mode of PARP 1 inhibition by 20 mM Bam.

In addition to the widely recognized role in energy metabolism ATP has emerged also as a PARP 1 allosteric inhibitor [12, 16]. In present study we were interested to test the ATP inhibitory effects alone and in combination with cisplatin in rat thymocyte nuclei. We found out that ATP at 5 mM concentration, which is considered close to the physiological intracellular levels [12], nearly completely abolished the PARP 1 activity under all experimental conditions (results not shown). Therefore we choose to test the lower (1 mM) concentration. The pattern of 1 mM ATP mediated PARP 1 inhibition in thymocyte nuclei displayed an opposite trend to the one elicited by Bam featuring a lower sensitivity to the inhibitor in the older age group of control animals (Fig. 3B). Although the pretreatment of rats with cisplatin had no impact on the level of inhibition in the 6 week old rats it markedly elevated ATP inhibitory efficiency in the 10 week old rats of both sexes.

4. Discussion

Although the sex and age-related differences in drug pharmacokinetics and pharmacodynamics are widely recognized, little is known whether these factors may also affect therapeutic potential of PARP 1 inhibitors used in combination chemotherapy [3-5, 30]. In cancer therapy PARPi is often used in combination with cisplatin, which necessitates understanding the role of drug-drug interaction on PARP inhibition. In present study we examined whether the *in vivo* treatment with cisplatin can influence efficiency of PARP 1 inhibitors in sex-/age-dependent manner in rat thymocytes, considering their crucial role in the defense against toxic xenobiotics. To make the picture more transparent we sought to eliminate interplay of pharmacokinetic and pharmacodynamic parameters stemming from co-treatment with cisplatin and PARP 1 inhibitors. For this purpose PARP 1 inhibition had been assayed in the nuclei *ex vivo*.

Albeit the age-dependent PARP 1down-regulation is well documented in mononuclear leukocytes and cerebellum of aging animals [19, 20] little is known about changes of the enzyme activity in young organisms. Our data demonstrate that diminution of PARP 1 activity occurs in the intra-thymicthymocytes in growing thymus glands of 6^{th}-10^{th}week old young rats. It has been shown previously that estrogens play important role in PARP 1inhibition regulating thus its intracellular activity [11, 18, 19]. Therefore we expected that thymocytes of sexually mature 10 week old male and female rats should display different baseline PARP 1 activity. However, the absence of sex-dependent differences in the PARP 1 activity in either age group demonstrates that factors other than estrogens may also contribute to PARP 1 suppression.

It is well documented that NAD^+ and ATP metabolism play pivotal role in maintenance of the PARP 1 activity [1, 2, 12]. It is also known that PARP 1 activity comprises two different components, i. e. auto-and trans-PARylating activities [13]. Taking into consideration that proliferating cells exhibit only auto- PARylating activity [14], we hypothesized that slowing down of the thymus growth and thymocyte proliferation in young adult animals [7, 8] can shift the balance between auto- and trans- PARylating activities in the thymocytes in favor of the latter, not inhibited by ATP [15, 16]. Indeed, in contrast to the 6 week old animals the nuclei of older rats demonstrated high resistance to the PARP 1 inhibition by 1 mM ATP.

Although there was no sex bias in the baseline PARP 1 activity in the thymocytes of either age group, the mode of PARP 1 inhibition by the competitive inhibitor Bam is different in puberty and in the sexually mature animals. Age-dependent increase in Bam efficiency indicates an important role of yet to be discovered developmental factors in the enzyme inhibition.

The other question that we have attempted to address in the present study was the role of age and sex variables under the conditions of combined PARP 1iandcisplatin treatment. It appears that the treatment with cisplatin leads to the sex-dependent toxic atrophy of thymus. Interestingly such atrophy seems to lack characteristic traits of apoptosis, the predominant form of thymocyte elimination in the gland. The cisplatin induced thymic atrophy was less pronounced in the female animals (Fig. 1). We suggest that the PARP 1 activation detected in the female thymocyte nuclei (Fig. 2) could be the reason for such increased thymocyte survival via the improved DNA damage repair by PARP 1.

It was reported earlier that cisplatin can down-regulate intracellular ATP by inhibiting glycolytic enzymes [26, 28, 29]. Assuming that PARP 1 is a nuclear ATP sensor [17] we suggest that the age-dependent and cisplatin induced oscillations in PARP 1 activity could result from the changes in the levels of ATP. We hypothesize that such modulations ofPARP 1activity can contribute to the development of adverse effects which are often elicited by the group of anticancer drugs recognized as ATP mimetics [5].

Conclusion: The data presented here suggest that sex and age-dependent variables should be considered of paramount importance in the design of personalized therapy regimen involving combination of such chemotherapeutic agents as cisplatinand the PARP 1 inhibitors.

Abbreviations: Poly ADP-ribose polymerase 1- PARP 1, polyADP-ribose polymerisation-PARylation, benzamide-Bam, cisplatin-cis-diammine-1, 1-cyclobutanedicarboxylate platinum (II)

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