OMTO, Volume *26*

Supplemental information

Berberine inhibits carcinogenesis through antagonizing the ATX-LPA-LPAR2-p38-leptin axis in a mouse hepatoma model

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Supplementary Table 1 Serum levels were detected of 12 cytokines and metabolic hormones from male mice 1 month after DEN injection by employing the Luminex assay.

Examinations	sham	normal chow	0.04% BBR	0.2% BBR
IL-1β	458.3 ± 87.86	210.1 ± 15.43$^{\#}$	244.5 ± 24.59	345.9 ± 50.39*
IL-6	7.99 ± 1.28	5.19 ± 0.845	7.05 ± 0.691	7.88 ± 0.998
IL-18	1048 ± 136.9	924.3 ± 122.8	847.1 ± 58.04	1055 ± 106.3
TNF-α	1478 ± 205.6	1175 ± 130.8	1383 ± 94.3	2013 ± 237.6**
M-CSF	3369 ± 160.5	3041 ± 101.1	3745 ± 156.8**	3439 ± 172.5
G-CSF	53.76 ± 10	53.48 ± 8.754	83.88 ± 10.21	94.41 ± 17.32
GM-CSF	14.66 ± 5.488	2.931 ± 0.6061	6.619 ± 1.266	5.528 ± 1.346
leptin	132.9 ± 14.21	203.9 ± 38.99	175.1 ± 30.27	163.3 ± 25.49
Adiponectin	5566 ± 247.2	5590 ± 221.5	5249 ± 205.5	5365 ± 146.3
PAI-1	4270 ± 126.7	4036 ± 120.6	4032 ± 219.8	4236 ± 221.8
Insulin	5092 ± 595.4	4320 ± 749.6	8509 ± 2009	7160 ± 1491
Resistin	29472 ± 4009	15818 ± 1525$^{\#\#}$	36014 ± 2940**	28202 ± 2144**

1. The concentration unit was pg/mL, except adiponectin as ng/mL.

2. Data are expressed as mean ± SEM, n=14-16. * P<0.05, ** P<0.01, as compared to that of DEN model group fed on normal chow, using the one-way ANOVA. $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, as compared to that of sham treated group, using the unpaired t-test.

Supplementary Table 2 Serum levels were detected of 12 cytokines and metabolic hormones from male mice 4 months after DEN injection by employing the Luminex assay.

Examinations	sham	normal chow	0.04% BBR	0.2% BBR
IL-1β	196.1 ± 29.4	658.4 ± 202.2$^{\#}$	212.9 ± 35.52*	307.2 ± 57.79
IL-6	3.75 ± 2.02	5.6 ± 2.22	2.71 ± 0.378	5.13 ± 0.861
IL-18	457.3 ± 111.6	352.2 ± 49.74	376.5 ± 22.91	352.1 ± 58.91
TNF-α	555.8 ± 108.6	639.9 ± 62.01	809.8 ± 104.5	1169 ± 201.1*
M-CSF	3972 ± 397.5	4237 ± 283.1	3662 ± 211.9	3714 ± 165.9
G-CSF	30.59 ± 7.528	46.61 ± 9.042	27.34 ± 4.266*	26.44 ± 2.058*
leptin	11834 ± 2134	2386 ± 495.8$^{\#\#}$	637.8 ± 147.1**	757.6 ± 273.1**
Adiponectin	10770 ± 578.3	7946 ± 346$^{\#\#}$	8149 ± 240.4	8192 ± 516.3
PAI-1	3347 ± 149.7	5900 ± 425.1$^{\#\#}$	4421 ± 216.8*	4621 ± 451.7*
Insulin	5511 ± 852.6	3402 ± 834.7	3703 ± 651.9	3664 ± 786.4
Resistin	51523 ± 4838	24308 ± 2390$^{\#\#}$	16886 ± 1512*	23133 ± 2168

1. The concentration unit was pg/mL, except adiponectin as ng/mL.

2. Data are expressed as mean ± SEM. Sham, n=11; the other group, n=14-15. * P<0.05, ** P<0.01, as compared to that of DEN model group fed on normal chow, using the one-way ANOVA.

$^{\#}$ P<0.05, $^{\#\#}$ P<0.01, as compared to that of sham treated group, using the unpaired t-test.

3. For GM-CSF, the level of most samples was under the detection limit and thus not shown.

Supplementary Table 3 Serum levels were detected of 12 cytokines and metabolic hormones from male mice 6 months after DEN injection by employing the Luminex assay.

Examinations	sham	normal chow	0.04% BBR	0.2% BBR
IL-1β	388.3 ± 40.22	756 ± 93.4$^{\#\#}$	547.5 ± 83.47	663.5 ± 78.12
IL-6	23.88 ± 3.519	24.42 ± 1.979	31.95 ± 7.565	35.03 ± 4.202
IL-18	481.4 ± 90.53	463.1 ± 47.65	530.6 ± 59.5	488.5 ± 51.11
TNF-α	3736 ± 457.3	4984 ± 419.3	4093 ± 534.2	4947 ± 362.2
M-CSF	4220 ± 1106	3512 ± 611.9	2408 ± 129.2	3166 ± 420
G-CSF	126.3 ± 18.61	141.2 ± 39.37	102.8 ± 23.78	113 ± 35.3
GM-CSF	31.95 ± 3.635	37.4 ± 3.974	36.18 ± 3.444	31.08 ± 3.843
leptin	1433 ± 338.9	4461 ± 697.9$^{\#\#}$	2659 ± 542.5	2104 ± 352.1*
Adiponectin	9369 ± 570.7	7558 ± 392.1$^{\#}$	6938 ± 349.8	6236 ± 250.1*
PAI-1	4098 ± 786.7	4785 ± 380.1	4541 ± 273.9	4083 ± 403.5
Insulin	4615 ± 493.1	8791 ± 1297$^{\#\#}$	10355 ± 2569	11673 ± 2144
Resistin	35898 ± 3416	51256 ± 4502$^{\#}$	36305 ± 3770*	32938 ± 3600**

1. The concentration unit was pg/mL, except adiponectin as ng/mL.

2. Data are expressed as mean ± SEM, n=12-14. * P<0.05, ** P<0.01, as compared to that of DEN model group fed on normal chow, using the one-way ANOVA. $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, as compared to that of sham treated group, using the unpaired t-test.

Supplementary Table 4 Serum levels were detected of 12 cytokines and metabolic hormones from male mice 8 months after DEN injection by employing the Luminex assay.

Examinations	sham	normal chow	0.04% BBR	0.2% BBR
IL-1β	492.2 ± 130.2	530.4 ± 81.3	302.8 ± 42.11*	322.9 ± 44.65*
IL-6	10.39 ± 4.723	26.06 ± 11.93	14.18 ± 3.271	41.8 ± 19.67
IL-18	261.9 ± 46.32	503.2 ± 42.68$^{\#\#}$	517.8 ± 66.56	397.4 ± 62.25
TNF-α	1551 ± 422.5	2180 ± 253.9	1370 ± 130.9	1803 ± 361.2
M-CSF	4617 ± 574.6	5154 ± 547.2	6639 ± 365.6	6628 ± 515
G-CSF	104.7 ± 16.36	137.3 ± 26.42	71.67 ± 8.948*	69.27 ± 9.023*
leptin	15145 ± 3872	11622 ± 1924	9906 ± 2025	6859 ± 1375
Adiponectin	6073 ± 391.6	4428 ± 297.1$^{\#\#}$	5912 ± 295.9**	5353 ± 377.3
PAI-1	6403 ± 1015	13839 ± 2747$^{\#}$	6286 ± 487.2**	8702 ± 845.8
Insulin	6715 ± 1250	8659 ± 1351	8467 ± 1701	9335 ± 1424
Resistin	64781 ± 7993	56518 ± 4364	52211 ± 5717	44932 ± 4530

1 The concentration unit was pg/mL, except adiponectin as ng/mL.

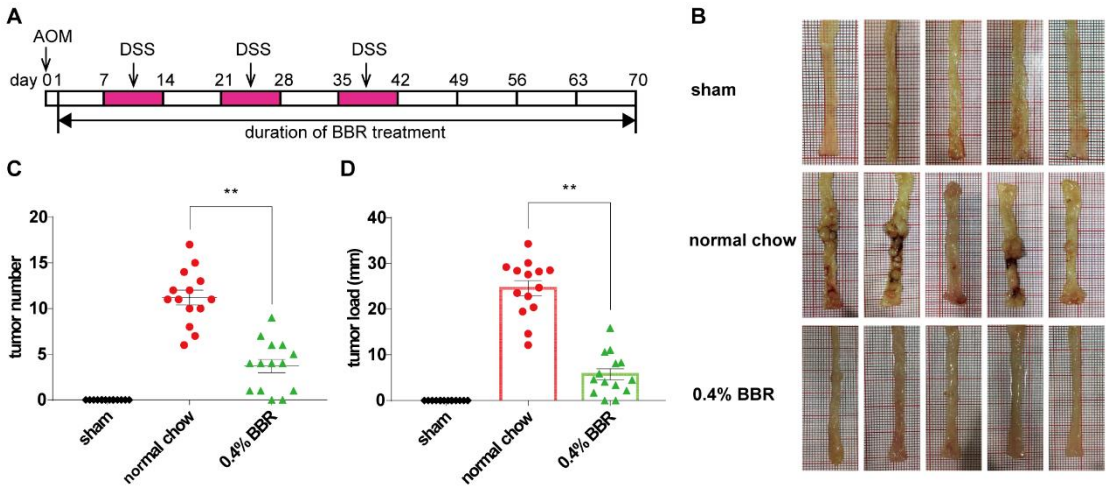
2 Data are expressed as mean ± SEM. Sham, n=13; the other group, n=23-24. * P<0.05, ** P<0.01, as compared to that of DEN model group fed on normal chow, using the one-way ANOVA.

$^{\#}$ P<0.05, $^{\#\#}$ P<0.01, as compared to that of sham treated group, using the unpaired t-test.

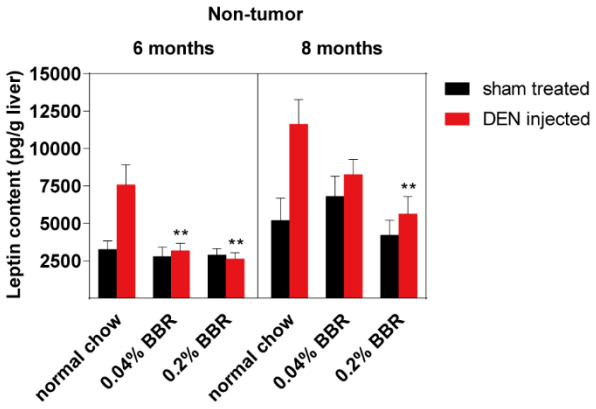
3. For GM-CSF, the level of most samples was under the detection limit and thus not shown.

Supplementary Table 5 Primers used in real-time RT PCR

Species	Gene names		Primer sequences (5'→3')
Mouse	ATX	left	tggcttacgtgacattgagg
		right	agtgggtagggacaggaatagag
	LPAR2	left	tctgccgcttgactggat
		right	gccgatggtctcgttgtagt
	leptin	left	cccaaaatgtgctgcagatag
		right	ccagcagatggaggaggtc
	β-actin	left	ccaaccgtgaaaagatgacc
		right	accagaggcatacagggaca
Human	LPAR2	left	ccgctaccgagagaccac
		right	tgtccagcagaccacgaac
	leptin	left	cagctgaacagccaaatgc
		right	cccctcagctcataccatttc
	C/EBPα	left	ggagctgagatcccgaca
		right	ttctaaggacaggcgtggag
	HIF1-α	left	ttttcaagcagtaggaattggaa
		right	gtgatgtagtagctgcatgatcg
	β-actin	left	tcaacaccccagccatgta
		right	agtacggccagaggtgtacg



Supplementary Figure 1 Berberine inhibited AOM-induced colon carcinogenesis. (A) Scheme of the experimental design: AOM-induced colon carcinogenesis and BBR treatment (n=14). The day of AOM-injection was set as the "day 0". (B) The AOM-induced tumors were reduced by BBR treatment (supplemented in diet, 0.4%, w/w) for 10 weeks. The representative images of colon were demonstrated for their group. (C, D) BBR significantly reduced the tumor number and load 10 weeks after AOM-injection in the mice. Statistics was done for the whole group (n=14), and data were expressed as the mean ± SEM. ** $P<0.01$, BBR treated group versus AOM model group.



Supplementary Figure 2 Berberine reduced the leptin content in DEN-injected but not sham treated mice. Liver leptin content was detected by ELISA assay of non-tumor liver tissue from male mice sacrificed at 6 and 8 months after DEN-injection, respectively. Values were the mean ± SEM (n=5). * $P<0.05$, ** $P<0.01$ VS that of the DEN model group fed with normal chow.

Methods

Animal studies

Azoxymethane (AOM, purity over 98%, A5486) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO). Dextran Sulfate Sodium Salt (DSS, purity over 99%, #0216011080) was purchased from MP Biomedicals Co., Ltd (Shanghai, China). To generate AOM-induced colon tumors, 10-week-old C57BL/6 mice were subjected to single intraperitoneal injection with AOM (10 mg kg−1 of body weight). On the next day, the mice were randomly grouped and fed with either normal chow diet (Keao Xieli Feed Co., Ltd, Beijing, China) or the diet supplement with BBR (0.4%, w/w). DSS was supplemented in the drinking water (1%, w/v) at the 2nd, 4th and 6th week of the experiment period. Mice were sacrificed to learn BBR's effect on colon carcinogenesis at the end of 10 weeks after AOM injection. All visible tumors were counted on the surface of colon, and the diameter of each tumor was measured using a sliding caliper. The tumor load was calculated through multiplying the tumor number by the average diameter per mouse, as reported previously (1).

Reference

1. Neufert C, Heichler C, Brabletz T, Scheibe K, Boonsanay V, Greten FR, *et al.* Inducible mouse models of colon cancer for the analysis of sporadic and inflammation-driven tumor progression and lymph node metastasis. Nat Protoc 2021;16(1):61-85.