SUPPLEMENTARY MATERIALS AND METHODS

Collection of melanoma samples

The ONTRAC study was approved by the human ethics committees of the University of Sydney and the Sydney Local Health District and all study participants provided written informed consent. Tumors collected from patients at the Royal Prince Alfred Hospital (RPAH) ONTRAC study site (**Table S1**) and processed by the Department of Tissue Pathology and Diagnostic Oncology at RPAH were analysed in this project. All tumor analysis was done by investigators blinded to the patients' treatment allocation to nicotinamide (NAM) or placebo.

Ex vivo experiments

Immunohistochemistry for immune cell markers

Formalin-fixed paraffin embedded tumor tissue, collected from RPAH was sectioned using a manual rotary microtome (Leica Biosystems, Wetzlar, Germany) at a thickness of 4μm. Immunohistochemistry for stains CD3, CD4, CD8 and CD68 were performed on the Leica Biosystems, BOND III Automated IHC stainer (Leica Biosystems) as is done for routine immunohistochemistry by the RPAH Department of Tissue Pathology and Diagnostic Oncology. HIER was conducted on the Automated stainer, using Epitope retrieval buffer 2 (EDTA buffer) for all stains. Clones for each marker were LN10 (CD3), SP35 (CD4), C8144B (CD8) and KP1 (CD68).

Staining for Ki67 was performed using the Ventana Benchmark Ultra (Ventana Medical Systems Inc. Tucsan, AZ, USA; RPAH Department of Tissue Pathology and Diagnostic Oncology). Pretreatment was performed on the stainer with Cell conditioning solution 1 (EDTA buffer). The clone used was 30-9. All slides were coverslipped using the Automated Dako coverslipper.

The Dako autostainer (Dako, Agilent Technologies) in our research laboratory was used to stain markers CD163, CD11c and FoxP3 independently. Isotype controls were conducted with IgG1 and IgG2a.

Immunodetection for markers CD68, CD163, FoxP3

The following reagents were made prior to antigen retrieval:

- 10x Tris buffered saline (TBST).
- 3% Hydrogen Peroxide (v/v) (Lab Vision Corporation, Thermo Fischer Scientific, Fremont, CA, USA).
- Antigen retrieval citrate buffer pH6: PT module buffer 1 (100x citrate solution pH6) (Lab Vision Corporation, Thermo Fischer Scientific).
- Antigen retrieval Tris-EDTA buffer pH9: PT module buffer 4 (100x Tris-EDTA solution pH 9) (Lab Vision Corporation, Thermo Fischer Scientific).
- Warp red chromogen kit (Biocare Medical, Concord, CA, United States).
- 3,3'-Diaminobenzidine (DAB) betazoid chromogen kit (Biocare Medical,).
- Haematoxylin (Sigma-Aldrich): was diluted to 30% v/v with TBST.

Slides were placed in a PT Link, pre-treatment module (Dako, Agilent Technologies, California, USA) to allow deparaffinisation, rehydration and epitope retrieval to be conducted at one time. Slides were stained with immune markers CD163, CD11c and FoxP3 using this method. CD163 and CD11c were optimized in an antigen retrieval citrate buffer (pH 6), whereas FoxP3 was optimized in antigen retrieval buffer pH 9.

The Dako autostainer (Dako, Agilent Technologies) was used to stain markers CD163, CD11c and FoxP3. The protocol was inserted into the software and optimised with positive

controls prior to staining of specimens. Once on the autostainer the slides were rinsed twice with TBST. 3% hydrogen peroxide was then added on top of the slide for 5 minutes. Slides were again rinsed twice with TBST. The primary antibodies were then added to the appropriate slides for 45 minutes (CD163, CD11c, FoxP3, isotype control IgG1 and isotype control IgG2a). Slides were again rinsed twice with TBST.

Probe solution from a secondary MACH 3 mouse HRP-polymer detection kit (Biocare Medical) was added for 10 minutes to each slide, for all slides stained with DAB), followed by another rinse step with TBST. HRP solution from the secondary MACH 3 mouse HRP-polymer detection kit (Biocare Medical) was then added for 10 minutes. Slides were then rinsed twice with distilled water.

Probe solution from a secondary MACH 3 mouse AP-polymer detection kit (Biocare Medical) was added to the slides stained with warp red. Probe and AP solutions were added for 10 minutes each to the slides in the same way as the MACH 3 mouse HRP-polymer detection kit. The warp red was added for 5 minutes for slides stained with FoxP3 and CD163. DAB was added to slides stained for CD11c. Subsequently, the slides were washed with distilled water. All slides were counterstained with diluted haematoxylin for 5minutes. On completion, the slides received a final wash with distilled water. Slides stained with warp red were dried in the dehydrating oven for 15minutes at 65°C. They were then transferred into an automated coverslipper (Dako) and left to dry for 24 hours prior to analysis.

Slides stained with DAB were rehydrated (washed in 70% ethanol for 5 minutes, then 95% ethanol for 5 minutes, then absolute ethanol for 5minutes). Slides were then incubated in two containers of xylene for 5 minutes each and transferred to the automated coverslipper.

Image capture and analysis

Images of all stains except for Ki67 were exported into ImageJ v.2.0.0. The tumoral and peritumoral areas were outlined in each H&E image using the 'paint brush' tool. The peritumoral area outline was drawn 0.25mm from the tumour in all directions using the 'straight line' tool. The scale in each image was calibrated to 'set measurements'. The 'magic wand' tool was then selected to fill each outlined area. The selected area was calculated in mm^2.

Within the tumor and peritumoral regions, the 'multipoint' tool in ImageJ was used to click on each individual cell. Once all the cells had been selected, ImageJ calculated the total number of cells clicked. Results for each tumor were documented on an excel spreadsheet. The result was divided by the area of each region to obtain a calculation for the number of cells/mm^2.

The InForm advanced imaging analysis software was used for the analysis of Ki67 stained melanomas. Ki67 stains all proliferating cells in the tumor and peritumoral area. The InForm software has pattern recognition algorithms that allow for specific cell biology of interest within a tissue section to be located with high sensitivity. The InForm was trained to locate all the proliferating melanocytes only within the tumoral region and mark these black. Images were exported into ImageJ and all cells marked by the software as black and by IHC as red were counted with the multipoint tool.

In vitro experiments

UV Radiation

The UVA dose was 1813mJ/cm^2 and the UVB dose 187mJ/cm^2. The solar simulated irradiance rate was measured to be 40.4mW/cm^2, the UVA irradiance was 36.6mW/cm^2 and UVB irradiance was 3.8mW/cm^2. An atmospheric attenuation filter (Oriel, Newport) was used to approximate the spectrum of the solar simulator to that of the UV component of natural sunlight

at sea level. monitoring of the UV output was as our research group has described previously (Halliday, 2013).

Cells were irradiated without lids for viability and proliferation assays. Cells were irridated within a perspex flask in the invasion assay. This was done because the cells could not be irradiated once they were in fluoroblok inserts. Further, keeping the cells in the flaks helped to prevent bacterial contamination and enabled use of large quantities of cells in each experiment. Total dose of ssUV delivered to the cells in the flask was 22% less than that delivered to the cells without the flask (20% reduction in the dose of UVA and 40% reduction in the dose of UVB).

Viability assay

5x104 primary melanocytes and 5x103 melanoma cells were seeded into 96 well plates. Cells were incubated for 24 hours with either 50μM, 5mM or 20mM of NAM or in medium 254 supplemented with 1% HMGS alone. After 24 hours half of the experimental groups were irradiated with 2J/cm^2 ssUV. After a further 24 hours the cells in half of each experimental group of wells were lysed with 9% Triton-X 100 (Sigma-Aldrich). Equal quantities of the Cytotox-one kit were pipetted into each well of the remaining experimental groups. The intensity of the fluorescent colour was in proportion to the release of LDH from dead cells produced and was quantitatively determined in counts per second by a microplate reader, Fluorostar Omega (BMG Labtech, Ortenberg, Germany). Cell viability for each experimental group was calculated by subtracting the cytotoxicity reading (unlysed cells) from the complete lysis reading in corresponding wells (millunit/ml) and therefore the proportion of viable cells was calculated for each cell type in each experiment. The assay was performed three independent times for each cell line with triplicate wells in each experimental group.

Proliferation assay

Primary melanocytes and melanoma cells were seeded in triplicate wells in Greiner black, 96 well plates (Greiner Bio One GmBh, Frickenhausen, Germany) at a cell density of 7.5x103 and 5x103 per well respectively. Triplicate wells were constructed for each experimental group. Cells were seeded onto seven separate plates. Each plate was analysed either at 0hrs (24hrs after seeding cells), 24hrs after time 0 or 48hrs after time 0. Cells were allowed to incubate for 24hrs prior to analysis to allow complete cell attachment. At 0hrs, three plates were exposed to UVR. The other three plates were washed with PBS twice and medium changed with or without supplemental NAM to standardise the experimental technique. Each plate at its corresponding analysis time was fixed with methanol. PBS was drained from the wells and 100µL 4',6-Diamidino-2-Phenylindole (DAPI) (Thermo Fischer Scientific, Fremont, CA, USA) was added to each. Quantitative measurement of the fluorescent intensity produced by the fixed cells was conducted by the Fluorostar omega, multiplate reader and measured in counts per second. Fluorescent intensity produced from triplicate wells was averaged and normalised to the control (no NAM or UVR) in each experiment for statistical analysis.

Invasion assay

Melanoma cells were seeded in a FluoroBlok insert (Corning Incorporated), which was placed in a 24 well companion plate (Corning Incorporated). FluoroBlok inserts are designed with a light-tight polyethylene terephthalate membrane, efficiently blocking the transmission of light from 400-700nm, making them suitable for analysis in a fluorescent plate reader. Each FluoroBlok insert was individually coated with a 100µl layer of matrigel (Corning Incorporated), to imitate the basement membrane within the skin.

For each cell line, two 25cm^2 tissue culture flasks were maintained, each at approximately 80% confluence. As cells could not be irradiated when inside the FluorBlok

inserts, flasks were used in this instance to maintain the cell lines. Melanoma cell lines appeared to adhere and grow more consistently in tissue culture flasks and were better able to be reach the large quantities required for each experiment without bacterial contamination. One flask was left unirradiated and the other was irradiated. Prior to irradiation, both flasks were washed with 2ml of PBS, twice, and 5ml of PBS was then added to each flask. One flask containing PBS was irradiated with ssUV at 1.57J/cm^2.

Cells were pre-dyed with a non-toxic lipophilic fluorescent dye, dilc12 (Thermo Fischer Scientific) used for labelling the membranes and other hydrophobic structures of the cell. $2.5x10^4$ melanoma cells and 350µL medium were seeded into the upper well of the FluoroBlok insert. Triplicate wells were used in each plate. In the bottom compartment 800µl of medium and 10% FCS was used as a chemoattractant for the cells to migrate through the Matrigel. Cells able to migrate through the Matrigel layer sat on the PET coating of the Fluoroblok insert. Fluorescent intensity produced by the cells was measured in counts per second every 24 hours using the Fluorostar omega multiplate reader and images were taken using Olympus CKX31/CKX41 Phase Contrast and Fluorescence Inverted Microscope with ProgRes C5 Cooled Colour CCD Camera (Olympus Corporation).

Statistical methods

Intratumoral and peritumoral data from ONTRAC were summed to form an aggregated value for each marker type, except for Ki67. Given the small sample size, the results for the placebo and NAM groups were compared using an exact implementation of the Wilcoxon rank-sum test.

For cell viability, proliferation, and invasion data, the four melanoma cell lines were analysed as a separate set to the two melanocyte cell lines. For each of these two sets, the initial full regression model comprised covariates for dose level, cell line, UV exposure, and all

possible interactions. A parsimonious model was sought using a backwards elimination approach from the full model. Evidence of interactions between cell line type and other factors (i.e. UVR and dose) was used as grounds for further partitioning of the two cell line sets into more homogeneous subsets for separate analysis.

References

Halliday GM, Byrne, S.N., Lyons, J.G. and Damian, D.L. . Photocarcinogenesis nonmelanoma skin cancer. In "Handbook of Photomedicine". Editors Michael R. Hamblin and Ying-Ying Huang. CRC Press, Taylor and Francis Group, Boca Raton Cat#: K13978. . 2013 (Chapter 7):69-79.

Treatment	Age	Gender	Body site	Diagnosis	Breslow thickness	Clark level	Ulceration	Dermal mitoses
NAM	70	M	Face	SSM	0.4mm	2	no	0
NAM	76	F	Upper limbs	SSM	0.6mm	2	yes	2/mm^2
NAM	70	M	Trunk	MIS	0	1	no	0
NAM	69	M	Upper limbs	MIS	0	1	no	0
NAM	47	M	Trunk	MIS	0	1	no	0
PBO	54	M	Lower limbs	MIS	0	1	no	0
PBO	65	F	Lower limbs	SSM	0.5mm	3	no	0
PBO	85	M	Face	LM	0	1	no	0
PBO	82	M	Face	LM	0	1	no	0
PBO	53	M	Trunk	SSM	0.93mm	4	no	1/mm^2

Table S1: Demographics of ten patients with melanoma

NAM, nicotinamide; PBO, Placebo; SSM, superficial spreading melanoma; MIS, melanoma in situ; LM: lentigo maligna

Table S2

Immunohistochemistry markers	Mean cell count/mm^2 Placebo	Mean cell count/mm^2 NAM	P value
CD68	**230.9**	**438.0**	**0.69**
CD163	**305.0**	**432.9**	**0.84**
CD11c	**56.5**	**158.3**	**0.22**
Ki67 (peritumoral)	**45.8**	**78.5**	**0.31**
Ki67 (intratumoral)	**449.2**	**395.1**	**0.6905**