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Role of free radicals in PARP inhibition of cervical carcinogenesis: PARylation of HeLa cells, and inhibition of hPARP-1 enzyme

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Keywords: PARP; PARP inhibitors; PARylation; cervical cancer HeLa cells; oxidative stress;

Abstract

A study of the in vitro inhibition of hPARP-1 enzyme and PARylation of HeLa cells by a wide range of PARP inhibitors has been conducted utilizing an established linear free energy methodology comprising the free energy of water desolvation and lipophilicity, dipole moment, molecular volume or electron affinity of the PARP inhibitors. It is shown that the IC₅₀ in vitro inhibition of the hPARP-1 enzyme is close related to the EC₅₀ values for PARylation in HeLa cells. The electron affinity of the inhibitors is strongly related to the EC₅₀ values, suggesting that inhibitor free radicals are involved in oxidative stress processes and PARylation in HeLa cells.

Abbreviations

PARP Poly (ADP-ribose) polymerase, PARylation protein poly ADP-ribosylation, hPARP-1 human PARP-1 enzyme, HeLa cells, BRCA Breast Cancer susceptibility gene, SSB Single Strand DNA breaks, DSB Double Strand DNA breaks, Synthetic lethality combination of deficiencies in the expression of two or more genes leads to cell death, whereas a deficiency in only one of these genes does not lead to cell death, EC₅₀ concentration of inhibitor that gives half-maximal response in HeLa cells, IC₅₀ concentration of an inhibitor where the binding to the hPARP-1 enzyme is reduced by half, ROS reactive oxygen species, $\Delta G_{\text{desolvation}}$ free energy of water desolvation, HR homologous recombination, $\Delta G_{\text{lipophilicity}}$ free energy of lipophilicity or hydrophobicity, $\Delta G_{\text{desolv,CDS}}$ free energy of water desolvation of the cavitation dispersion solvent structure (CDS), $\Delta G_{\text{lipo,CDS}}$ free energy of lipophilicity or hydrophobicity for the CDS, DM dipole moment, MV molecular volume, EA electron affinity, R² multiple correlation coefficient, HOMO highest occupied molecular orbital, F the F test of significance, SEE standard errors for the estimates, SE($\Delta G_{\text{desolvation}}$) standard errors of $\Delta G_{\text{desolvation}}$, SE($\Delta G_{\text{desolv,CDS}}$) standard errors of $\Delta G_{\text{desolv,CDS}}$, SE($\Delta G_{\text{lipophilicity}}$), standard errors of $\Delta G_{\text{lipophilicity}}$, SE(Dipole Moment) standard errors for dipole moments, SE (Molecular Volume) standard errors for molecular volumes as calculated from "t" distribution statistics

Introduction

PARP (Poly (ADP-ribose) polymerase) is a protein family involved in a number of cellular processes such as DNA repair, genomic stability, and programmed cell death. PARP's main function is to detect and initiate an immediate cellular repair response to metabolic, chemical, oxidative stress, or radiation-induced single-strand DNA breaks. PARylation (protein poly ADP-ribosylation) is a widespread post-translational modification at DNA lesions catalyzed by PARPs. This modification regulates a number of biological processes particularly DNA damage response but also transcriptional regulation, apoptosis, and mitosis. PARP can function as a DNA damage sensor activated by DNA lesions, thereby forming PAR chains that serve as a docking platform for DNA repair factors. [1,2]

PARP is a key facilitator of DNA damage repair and has a key role in tumorigenesis associated with dysfunctional DNA repair pathways. Transcript, protein, and enzyme activity of PARP is increased in breast cancer, ovarian cancer, hepatocellular cancer, colorectal cancer, and leukemia. Treating cancers by targeting PARP through increasing tumour sensitivity to

chemotherapeutic agents and also by inducing “synthetic lethality” is now common in the clinic. PARP inhibitors have been shown to be successful in the treatment of various cancers, attributed to impaired DNA repair of cancerous cells. It has been shown that PARP inhibition specifically kills BRCA1 and BRCA2 mutant cells, demonstrating synthetic lethality of PARP inhibition with the homologous recombination defect. It is thought that inhibitor-inactivated PARP-1 is trapped on DNA, and that trapped {PARP-1:DNA} complexes are cytotoxic. In addition, it is thought that loss of PARylation impairs early recruitment of both BRCA1 and BRCA2 to DNA lesions, suggesting that the PARylation directly contributes to homologous recombination. [3-9]

PARP inhibitors are clinically effective in a number of tumour types, including platinum-sensitive epithelial ovarian cancer, breast cancer with mutation in BRCA-1 or BRCA-2, and prostate cancer. Olaparib is a PARP inhibitor which has been approved as a monotherapy for the treatment of patients with germline BRCA mutated advanced ovarian cancer who have been treated with three or more prior lines of chemotherapy. Olaparib has been approved in Europe for maintenance therapy in both germline and somatic BRCA-mutant platinum-sensitive ovarian cancer. Rucaparib has been approved for previously treated BRCA-mutant ovarian cancer. Niraparib, a PARP-1 and PARP-2 inhibitor, is approved for epithelial ovarian, fallopian tube, and primary peritoneal cancer.

PARPs are also involved in oxidative stress, cell death, and oxidative-stress-related diseases. Oxidative stress can cause DNA breaks which results in activation of the DNA nick sensor enzyme PARP-1. Normal PARP activation facilitates DNA repair, but extensive PARP activation induces mitochondrial transition and mitochondrial damage that culminates in cell death. Mitochondrial dysfunction is strongly associated with PARP-mediated apoptotic cell death. PARP inhibition converts necrosis to apoptosis. The majority of PARP activity is located in the nucleus, but some PARP activity is found in the cytoplasm. [10] Inhibition or depletion of PARP leads to not only an increase in DNA damage, but also an elevation in the levels of reactive oxygen species (ROS). Oxidative stress is also increased by PARP inhibition and mediates the antitumor effect. The antioxidant N-acetylcysteine (NAC) attenuated the induction of DNA damage and the proliferation by PARP inhibition. NADPH oxidases 1 and 4 were upregulated by PARP inhibition and were partially responsible for the induction of oxidative stress. Thus in addition to compromising the repair of DNA damage, PARP inhibition also exerts an extra antitumor effect by elevating oxidative stress in ovarian cancer cells. [11,12]

Hypoxic cells with decreased homologous recombination (HR) protein expression showed increased clonogenic killing following chemical inhibition of PARP1. Selective cell killing of HR-defective hypoxic cells in vivo as a consequence of microenvironment-mediated “contextual synthetic lethality” has been demonstrated. [13,14] PARP inhibitors can potentiate the effects of radiotherapy and chemotherapy in cancer cells. Inhibition of PARP activity can sensitize hypoxic cancer cells and the combination of ionizing radiation with PARP inhibitors can improve the therapeutic ratio of radiotherapy. [15] Olaparib is a potent radiosensitizer for head and neck cancer therapy. [16] Hypoxia and ease of reaction with ionizing radiation are hallmarks of free radical drug formation in chemoradiotherapy regimes. Drug-induced oxidative stress is implicated as a mechanism of toxicity in numerous tissues and organ systems. [17-23] The metabolism of a drug may generate a reactive intermediate that can reduce molecular oxygen directly to generate ROS. [24]

Many currently prescribed antineoplastic drugs induce high levels of free radicals and OS, with patients showing ROS-induced lipid peroxidation in their plasma, have reduced blood levels of vitamin E, vitamin C and β -carotene, and decreased tissue *glutathione* levels. Many anticancer drugs are also capable of generating ROS such as superoxide, typically by redox cycling with oxygen in mitochondrial systems or in the endoplasmic reticulum. These drugs can be readily bio-reduced by various enzymes, such as NADPH-cytochrome P450 reductases and FADH₂ etc. [25-30] Amongst the common antineoplastic drugs, the severity of oxidative stress varies from: (a) *very high*, anthracyclines: (b) *moderately high*, Pt-complexes, alkylating agents, epipodophyllotoxins (c) *low*, purines/pyrimidines, antimetabolites, taxanes, vinca alkaloids. [25-31] PARP inhibitors also involve free radicals and oxidative stress in their chemotherapeutic regimes. [11,32]

Figure 1 schematically illustrates the various processes known to be involved in the inhibition of PARP-1 enzymes and PARylation and accompanying oxidative stress induced by PARP activation.

We have previously shown that an equation of the general form shown below, eq 1, applies to cell membrane transport processes for various drugs as well as drug-enzyme binding interactions. [17,33-39]

Transport or Binding = $\Delta G_{\text{desolv,CDS}}$ + $\Delta G_{\text{lipo,CDS}}$ + Dipole Moment + Molecular Volume or Electron Affinity

This equation 1 uses the free energy of water desolvation ($\Delta G_{\text{desolv,CDS}}$) and the lipophilicity free energy ($\Delta G_{\text{lipo,CDS}}$) where CDS represents the non-electrostatic first solvation shell solvent properties, may be a better approximation of the cybotactic environment around the drug approaching or within the protein receptor pocket, or the cell membrane surface or the surface of a drug transporter, than the bulk water environment outside the receptor pocket or cell membrane surface. The CDS includes dispersion, cavitation, and covalent components of hydrogen bonding, hydrophobic effects. Desolvation of water from the drug ($\Delta G_{\text{desolv,CDS}}$) before binding in the receptor pocket is required, and hydrophobic interactions between the drug and protein ($\Delta G_{\text{lipo,CDS}}$) is a positive contribution to binding. $\Delta G_{\text{lipo,CDS}}$ is calculated from the solvation energy in n-octane. In some biological processes, where biological redox processes may be occurring, and the influence of molecular volume is small, the electron affinity EA has been included in place of the molecular volume, MV. In other processes, the influence of some of the independent variables is small and can be eliminated to focus on the major determinants of biological activity.

We have recently used this model to develop a predictive model of the transport and efficacy of hypoxia specific cytotoxic analogues of tirapazmine and the effect on the extravascular penetration of tirapazmine into tumours. [17] It was found that the multiparameter model of the diffusion, antiproliferative assays IC₅₀ and aerobic and hypoxic clonogenic assays for a wide range of neutral and radical anion forms of tirapazmine (TPZ) analogues showed: (a)

extravascular diffusion is governed by the desolvation, lipophilicity, dipole moment and molecular volume, similar to passive and facilitated permeation through the blood brain barrier and other cellular membranes, (b) hypoxic assay properties of the TPZ analogues showed dependencies on the electron affinity, as well as lipophilicity and dipole moment and desolvation, similar to other biological processes involving permeation of cellular membranes, including nuclear membranes, (c) aerobic assay properties were dependent almost exclusively on the electron affinity, consistent with electron transfer involving free radicals being the dominant species.

Results

The general equation (1) has been applied to the IC_{50} and EC_{50} data from the Table for the hexahydrobenzothiazidinone PARP inhibitors. All IC_{50} and EC_{50} data are from the same study and laboratory source [40] (see Figure 2) to minimize experimental data error. NB: Raw MV values have been normalized by factor of 0.1, and raw EA values normalized by 10.0 to allow direct comparison of coefficients in the equations.

Since there is literature evidence [11,25-32] that the free radical form of various anticancer drugs involve the free radical form of the drug (as well as some PARP inhibitors) in their chemotherapy, the experimental data in the Table has been tested against the free radical form, as well as the neutral (or protonated) form of the drugs. Applying the general equation (1) to the anion radical forms of the hexahydrobenzothiazidinone inhibitors:

EC_{50} values for 25 hexahydrobenzothiazidinone anion radical PARP inhibitors **Eq 2**

$$EC_{50} = 449.76\Delta G_{desol,CDS} + 439.68\Delta G_{lipo,CDS} - 4.56DM + 1739.15EA - 20464.4$$

Where $R^2 = 0.520$, $SEE = 1197.8$, $SE(\Delta G_{desol,CDS}) = 252.25$, $SE(\Delta G_{lipo,CDS}) = 332.51$, $SE(DM) = 29.89$, $SE(EA) = 596.82$, $F=5.15$, Significance $F = 0.0055$

Or by eliminating the non-significant dependency on DM in eq 2, gives eq 2(a):

EC_{50} values for 25 hexahydrobenzothiazidinone anion radical PARP inhibitors **Eq 2(a)**

$$EC_{50} = 453.76\Delta G_{desol,CDS} + 452.88\Delta G_{lipo,CDS} + 1706.6EA - 20007.7$$

Where $R^2 = 0.520$, $SEE = 1167.7$, $SE(\Delta G_{desol,CDS}) = 245.0$, $SE(\Delta G_{lipo,CDS}) = 313.1$, $SE(EA) = 543.5$, $F=7.21$, Significance $F = 0.0018$

However it is possible that PARylation in the HeLa cells may involve non-redox processes, and that inhibition occurs through the neutral or protonated at physiological pH levels. This is analysed in eq 3:

EC_{50} values for 23 hexahydrobenzothiazidinone neutral or protonated PARP inhibitors **Eq 3** (excluding #7 benzyl)

$$EC_{50} = 288.51\Delta G_{desol,CDS} + 848.6\Delta G_{lipo,CDS} + 56.43DM + 281.7MV + 363.2$$

Where $R^2 = 0.597$, $SEE = 831.1$, $SE(\Delta G_{desol,CDS}) = 192.8$, $SE(\Delta G_{lipo,CDS}) = 247.1$, $SE(DM) = 27.19$, $SE(MV) = 73.6$, $F=6.30$, Significance $F = 0.0026$

Analysis of the same data set used in eq 3 for the in vivo EC_{50} data gives eq 4 which shows the correlation with the in vitro IC_{50} data:

IC₅₀ values for 23 hexahydrobenzothiazopyridinone neutral or protonated PARP inhibitors Eq 4 (excluding #7 benzyl)

$$IC_{50} = 3.71\Delta G_{\text{desol,CDS}} + 9.75\Delta G_{\text{lipo,CDS}} + 2.45DM + 3.62MV - 20.12$$

Where R² = 0.747, SEE = 21.55, SE(ΔG_{desol,CDS}) = 3.17, SE(ΔG_{lipo,CDS}) = 2.17, SE(DM) = 0.50, SE(MV) = 1.7, F=16.27, Significance F = 0.0000

Direct comparison of the coefficients for eqs 3 and 4 gives:

Eq 3 Ratios: ΔG_{lipo,CDS} / ΔG_{desol,CDS} = 2.94, ΔG_{lipo,CDS} / DM = 15.04, ΔG_{lipo,CDS} / MV = 3.01

Eq 4 Ratios: ΔG_{lipo,CDS} / ΔG_{desol,CDS} = 2.63, ΔG_{lipo,CDS} / DM = 3.98, ΔG_{lipo,CDS} / MV = 2.69

Comparison of eq 3 with eq 4 shows that DM has greater influence for eq 3 for the in vitro hPARP-1 data, but otherwise the equations are very similar in terms of water desolvation, lipophilicity and molecular volumes of the inhibitors in the cellular environment compared to the in vitro environment.

Discussion

Figure 1 illustrates the various processes involved in the inhibition of PARP-1 enzymes and PARylation of HeLa cells and the accompanying oxidative stress induced by PARP activation. Oxidative stress is also increased by PARP inhibition and mediates the antitumor effect. The IC₅₀ data for the in vitro inhibition of the hPARP-1 enzyme can be taken as a close substitute of the inhibition of PARP-1 in cells (if membrane transport processes are comparatively insignificant), while the EC₅₀ data for the inhibition of the HeLa cells would include the inhibition of the PARP-1 enzyme preceding the inhibition of PARylation (and any membrane transport processes). It has been recently shown it is the kinetics of poly(ADP-ribosyl)ation, but not PARP-1 itself, that determines the cell fate in response to DNA damage in vitro and in vivo. Thus the homeostasis and dynamics of PAR formation is critical to the cells and organisms fate in coping with acute DNA damage, whereas the full PARylation response is essential for organism survival under genotoxic stress. PARylation involves the PARP catalysed transfer of the ADP-ribose moiety from β-nicotinamide-adenine-dinucleotide (β-NAD⁺) to acceptor amino acid residues of target proteins and the creation of long PAR chains. [41]

The EC₅₀ and IC₅₀ data of Torrisi [40] for the hexahydrobenzothiazopyridinone PARP inhibitors are valuable since it allows comparison of the cellular processes involved in PARylation of HeLa cells with the in vitro inhibitor binding to the hPARP-1 enzyme. The IC₅₀ inhibitory data could involve the neutral or protonated forms of the hexahydrobenzothiazopyridinones, since many of the inhibitors are likely to be partially protonated at physiological pH levels. There is strong evidence from this study, and literature sources [10-12], that oxidative stress may be involved in PARylation processes, and that the anion radical form of the inhibitors could be involved in the EC₅₀ PARylation processes in the HeLa cells. These possibilities have been tested in the results for equations 2 - 4 using the neutral, protonated or anion radical form of the neutral or protonated species of the inhibitors as shown in the Table. It is noted that the experimental conditions used by Torrisi [40] for the PARylation of HeLa cells involved stimulation of DNA damage by hydrogen peroxide.

We have previously investigated PARP inhibitors using similar equations [42] to those in this study, including an investigation of the IC_{50} values for more limited number of hexahydrobenzonaphthyridinones. It was also shown that the molecular docking scores [43] for a wide range of PARP-1 inhibitors were also well described by these equations. [42]

Conclusions

A study of the in vitro inhibition of hPARP-1 enzyme and PARylation of HeLa cells by a wide range of PARP inhibitors has been conducted utilizing an established linear free energy methodology comprising the free energy of water desolvation and lipophilicity, dipole moment, molecular volume or electron affinity of the PARP inhibitors. It is shown that the IC_{50} in vitro inhibition of the hPARP-1 enzyme is closely related to the EC_{50} values for PARylation in HeLa cells. The electron affinity of the inhibitors is strongly related to the EC_{50} values, suggesting that inhibitor free radicals are involved in oxidative stress processes and PARylation in HeLa cells.

Materials and Methods

All calculations were carried out using the Gaussian 09 package. Energy optimisations were at the DFT/B3LYP/6-31G(d,p) (6d, 7f) level of theory for all atoms. Selected optimisations at the DFT/B3LYP/6-311⁺G(d,p) (6d, 7f) level of theory gave very similar results to those at the lower level. Optimized structures were checked to ensure energy minima were located, with no negative frequencies. Energy calculations were conducted at the DFT/B3LYP/6-311+G(d,p) (6d, 7f) level of theory with optimised geometries in water, using the IEFPCM/SMD solvent model. With the 6-31G* basis set, the SMD model achieves mean unsigned errors of 0.6 - 1.0 kcal/mol in the solvation free energies of tested neutrals and mean unsigned errors of 4 kcal/mol on average for ions. [44] The 6-31G(d,p) basis set has been used to calculate absolute free energies of solvation and compare these data with experimental results for more than 500 neutral and charged compounds. The calculated values were in good agreement with experimental results across a wide range of compounds. [45,46] Adding diffuse functions to the 6-31G(d) basis set (ie 6-31⁺G(d,p)) had no significant effect on the solvation energies with a difference of less than 1% observed in solvents, which is within the literature error range for the IEFPCM/SMD solvent model. HOMO and LUMO calculations included both delocalized and localized orbitals (NBO).

Electron affinities (AEA) in eV in water were calculated by the SCF difference between the optimised/relaxed neutral and optimised radical species method as previously described. [17] It has been shown that the B3LYP functional gives accurate electron affinities when tested against a large range of molecules, atoms, ions and radicals with an absolute maximum error of 0.2 eV. [47-49] Raw MV values have been normalized by factor of 0.1, and raw EA values normalized by 10.0 to allow direct comparison of coefficients in the equations.

It is noted that high computational accuracy for each species in different environments is not the focus of this study, but comparative differences between various species is the aim of the study. The literature values for the EC_{50} and IC_{50} values used to develop the multiple regression LFER equations have much higher experimental uncertainties than the calculated molecular properties. The statistical analyses include the multiple correlation coefficient R^2 , the F test of significance, standards errors for the estimates (SEE) and each of the variables $SE(\Delta G_{desolCDS})$, $SE(\Delta G_{lipoCDS})$,

SE(Dipole Moment), SE (Molecular Volume), SE (AEA) as calculated from “t” distribution statistics. Residual analysis was used to identify outliers.

Table

Hexahydrobenzo-naphthyridinone PARP Inhibitors See Figure 2	$\Delta G_{\text{desolvation}}$ kcal/mol (CDS) Water	$\Delta G_{\text{lipophilicity}}$ kcal/mol (CDS) n-Octane	Dipole Moment D Water	Molecular Volume cm ³ /mol Water	Electron Affinity eV Water
4 Parent Ion	-4.30	-5.08	16.8	116	
4 Ion + e ⁻	-4.3	-5.08	16.8	116	1.54
7 Ion	-6.00	-8.35	9.3	194	
7 Ion + e ⁻	-6.00	-8.35	22.6	215	1.51
8	-5.22	-8.44	9	197	
8 + e ⁻	-5.22	-8.44	8.95	197	1.31
9	-5.3	-8.97	9	174	
9 + e ⁻	-5.43	-8.97	21	194	1.31
11	-5.28	-9.24	9.55	238	
11 + e ⁻	-5.28	-9.24	22.3	229	1.31
15	-4.85	-8.64	10.4	242	
15 + e ⁻	-4.05	-8.64	22.0	247	1.42
16	-5.54	-8.95	6.6	244	
16 + e ⁻	-5.54	-8.95	21.0	204	1.34
17	-5.15	-8.85	8.8	255	
17 + e ⁻	-5.15	-8.85	22.0	244	1.37
18	-5.51	-8.83	9.73	216	
18 + e ⁻	-5.51	-8.83	21.8	214	1.34
20 Ion	-8.19	-10.72	42.7	302	
20 Ion + e ⁻	-8.19	-10.72	37.5	320	1.78
22	-5.94	-9.49	9.09	212	
22 + e ⁻	-5.94	-9.49	21.89	264	1.34
23	-5.97	-9.51	8.78	235	
23 + e ⁻	-5.97	-9.51	21.81	246	1.33
24	-5.85	-9.39	8.91	243	
24 + e ⁻	-5.85	-9.39	23.8	234	1.33
29	-7.84	-12.02	9.8	293	
29 + e ⁻	-7.84	-12.02	31.1	341	1.34
33	-3.98	-7.87	10.1	186	
33 + e ⁻	-3.98	-7.87	21.0	196	1.34
36	-2.93	-7.47	7.9	192	
36 + e ⁻	-2.93	-7.47	13.6	174	1.34
39	-4.36	-8.07	9.1	180	
39 + e ⁻	-4.36	-8.07	24.2	175	1.31

40	-1.33	-7.22	3.43	196	
40 + e ⁻	-1.33	-6.86	13.35	215	1.375
40 Ion	-4.85	-7.16	15.71	168	
40 + e ⁻	-4.85	-7.16	26.0	196	1.44
41	-6.07	-8.50	4.36	213	
41 + e ⁻	-6.07	-8.50	10.94	214	1.39
42	-1.84	-6.80	2.71	239	
42 + e ⁻	-1.84	-6.80	14.66	238	1.38
42 Ion	-5.33	-7.08	22.42	210	
42 Ion + e ⁻	-5.33	-7.08	34.0	233	1.42
43	-2.16	-6.47	2.27	213	
43 + e ⁻	-2.16	-6.47	12.00	214	1.32
44	-5.08	-8.99	5.84	219	
44 + e ⁻	-5.08	-8.99	17.97	219	1.31
45 Ion	-4.33	-8.31	33	237	
45 Ion + e ⁻	-4.33	-8.31	50.9	252	1.39
47	-6.58	-11.38	3.89	253	
47 + e ⁻	-6.58	-11.38	16.25	314	1.31
48	-6.30	-8.13	2.98	259	
48 + e ⁻	-6.30	-8.13	10.76	186	1.35
49	-6.52	-7.96	1.38	206	
49 + e ⁻	-6.52	-7.96	11.97	215	1.37

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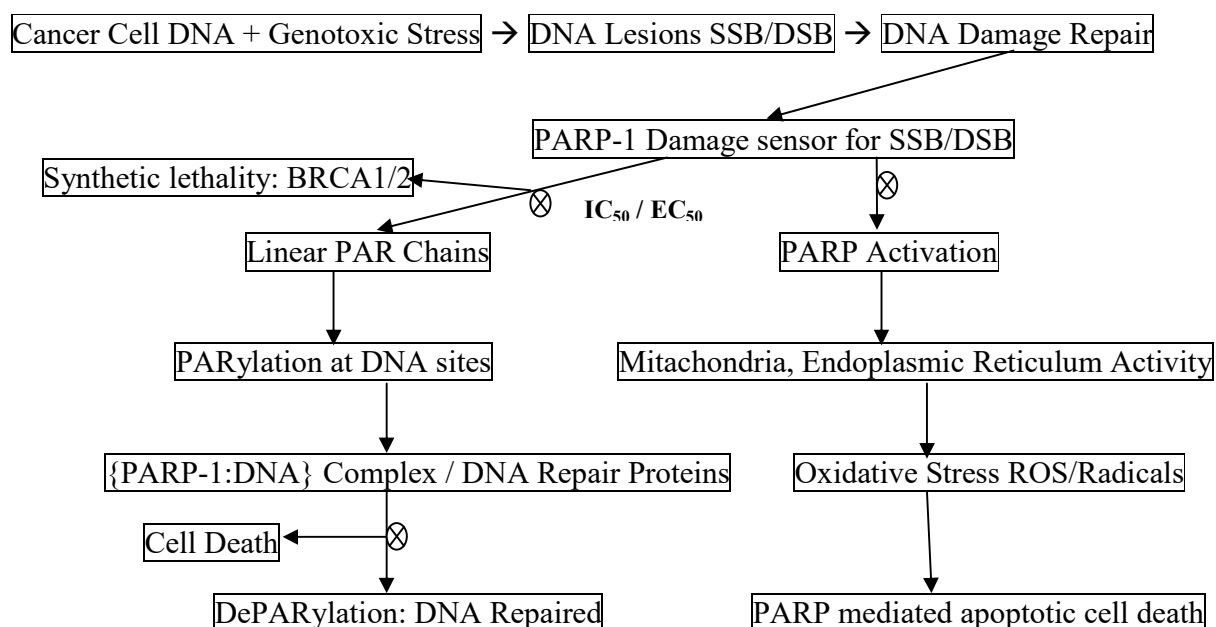
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Figure 1. Schematic flow chart of effect of inhibitors on PARP-1 enzyme and PARylation reactions and accompanying oxidative stress



⊗ Inhibitors, SSB/DSB Single Strand and Double Strand DNA Breaks, ROS Reactive Oxygen Species
Genotoxic Stresses: Mutagenic chemicals, UV, Oxidative Stress, ROS, Radicals, Ionizing Radiation etc

Figure 2. Hexahydrobenzothiazidinone PARP Inhibitors

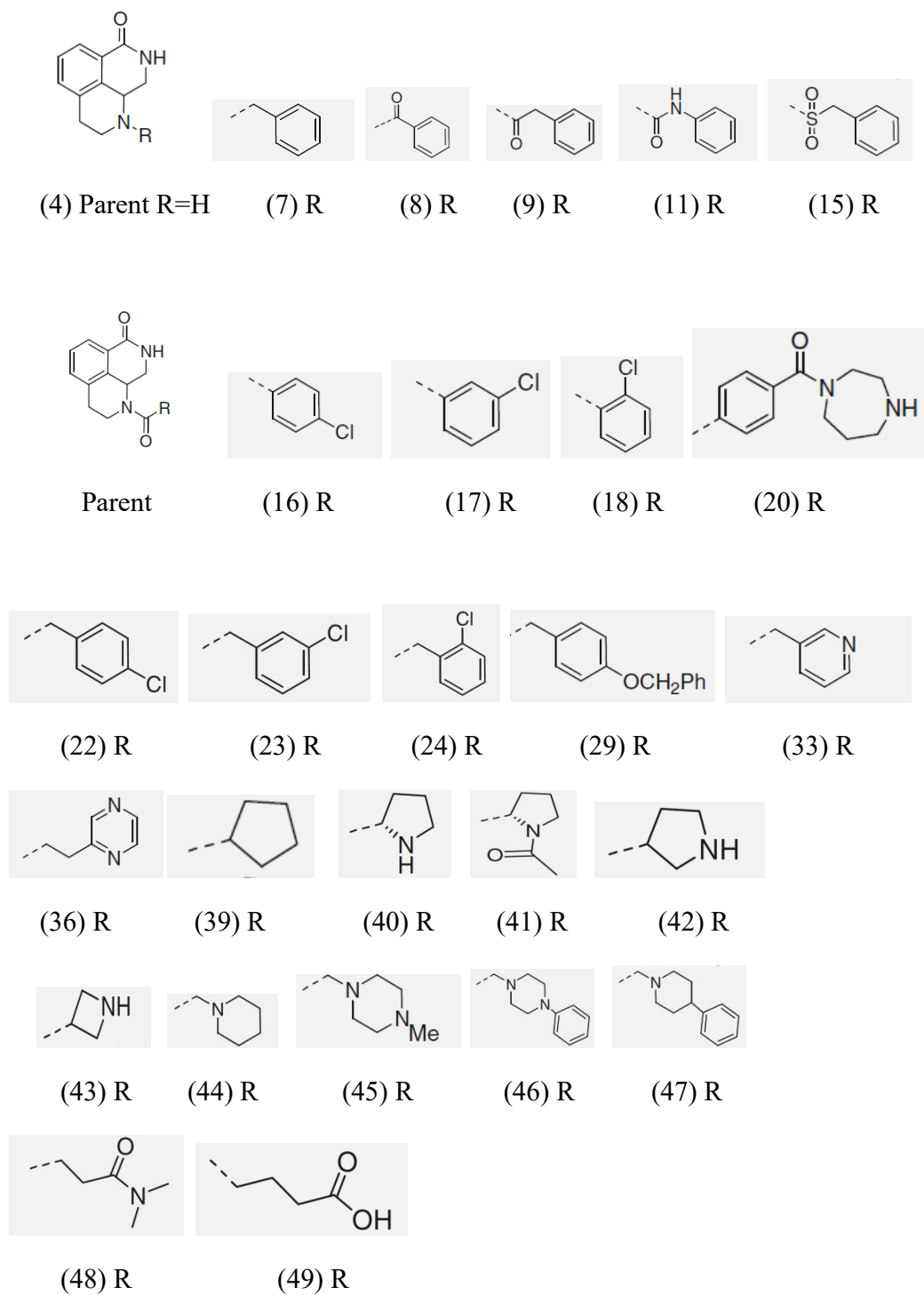


Figure 2 Footnote: Numbering scheme of hexahydrobenzothiazidinone inhibitors refers to numbering scheme in Torissi 2010.