Analysis of the mechanism of Aldo-keto reductase dependent cis-platin resistance in HepG2 based on transcriptomic and NADH metabolic analysis

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Abstract

Aldo-keto oxidoreductase (AKR) inhibitors could reverse several cancer cells' resistance to Cis-platin, but their role in resistance remains unclear. Our RNA-seq results showed de novo NAD biosynthesis-related genes, and NAD(P)H-dependent oxidoreductases were significantly upregulated in Cis-platin-resistant HepG2 hepatic cancer cells (HepG2-RC cells) compared with HepG2 cells. Knockdown of AKR1Cs could increase Cis-platin sensitivity in HepG2-RC cells about two-fold. Interestingly, the AKR1C inhibitor meclofenamic acid could increase Cis-platin sensitivity of HepG2-RC cells about eight-fold, indicating that knockdown of AKR1Cs only partially reversed the resistance. Meanwhile, the amount of total NAD and the ratio of NADH/NAD⁺ were increased in HepG2-RC cells compared with HepG2 cells. The increased NADH could be explained as a directly operating antioxidant to scavenge radicals induced by Cis-platin. We report here that NADH, which is produced by NAD(P)H-dependent oxidoreductases, plays a key role in the AKR-associated Cis-platin resistance of HepG2 hepatic cancer cells.

1 Introduction

Aldo-keto reductases (AKRs) are NAD(P)H-dependent oxidoreductases, which reduce carbonyl substrates with NAD(P)H and are present in all three domains of life. Human AKRs are important in metabolic pathways such as steroid biosynthesis, alcohol oxidation, and xenobiotic elimination[1]. Some AKRs, such as AKR1B10[2] and AKR1Cs[3,4], are correlated with carcinogenesis. AKR1Cs have also been related to Cis-platin resistance in gastric carcinoma TSGH-S3 cells[5] and metastatic bladder cancer cells[6].

Cis-platin is a front-line chemotherapy agent for cancer treatment[7]. The AKR1C inhibitor flufenamic acid was found useful to reverse Cis-platin resistance of bladder cancer[6]. It was speculated that AKR1Cs could reduce some cytotoxic lipid peroxidative products from aldehydes[5]. Hepatic cancer cells with high expression levels of AKRs usually show a rather high tolerance to Cis-platin treatment. However, the target molecule of AKRs remains unknown. Here we report that NADH, a product of Aldo-keto oxidation-reduction, plays a key role in the Cis-platin resistance of HepG2 hepatic cancer cells.

2 Materials and Methods

2.1 Cell culture

Human hepatic HL-7702 cells and the hepatic cancer cell lines HepG2 and its Cis-platin-resistant strain HepG2-RC were purchased from Fusheng Biotechnology (Shanghai, China). HL-7702 cells were cultured at 5% CO₂ in RPMI-1640 medium. HepG2 cells were cultured at 5% CO₂ in DMEM medium. HepG2-RC cells were cultured at 5% CO₂ in MEME medium with increasing Cis-platin concentration until 50% of cells died.

2.2 Quantitative real-time polymerase chain reaction

Total RNA isolation and first-strand cDNA synthesis from HL-7702, HepG2, and HepG2-RC cells were performed by a SuperReal Kit (Tiangen, Beijing, China). The primer sequences are listed in Table S1. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using ABI7900HTFast (Thermofisher, Waltham, MA). The data were normalized to the β -actin expression level and are expressed as the fold change relative to control ($2^{-\Delta\Delta C_t}$).

2.3 Western blot

Cells were washed twice with cold PBS and lysed in a buffer containing 0.5% NP-40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM PMSF, and 1 mM Na₃VO₄, and the lysate was clarified by centrifugation at 15,000 rpm for 10 min. The supernatants were then subjected to 8%–12% SDS-PAGE. Separated proteins were transferred to polyvinylidene difluoride membranes and blocked using Tris-buffered saline containing Tween-20 (TBS-T) with 5% skim milk at room temperature for 1 h. Membranes were incubated with the following primary antibodies: anti-AKR1C1 (Abnova, Taiwan, China), anti-AKR1C2 (Abcam, Cambridge, UK), anti-AKR1C3 (Abcam, Cambridge, UK), and AKR1C4 (Abnova, Taiwan, China) at 4°C overnight. Membranes were washed with TBS-T, followed by incubation with responding secondary antibodies. The membranes were washed three times in TBS-T, and the signal was developed using ECL (GE Healthcare, Little Chalfont, UK), followed by detection using an AzureC600 detection system (Azure Biosystems, Dublin, CA).

2.4 Measuring IC₅₀ of AKR1C inhibitors

73 The activity of AKR1C3 was estimated by measuring the OD340 (NADH) during the
74 conversion of glyceraldehyde to glycerol by the Vallee–Hoch method. Three
75 inhibitors, medroxyprogesterone acetate (MPA), meclofenamic acid (MCFLA), and
76 methylasmonate (MLS), were applied to inhibit AKR1C3 activity. The IC₅₀ of the
77 inhibitors was calculated from the plots of AKR1C3 activity vs concentration of
78 inhibitors. Each sample was measured in triplicate.

79 **2.5 Reverse Cis-platin resistance**

80 MPA, MCFLA, and MLS were applied to reverse Cis-platin resistance of HepG2-RC.
81 The inhibitors at given concentrations (MPA: 0.31 mM, MCFLA: 0.12 mM, MLS:
82 0.13 mM) were co-incubated with HepG2-RC cells under a gradient concentration of
83 Cis-platin. The Cis-platin IC₅₀ of each inhibitor-treated HepG2-RC sample was then
84 calculated from the MTT assay.

85 For the knockdown of AKR1Cs in HepG2-RC cells. Small interfering RNAs (siRNAs)
86 targeting human AKR1C1–4 were synthesized by Qiagen (Valencia, CA). HepG2-RC
87 cells (5×10⁶ cells/well) were transfected with 50 nM of si-AKR1Cs or si-scramble as
88 control (sequences are provided in Table S1) using HiPerfect transfection reagent
89 (Qiagen, Valencia, CA). After 24 or 48 h transfection, cells were subjected to RT-PCR
90 and immunoblotting to identify whether AKR1Cs were knocked down. The
91 HepG2-RC cells in which knockdown of AKR1Cs was successful then underwent a
92 Cis-platin IC₅₀ test as described above.

93 **2.6 RNA-sequencing and data analysis**

94 Approximately 106 HepG2 and HepG2-RC cells were frozen on dry ice. RNA
95 extraction, library preparation, RNA-seq, and bioinformatics analysis were performed
96 at BGI (Shenzhen, China). Each set of cell samples was sequenced in three
97 independent experiments. Image analysis, base-calling, and filtering based on
98 fluorescence purity and output of filtered sequencing files were performed through the
99 Illumina analysis pipeline.

100 The obtained raw reads of HepG2 and HepG2-RC cells were preprocessed by
101 removing reads containing adapter sequences, reads containing poly-N, and
102 low-quality reads. Q20 and Q30 were calculated. All six runs of HepG2 and
103 HepG2-RC samples showed that at least 96% of reads was Q20, and at least 87% was
104 Q30. All downstream analyses were based on high-quality clean reads. Gene function
105 was annotated based on Gene Ontology (GO) and the Kyoto Encyclopedia of Genes
106 and Genomes (KEGG). Genes with log₂(fold change) > 1 and FPKM > 0.1 were
107 selected as upregulated genes. Genes with log₂(fold change) < -1 and FPKM > 0.1
108 were selected as downregulated genes. Finally, 1486 upregulated genes and 270
109 downregulated genes were identified.

110 **2.7 Assay of NADH and NAD⁺ content**

111 The amounts of NADH and NAD⁺ were measured by NAD⁺/NADH Assay Kit with
112 WST-8 (Beyotime, Shanghai, China). Approximately 106 HL-7702, HepG2, and
113 HepG2-RC cells were collected for NAD⁺ or NADH extraction. Each sample was
114 divided into two equal parts. One part was used for measuring the total amount of
115 NAD (NAD⁺+NADH), and the other part was used for measuring the amount of
116 NADH after 60°C heat treatment. In the assay, NAD⁺ was first converted to NADH by
117 adding alcohol dehydrogenase and ethanol. NADH then reduced WST-8 to formazan,
118 and the amount of NADH could be measured by monitoring the OD450 value. The
119 ratio of NAD⁺/NADH was calculated by the formula $ratio = ([NAD]_{total} -$
120 $[NADH])/([NADH])$. Each sample was measured in three independent experiments.

121 2.8 Docking experiment

122 A protein docking model of AKR1C3 and its inhibitors was simulated by AutoDock
123 4.2.6 (The Scripps Research Institute, San Diego, CA). The crystal structure of
124 AKR1C3 was derived from the RCSB protein data bank (ID: 4DBW). Ligands were
125 initially drawn using ChemBioDraw Ultra 13.0 (PerkinElmer, Waltham, MA), and
126 energy minimization was performed with ChemBio3D Ultra 13.0 using the MMFF94
127 force field (PerkinElmer, Waltham, CA). Optimized ligand candidates were saved in
128 PDBQT format. The dimensions of the grid box were set at 110, 110, 85 (x, y, z), and
129 the center of the box was placed on Tyr-55 in the A-chain. MPA, MCFLA, and MLS
130 were designed and docked onto the AKR1C3 model with or without NADP⁺, and their
131 binding energy was estimated from docking models.

132 3 Results

133 3.1 AKR1C3 was upregulated in HepG2-RC cells compared with HepG2 cells

134 To investigate the differential expression of AKR1Cs in HepG2 and HepG2-RC cells,
135 we evaluated their mRNA and protein levels. According to the qRT-PCR results
136 (Table 1), the mRNA levels of all AKR1C isoenzymes were higher in HepG2 cells
137 compared with human hepatic HL-7702 cells. However, only AKR1C3 was
138 upregulated in HepG2-RC cells compared with HepG2 cells (~50-fold), while the other
139 isoenzymes showed decreased levels in HepG2-RC cells.

140 According to our Western blot results (Fig.1), AKR1C1, AKR1C2, and AKR1C4
141 levels were almost equal between HepG2 and HepG2-RC cells (1.2-fold differences).
142 AKR1C3 levels in HepG2-RC cells were as much as 1.5-fold higher than in HepG2
143 cells, which was consistent with our qRT-PCR results.

144 3.2 AKR1C inhibitors could reverse Cis-platin resistance of HepG2-RC cells

145 Three AKR1C inhibitors, MPA, MCFLA, and MLS, were applied to reverse Cis-platin
146 resistance in HepG2-RC cells. First, the IC₅₀ value of each AKR1C3 inhibitor was
147 determined (Fig. 2A). MPA showed the lowest IC₅₀ value (2.1 μM). The IC₅₀ values
148 of the other inhibitors were 3.3 μM (MCFLA) and 16.3 μM (MLS). However, MCFLA
149 caused the strongest increase in Cis-platin sensitivity (~8-fold). MPA and MLS

150 increased Cis-platin sensitivity almost 2.5-fold and approximately 1.5-fold,
151 respectively (Fig. 2B).

152 **3.3 SiRNA of AKR1Cs could partially reverse resistance of HepG2-RC cells**

153 Since AKR1C inhibitors could reverse Cis-platin resistance of HepG2-RC cells, RNAi
154 knockdown experiments of all AKR1Cs were performed. Western blot results showed
155 that AKR1C1 and AKR1C3 protein levels were strongly reduced, while these
156 remained unchanged in control-siRNA HepG2-RC cells (Fig. 2C). These observations
157 confirm that AKR1C1 and AKR1C3 were successfully knocked down in HepG2-RC
158 cells.

159 Cis-platin resistance reversal experiments showed that the AKR1C knockdown
160 HepG2-RC cells could tolerate two-fold lower Cis-platin concentrations than control
161 HepG2-RC cells (Fig. 2D). The effects of AKR1C knockdown on Cis-platin resistance
162 were equivalent to the effects of MPA (~2.5-fold reversal), but were much weaker than
163 the effects of MCFLA (~8-fold reversal), indicating that knockdown of AKR1Cs could
164 partially reverse Cis-platin resistance of HepG2-RC cells.

165 **3.4 Most NAD(P)H-dependent reductase/oxidases were upregulated in 166 HepG2-RC cells**

167 According to our RNA-seq results, AKR1C levels were not greatly changed in
168 HepG2-RC cells ($\log_2(\text{HEPG2-RC}/\text{HEPG2}) < 2$; Table 2), and only AKR1C3 showed
169 a slight increase, while the other three showed a slight decrease. This tendency was
170 consistent with the above qRT-PCR results. Among the 16 human AKR enzymes, four
171 (AKR1B10, AKR1B15, AKR1D1, and AKR1B1) were upregulated about four-fold,
172 while two (AKR1E2 and AKR1C4) were downregulated about twofold; the remaining
173 nine enzymes showed almost no change.

174 Moreover, many other NAD(P)H-dependent reductase/oxidases were upregulated four-
175 to eight-fold (Table 2). The strongest upregulation was observed for RFTN1
176 ($\log_2(\text{HEPG2-RC}/\text{HEPG2}) = 6.55$), which encodes raftlin, which bears NAD(P)H
177 cytochrome-b5 reductase activity. Comparing RNA-seq results from HepG2 and
178 HepG2-RC cells, 63 NAD(P)H-dependent reductase/oxidases were upregulated in
179 HepG2-RC cells at least twofold, while only 23 were downregulated at
180 least/approximately twofold. Moreover, 23 of those 63 upregulated genes had at least
181 four-fold higher transcription in HepG2-RC cells compared with HepG2 cells, while
182 only two of those 23 downregulated genes showed a reduction to less than 25% of
183 HepG2 levels. In other words, even though no NAD(P)H-dependent
184 reductase/oxidases were present in the top 10 upregulated genes (Tables S2 and S3),
185 they were generally upregulated.

186 **3.5 Ratio of NADH/NAD⁺ in HepG2-RC cells was higher than in HepG2 cells**

187 As shown in Fig. 3, the amount of total NAD in HepG2 and HepG2-RC cells was
188 approximately four-fold higher than in HL-7702 cells. Interestingly, the ratio of

189 NADH/NAD⁺ in HepG2-RC cells was almost seven-fold higher than in HepG2 or
190 HL-7702 cells. These results could be explained by the RNA-seq results that de novo
191 NAD biosynthesis-related genes were upregulated in HepG2-RC cells (Table 3).
192 Especially, TDO2, encoding tryptophan dioxygenase; KYNU, encoding kynureninase;
193 and NMNAT2, encoding nicotinamide nucleotide adenylyl transferase, showed at least
194 four-fold and at most 32-fold higher mRNA levels in HepG2-RC than in HepG2 cells.
195 Moreover, almost all genes involved in NAD degradation did not show altered mRNA
196 levels, except the ART family. All ART family genes were upregulated in HepG2-RC
197 compared with HepG2; in particular, ART1 mRNA levels in HepG2-RC cells
198 increased 32-fold compared with HepG2 cells. It has been reported that ART1
199 overexpression is closely related to some kinds of cancer[8-10]. In summary, the de
200 novo NAD biosynthesis pathway was upregulated in HepG2-RC, and total NAD levels
201 were increased in HepG2 and HepG2-RC cells compared with HL-7702 cells.

202 **3.6 AKR1C inhibitors could bind with AKR1C3 at different locations, as** 203 **predicted by molecular docking stimulations**

204 Docking simulations with an NADP⁺-bound AKR1C3 model (Fig. 4A–C) showed that
205 the highest affinity docking inhibitor was MPA, with the lowest binding free energy
206 ($\Delta G = -11.54$ kcal/mol). MCFLA showed the second highest affinity, with $\Delta G =$
207 -7.06 kcal/mol, and MLS showed the lowest affinity (-6.47 kcal/mol). These results
208 are consistent with the IC₅₀ values of the inhibitors on AKR1C3, with MPA having
209 the lowest and MLS having the highest IC₅₀ value (Fig. 2A).

210 If NADP⁺ was removed from the AKR1C3 structure and only inhibitors were docked
211 with AKR1C3 apoenzyme, the order did not change, but we could see considerable
212 differences in their inhibitor binding location (Fig. 4A–C). MCFLA and MLS occupied
213 the oxygen site composed by Tyr-55, His-117, and NADP⁺, while MPA was tightly
214 bound to the steroid-binding site. In the NADP⁺-bound AKR1C3 model, the distance
215 between the nitrogen atom of the nicotinamide ring of NADP⁺ and the carbonyl
216 oxygen of the inhibitors was consistent with the notion that MCFLA was closer to
217 NADP⁺ (~ 6.57 Å) than MPA (~ 7.68 Å). These results indicate that MPA is the best
218 selective inhibitor of AKR1C3, while MCFLA partially interacts with the NADP⁺
219 binding site, showing a relatively poor selectivity.

220 **4 Discussion**

221 It has been reported that AKR1C enzymes are overexpressed in several cancer cell
222 types, such as bladder cancer cells and gastric cancer cells, contributing to the
223 resistance to Cis-platin treatment[2-6,11]. Some cytotoxic lipid peroxidative products
224 have been mentioned as targets of AKR1Cs that are involved in Cis-platin
225 resistance[6]. However, the role of AKR1Cs in the mechanism underlying Cis-platin
226 resistance remains unclear. We found that AKR1C inhibitors could reverse resistance
227 of HepG2-RC cells, even though AKR1Cs were not significantly upregulated in
228 HepG2-RC cells. The effects of siAKR1Cs on HepG2-RC cells were comparable to

those of the inhibitor MPA, but could only partially explain the effects of MCFLA. This fact could be explained by that MPA is a steroid analog, which shows a high selectivity, while MCFLA inhibits not only AKR1C enzymes but also other AKR enzymes. MCFLA belongs to the class of non-steroid anti-inflammatory inhibitors, which have been reported to also inhibit cyclooxygenases besides AKR1Cs, indicating its poor selectivity[12]. As shown in Fig. 4D, all AKR enzymes share a common set of residues to bind NADP(H). As shown in Fig. 4B, MCFLA preferred the oxygen site rather than the steroid pocket, indicating that MCFLA inhibits AKR1C3 by disturbing NAD(P) (H) binding.

These results indicate that MCFLA could inhibit some other AKR enzymes besides AKR1Cs. This could explain why MCFLA causes a stronger reversal of Cis-platin resistance of HepG2-RC cells than MPA. It is likely that NAD(P)H plays a key role here because it is a reducing force and a product of NAD(P)H-dependent oxidoreductases. Reprogramming energy metabolism is considered as a hallmark of cancer cells in which NAD(P) or NAD(P)H levels are increased[13-15]. It has been reported that some NAD(P)H-dependent oxidoreductases, such as ALDHs, increase NAD(P)H levels in the cytosol of cancer cells, which then serves as an electron source[16].

We also found that the amount of total NAD (NAD++NADH) in both HepG2 and HepG2-RC cells was approximately four-fold higher than in HL-7702 cells. Moreover, the ratio of NADH/NAD+ in HepG2-RC cells was increased as much as seven-fold compared to HepG2 cells. The increased ratio could be explained by two steps (Fig. 5). First, NADH is produced continuously in hepatic cancer cells. This is in contrast to the situation in normal cells, where AKRs function as reductases[1]. However, the continuously biosynthesized NAD in hepatic cancer cells would make NAD(P)H-dependent enzymes catalyzing reactions in one direction, from NAD(P) to NAD(P)H. Second, the produced NAD(P)H would deal with Cis-platin-induced peroxidative products directly or indirectly. Usually, NADPH maintains glutathione at the reduced state, which could detoxify reactive oxygen species. However, this could not alter the NAD(P)H/NAD(P) ratio too much, because each reaction is reversible. It has also been reported that no alteration of resistance to Cis-platin or oxaliplatin occurred after GSH depletion in oxaliplatin-resistant human gastric adenocarcinoma TSGH cells[17], indicating GSH is not involved in the Cis-platin resistance.

Besides GSH, NAD(P)H could also react rapidly with moderately oxidizing radicals to repair biomolecules[18]. So, NAD(P)H could also work as a directly operating antioxidant that scavenges radicals as NAD(P)H* forms. These forms would exist in the cell for a relatively long time and keep the intracellular concentration of free NAD(P)H very low. Therefore, NAD(P)H-dependent enzymes could catalyze NAD(P) to NAD(P)H continuously, resulting in a high ratio of NAD(P)H/NAD(P). If these enzymes were widely inhibited by a poor selective inhibitor, such as MCFLA, NADH would not accumulate anymore, and Cis-platin resistance would be suppressed as well.

270 **5 Conclusions**

271 In summary, it is believed that NAD(P)H-dependent oxidoreductases, especially
272 AKRs, produce NADH in HepG2 cells to overcome Cis-platin-induced cytotoxicity.
273 According to this notion, chemotherapy with inhibitors, which could compete with
274 NAD(P) in most oxidoreductases, could lead to a better reversal of Cis-platin
275 resistance in Cis-platin-resistant cancer cells.

276 **6 Conflict of Interest**

277 The authors declare that the research was conducted in the absence of any commercial
278 or financial relationships that could be construed as a potential conflict of interest.

279 **7 Author Contributions**

280 Yuanhua Yu conceived-designed experiment. Tingting Sun and Le Gao analyzed data
281 and wrote and edited the main manuscript text. Xin Wang, Xue Sun and Rui Guo
282 performed in vitro experiments. All authors read and approval of the manuscript.

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290 **10 Supplementary Material**

291 Additional tables(Table.S1., Table.S2., Table.S3.)

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352 12 Figure Legends

353 Fig. 1. Differential expression of AKR1Cs in hepatic cell lines HL-7702, HepG2, and
 354 HepG2-RC, as measured with western blot. A, AKR1C1; B, AKR1C2; C, AKR1C3;
 355 D, AKR1C4. β -actin was selected as an internal reference. The signal intensity of each
 356 band was assessed with ImageJ software and divided by the value of β -actin. The ratios
 357 of AKR1Cs and β -actin are shown at the top of the figure.

358 Fig. 2. Reversion of Cis-platin sensitivity in HepG2-RC. A, IC₅₀ value of three
 359 inhibitors acting on AKR1C3. MPA, medroxyprogesterone acetate; MCFLA,
 360 meclofenamic acid; MLS, methyliasmonate. B, Reversion ratio of Cis-platin sensitivity
 361 in HepG2-RC cells caused by three inhibitors. C, Knockdown of AKR1Cs by siRNA.
 362 Successful knockdown was confirmed with western blot. D, Reversion of Cis-platin
 363 sensitivity in AKR1C knockdown cells.

364 Fig. 3. Ratio of NADH/NAD⁺ in HL-7702, HepG2, and HepG2-RC cells. The
 365 amounts of total (NAD⁺⁺+NADH) and NADH were measured with an NAD/NADH
 366 quantitation kit. The amount of NAD was calculated from these two values. Finally, a
 367 ratio of NADH vs NAD was obtained.

368 Fig. 4. Molecular docking simulation results of AKR1C3 and three inhibitors. A,
 369 Meclofenamic acid (MCFLA) with AKR1C3 alone or AKR1C3 and NADP⁺. B,
 370 Methyliasmonate (MLS) with AKR1C3 alone or AKR1C3 and NADP⁺. C,
 371 Medroxyprogesterone acetate (MPA) with AKR1C3 alone or AKR1C3 and NADP⁺.
 372 OS, oxygen site; SP, steroid-binding pocket. Distance was measured between the
 373 nitrogen atom of the nicotinamide ring and the carbonyl oxygen of the inhibitor.
 374 AKR1C3 is represented in cartoon style. NADP⁺ is shown as a stick model, and
 375 Tyr-55 and His-117 are shown as a stick-ball model. NADP⁺, Tyr-55, and His-117
 376 contain an oxygen site (OS). The steroid pockets (SPs) are displayed in surface style.
 377 D, The alignment of representative members of the AKR enzyme family (AKR1A1,
 378 AKR1B1, AKR1C3, AKR1D1, AKR1E2, and AKR7A2). The residues involved in the
 379 NADP⁺ binding are placed in the box.

380 Fig. 5. A possible role of NAD(P)H/NAD(P)-dependent reductase/oxidase in the
 381 mechanism underlying Cis-platin resistance. Arrows pointing upwards indicate
 382 upregulation in HepG2-RC cells; arrows pointing downwards indicate downregulation
 383 in HepG2-RC cells.

384 Table.1. The mRNA amount (2- Δ Ct) of AKR1Cs in different hepatic cell lines
 385 measured by qRT-PCR.

Cell lines Enzymes	HL-7702	HepG2	HepG2-RC
AKR1C1	10.3	3.1×10^3	0.3×10^3
AKR1C2	6.8	0.3×10^3	0.1×10^3
AKR1C3	16.1	6.1×10^4	2.9×10^6
AKR1C4	4.6	6.6	1.6

386 Table.2. Regulation of AKR enzymes measured by RNA-seq.

Gene	log2(HepG2-RC/HepG2)	Annotation
AKR1B10	3.12	All-trans-retinol + NADP+ $\rightleftharpoons$ all-trans-retinal + NADPH
AKR1B15	2.63	L-Arabitol:NADP + 1-oxidoreductase L-Arabitol + NADP+ $\rightleftharpoons$ L-Arabinose + NADPH + H+
AKR1D1	2.16	5 β -androstane-3,17-dione:NADP + 4,5-oxidoreductase 5 β -Androstane-3,17-dione + NADP+ $\rightleftharpoons$ H+ + NADPH + Androstenedione
AKR1B1	2.14	glycerol:NADP + oxidoreductase Glycerol + NADP+ $\rightleftharpoons$ D-Glyceraldehyde + NADPH + H+
AKR1C3	0.41	NADP+ + trans-1,2-dihydrobenzene-1,2-diol $\rightleftharpoons$ catechol + H+ + NADPH
AKR6A5	0.21	NADPH
AKR6A9	0.18	potassium voltage-gated channel subfamily A regulatory beta subunit 2
AKR7A3	-0.02	potassium voltage-gated channel subfamily A regulatory beta subunit 2
AKR6A3	-0.09	potassium voltage-gated channel subfamily A regulatory beta subunit 3
AKR1C1	-0.43	aflatoxin B1 + NAD(P)+ $\rightleftharpoons$ aflatoxin B1-dialcohol + NAD(P)H
AKR1C2	-0.44	potassium voltage-gated channel subfamily A regulatory beta subunit 1
AKR7A2	-0.55	17 α ,20 α -dihydroxypregn-4-en-3-one + NAD(P)+ $\rightleftharpoons$ 17 α -hydroxyprogesterone + H+ + NAD(P)H
AKR1A1	-0.68	3 α -hydroxysteroid + NADP+ $\rightleftharpoons$ 3-oxosteroid + H+ + NADPH
AKR7A4	-0.94	4-hydroxybutanoate + NADP+ $\rightleftharpoons$ H+ + NADPH + succinate semialdehyde
AKR1E2	-1.03	allyl alcohol + NADP+ $\rightleftharpoons$ acrolein + H+ + NADPH
AKR1C4	-1.03	aflatoxin B1 + NAD(P)+ $\rightleftharpoons$ aflatoxin B1-dialcohol + NAD(P)H
		testosterone:NAD+ 17-oxidoreductase Testosterone + NAD+ $\rightleftharpoons$ Androstenedione + NADH + H+
		Androsterone:NADP+ oxidoreductase Androsterone + NADP+ $\rightleftharpoons$ 5 α -Androstane-3,17-dione + NADPH + H+

387 Table.3. Regulation of NAD metabolism related genes in HepG2 and HepG2-RC
388 measured by RNA-seq.

Gene	Log2(HepG2-RC/HepG2)	Annotation
IDO1	0.41	indoleamine 2,3-dioxygenase 1
IDO2	NA	indoleamine 2,3-dioxygenase 2
TDO2	3.97	tryptophan 2,3-dioxygenase
KMO	-0.42	kynurenine 3-monooxygenase
KYNU	2.78	kynureninase
HAAO	-0.03	3-hydroxyanthranilate 3,4-dioxygenase
QPRT	0.076	quinolinate phosphoribosyltransferase
NMRK1	0.33	nicotinamide riboside kinase 1
NMRK2	NA	nicotinamide riboside kinase 2
NMNAT1	0.03	nicotinamide nucleotide adenylyltransferase 1
NMNAT2	3.76	nicotinamide nucleotide adenylyltransferase 2
NMNAT3	-0.10	nicotinamide nucleotide adenylyltransferase 3
Naprt	-0.71	nicotinate phosphoribosyltransferase
NAMPT	0.83	nicotinamide phosphoribosyltransferase
Nadsyn1	0.4	NAD synthetase 1
SIRT1	-0.16	sirtuin 1
SIRT2	0.30	sirtuin 2
SIRT3	-0.61	sirtuin 3
SIRT4	-0.012	sirtuin 4
SIRT5	-0.23	sirtuin 5
SIRT6	-0.11	sirtuin 6
SIRT7	0.27	sirtuin 7
CD38	-1.71	CD38 molecule
PARP1	-0.20	poly(ADP-ribose) polymerase 1
PARP2	-0.68	poly(ADP-ribose) polymerase 2
PARP3	0.04	poly(ADP-ribose) polymerase 3
PARP4	0.48	poly(ADP-ribose) polymerase 4
PARP6	-0.33	poly(ADP-ribose) polymerase 6
PARP8	0.22	poly(ADP-ribose) polymerase 8
PARP9	1.64	poly(ADP-ribose) polymerase 9
PARP10	1.1	poly(ADP-ribose) polymerase 10
PARP11	NA	poly(ADP-ribose) polymerase 11
PARP12	1.14	poly(ADP-ribose) polymerase 12
PARP14	1.22	poly(ADP-ribose) polymerase 13
PARP15	0.96	poly(ADP-ribose) polymerase 15
PARP16	-0.49	poly(ADP-ribose) polymerase 16
ART1	4.06	ADP-ribosyltransferase 1
ART3	1.24	ADP-ribosyltransferase 3
ART4	1.29	ADP-ribosyltransferase 4
ART5	3.73	ADP-ribosyltransferase 5

Fig 1

Fig 3

A

D

[illegible]

Fig 5

Fig 2