Supplementary information for " Anti-tumor Effects and Potential Therapeutic Response Biomarkers in α-Emitting *meta*-^{211}At-Astato-Benzylguanidine Therapy for Malignant Pheochromocytoma Explored by RNA-sequencing "

Supplementary materials and methods

PC12 cell culture

PC12 cells were cultured in RPMI1640 (Wako Pure Chemical Industries, Osaka, Japan) containing 10% heat-inactivated horse serum (Thermo Fisher Scientific, Inc., Waltham, MA), 5% heat-inactivated fetal bovine serum (AusGeneX, Loganholme, OLD, Australia), penicillin (100 units/ml) and streptomycin (100 μg/ml) and L-glutamine (2 mM). Incubation conditions of PC12 were 37°C, 5% CO_2 and 95% air.

Cell survival assay

Cells treated with ^{211}At-MABG or irradiated with γ-rays were washed with phosphate buffered saline (PBS), suspended in growth medium, and seeded at 400 cells/well in a 96-well plate for 2 weeks incubation. After incubation, cell survival was evaluated by the

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported [1].

Absorbed dose for ^{211}At-MABG treatment

Absorbed dose of ^{211}At-MABG treated cells was estimated from the cellular uptake and release experiments. For uptake, 10^5 cells were incubated with 5.0 kBq of ^{211}At-MABG in 5 mL growth medium (1.0 kBq/mL) for 0, 1, 3, 6 and 12 h. Just after incubation, cells were washed with ice-cold PBS, and dissolved in 0.1 N NaOH. Radioactivity of ^{211}At in the solution was measured by γ-counter. On the other hand, the cellular release was examined as follows: (i) Cells were treated with ^{211}At-MABG for 1 h, (ii) after ^{211}At-MABG exposure, cells were washed by PBS and (iii) cells were incubated in growth medium for 1, 3, 6, 12 and 24 h. Cells were washed by PBS after incubation, and ^{211}At radioactivity of cells was measured.

Absorbed dose of ^{211}At-MABG treated cells was basically estimated by the published method [2] with some modifications. Time activity curves (TACs) of cellular uptake and release experiments were fitted by the following functions using real-coded genetic algorithm (Real-GA) [3] (**Figure S1A**):

$$\Delta (\%AD_1) = -k_{12} * \%AD_1 * \Delta t + k_{21} * \%AD_2 * \Delta t \quad \text{(a1) and}$$

$$\Delta (\%AD_2) = -k_{21} * \%AD_2 * \Delta t + k_{12} * \%AD_1 * \Delta t \quad \text{(a2).}$$

Here, $\%AD_1$ and $\%AD_2$ are the percent applied dose of growth medium (5 mL) and cells (10^5), respectively. k_{12} and k_{21} are transport coefficients in s^{-1}. Δ indicates the difference and Δt is the time step of 1 s. For uptake, when the calculation time was 0, $\%AD_2$ was set to be 0, and $\%AD_1$ was 0 for release. Using the estimated values of k_{12} (1.50×10^{-5}) and k_{21} (4.18×10^{-5}), we simulated well both uptake and release experiments (**Figure S1B-C**).

These parameters made our model simulate TAC in the survival experiment. **Figure S2A-B** show the TACs during the incubation for ^{211}At-MABG exposure (1.0 kBq/ml at the start time) and 10 times of half-life in ^{211}At ($7.2 * 10 = 72$ h).

Western blot analysis

Cells were dissolved in sample buffer and incubated at 95°C for 15 min. Aliquots of samples containing 40 μg protein were analyzed by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membranes. Blots were incubated at 4°C overnight in tris-buffered saline and 0.1% polysorbate-20 (TBST) containing 5% w/v milk. Blots were then incubated with rabbit anti-TSPO antibody (1/200, Biorbyt, Cambridge, UK) or rabbit anti-β-actin

antibody (1/1000, Cell Signaling Technology, Beverly, MA) at room temperature for 2.5 h. After washing with TBST, the blots were incubated with horseradish peroxidase conjugated anti-rabbit IgG antibody for 1.5 h at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

MIBG-control experiment

Since there is no stable isotope in astatine, nonradioactive MIBG which shows a similar biological kinetics to ^{211}At-MABG was used for the control experiments [1]. The radionuclidic purity of ^{211}At was over 99% in our study. Therefore, molar concentrations of ^{211}At-MABG were 49.7 fM for 10% survival dose (0.8 kBq/ml) and 6.2 fM for 80% survival dose (0.1 kBq/ml) according to the following formula; $A=0.693/T\times6.02\times1023\times M$ (A: radioactivity, T: half-life (sec), M: molar concentration). PC-12 cells were treated with culture medium or 80% and 10% survival equivalent dose of MIBG for 0, 3, 6, 12 h. After harvesting cells, RNA extraction, sequencing, differential expression analysis were performed according to Materials and Methods.

Supplementary result

Low fluence rate

The doses for 10% survival were 10 Gy and 3.5 Gy for γ-ray and ^{211}At-MABG, respectively. The relative biological effectiveness (RBE) at 10% survival was thus approximately 2.9, suggesting the strong anti-tumor effect of the α-particles emitted from ^{211}At-MABG. Because nuclear DNA damage is the main cause of IR-induced cell death, the number of α-particles passing through the nucleus is an important concern [4]. The maximum number of α-particles emitted was approximately 25 per cell (Figure S2D), and 10 α-particles passing through the nucleus can induce 10% survival in mammalian cells [5]. Overall, the number was one-third to one-half of the maximum α-particles emitted by ^{211}At-MABG. Thus, adjacent cellular ^{211}At-MABG may contribute to nuclear-penetrating α-particles because the probability of α-particles emitted in all directions by ^{211}At-MABG passing through the nucleus is expected to be slightly lower than one-third. Taken together, the number of α-particles derived from 0.8 kBq/mL of ^{211}At-MABG-exposure would be sufficient for 10% survival in PC12 cells.

Supplementary references

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3. Herrera F, Lozano M, Verdegay JL. Tackling real-coded genetic algorithms: operators and tools for behavioural analysis. Artif Intell Rev. 1998; 12(4): 265–319.

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Table S1 Representative DEGs between ^{211}At-MABG treatment and γ-irradiation.

Rank	*Gene name*	LogFC	Rank	*Gene name*	LogFC	Rank	*Gene name*	LogFC
1	*Snrpg*	4.075	**56**	*Ero1b*	1.615	**111**	*Nup93*	1.311
2	*Mien1*	3.665	**57**	*Csrp2*	1.612	**112**	*Slirp*	1.306
3	*Hnrnpa3*	2.925	**58**	*Shfm1*	1.606	**113**	*Timm8b*	1.304
4	*Vdac1*	2.897	**59**	*Dbi*	1.587	**114**	*Siva1*	1.304
5	*Otub1*	2.697	**60**	*Sass6*	1.584	**115**	*Hnrnpf*	1.3
6	*Ppial4d*	2.672	**61**	*Cdkn2aipnl*	1.563	**116**	*Map1s*	1.287
7	*Gm5471*	2.644	**62**	*Cox6c*	1.562	**117**	*Ankfy1*	1.286
8	*Tuba1a*	2.623	**63**	*Btf3*	1.556	**118**	*Slbp*	1.285
9	*Sos2*	2.551	**64**	*App*	1.555	**119**	*Hdac1l*	1.282
10	*Dmap1*	2.443	**65**	*Fkbp5*	1.539	**120**	*Txn1*	1.28
11	*Snrpf*	2.377	**66**	*Uap1*	1.535	**121**	*Rbx1*	1.261
12	*Usmg5*	2.339	**67**	*Slc16a1*	1.51	**122**	*Med21*	1.261
13	*Vegfa*	2.328	**68**	*Ercc4*	1.51	**123**	*Bre*	1.259
14	*Uqcrb*	2.305	**69**	*Epm2a*	1.506	**124**	*Paip2b*	1.252
15	*Fam222b*	2.305	**70**	*Snrpe*	1.498	**125**	*Gtf2f2*	1.242
16	*Cilp2*	2.299	**71**	*Fkbp11*	1.491	**126**	*Atg14*	1.241
17	*Adk*	2.279	**72**	*Sgol2*	1.49	**127**	*Pole4*	1.24
18	*Lsm5*	2.277	**73**	*Clybl*	1.489	**128**	*Adck1*	1.231
19	*Eif3h*	2.24	**74**	*Zdhhc13*	1.48	**129**	*Morf4l1*	1.223
20	*Ybx1-ps3*	2.22	**75**	*Fam83d*	1.477	**130**	*Sub1*	1.217
21	*Fen1*	2.218	**76**	*Pitpnb*	1.476	**131**	*Rgs7*	1.217
22	*Slc6a17*	2.208	**77**	*7-Sep*	1.474	**132**	*Hnrnpa2b1*	1.214
23	*Zbed5*	2.195	**78**	*Exoc6b*	1.473	**133**	*Kntc1*	1.206
24	*Atp5i*	2.17	**79**	*SNORA44*	1.462	**134**	*Chchd1*	1.2
25	*Cdk5r2*	2.078	**80**	*Trmt112*	1.457	**135**	*Ahcyl2*	1.197
26	*Atp5j2*	2.061	**81**	*Fam64a*	1.447	**136**	*Sdhaf3*	1.194
27	*Ska2*	1.978	**82**	*Cdca5*	1.44	**137**	*Mb21d1*	1.191
28	*Msh2*	1.975	**83**	*Nuf2*	1.436	**138**	*Rtcb*	1.187
29	*Atp5e*	1.97	**84**	*Galnt2*	1.436	**139**	*Ddx19b*	1.187
30	*Acp1*	1.93	**85**	*Ncam1*	1.434	**140**	*Srsf7*	1.177
31	*Chmp6*	1.928	**86**	*Abi1*	1.427	**141**	*Ccne2*	1.175
32	*Tubg1*	1.865	**87**	*Cox7b*	1.417	**142**	*Rell1*	1.168
33	*Cwc22*	1.864	**88**	*Vrk2*	1.406	**143**	*Slc36a1*	1.145
34	*Lin9*	1.857	**89**	*Dazap1*	1.406	**144**	*Ndufb3*	1.141
35	*Ddx6*	1.848	**90**	*Clspn*	1.402	**145**	*Parp2*	1.135
36	*Csde1*	1.832	**91**	*Ppih*	1.399	**146**	*Cox7c*	1.133
37	*Ptma*	1.812	**92**	*Exosc8*	1.399	**147**	*Actr2*	1.129
38	*Lims1*	1.808	**93**	*Gabpb1l*	1.39	**148**	*Atp11b*	1.124
39	*MGC95208*	1.795	**94**	*Tuba1a*	1.388	**149**	*Gins3*	1.116
40	*Phb*	1.794	**95**	*SNORA24*	1.377	**150**	*Osbpl8*	1.112
41	*Zwilch*	1.774	**96**	*Nt5c3b*	1.377	**151**	*Strbp*	1.11
42	*Matr3*	1.766	**97**	*Usp12*	1.368	**152**	*Dhx40*	1.108
43	*Map4*	1.764	**98**	*Mpv17l*	1.365	**153**	*Eif4h*	1.106
44	*Glis2*	1.752	**99**	*Rpa3*	1.362	**154**	*Dpy19l1*	1.105
45	*Mgst3*	1.751	**100**	*Cycs*	1.362	**155**	*Snx4*	1.094
46	*Tmem161b*	1.726	**101**	*RT1-A1*	1.355	**156**	*Racgap1*	1.07
47	*Brca1*	1.716	**102**	*Sbf2*	1.349	**157**	*Cpd*	1.07
48	*Pkm*	1.705	**103**	*Magohb*	1.348	**158**	*Smim20*	1.068
49	*Ppil2*	1.678	**104**	*Tgds*	1.343	**159**	*Cry2*	1.066
50	*Set*	1.661	**105**	*Arhgap11a*	1.341	**160**	*Smarcc1*	1.064
51	*Eif3i*	1.647	**106**	*Crnkl1*	1.328	**161**	*Cox8a*	1.064
52	*Mboat1*	1.646	**107**	*Tipinl1*	1.327			
53	*S100a6*	1.643	**108**	*Arhgef3*	1.324			
54	*Ndufab1*	1.631	**109**	*Selk*	1.321			
55	*Sap18*	1.625	**110**	*Suclg2*	1.316			

Supplementary figure legends

Figure S1 Pharmacokinetics of *in vitro* ^{211}At-MABG treatment. (A) 2 compartment model which consists of "growth medium" and "cells" compartments. k_{12} and k_{21} parameters are transport coefficients in h. $\%AD_1$ and $\%AD_2$ indicate the percent applied dose. **(B)** Cellular uptake simulation of ^{211}At-MABG. **(C)** Cellular release simulation of ^{211}At-MABG.

Figure S2 Time activity curves (TACs) of *in vitro* ^{211}At-MABG treatment. (A) % Applied dose of the "cells" compartment. **(B)** TAC of the "cells" compartment in kBq treated with the applied dose of 1.0 kBq/mL. **(C)** Dose rate in 10^{-5} Gy/s for ^{211}At-MABG treatment and γ-ray irradiation. **(D)** Cumulated activity in Bq s per cell for 0.8 kBq/mL ^{211}At-MABG treatment.

Figure S3 Vastly different transcriptional profiles. A pair comparison of all treatment conditions was performed to test the correlation. There were similar expression levels among γ-ray irradiated samples, in which early (3 h) response of weak irradiation (80% survival) showed very weak correlation (around 0.2 in pearson correlation) to 3 h control conditions

Figure S4 Number of DEGs of γ-ray irradiation and ^{211}At-MABG treatment. Number of DEGs was summarized in each condition, 80% and 10% survival rates, 3, 6 and 12 h post treatments. Yellow and purple circles with light or dark filled color indicate the conditions compared for DEGs. Here the DEGs are FDR < 0.05. Number with a gray bar demonstrates the number of DEGs significantly expressed in all selected conditions.

Figure S5 Heatmap of clustered correlations between MIBG-control experiments. The expression level of all genes with log-normalized TPM (Transcript Per Million, +1 to avoid taking log of zero) is used to cluster samples according to overall Pearson correlation. Regardless of the time point or survival rate, the two conditions (control, stable-iodine labeled MIBG treatment) showed similar expression pattern.

Figure S6 Number of DEGs of stable-iodine labeled MIBG treatment. Number of DEGs was summarized for each comparisons, where DEGs > two fold change (FC) shown in orange, and those below in blue. Actual numbers are displayed above the bars. Overall, comparison between control conditions and MIBG treatment resulted in extremely low number of DEGs exceeding FC > 2, indicating very limited effects of the compounds themselves.

Figure S7 Gene expressions of the regulatory network for cell cycle checkpoints.

Expressions (FPKM) of all genes configured at the pathway map of **Figure 4A** are shown at 15 panels. Error bars represent standard deviation among the three replicates, and median values are represented by the symbols.