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Evaluation of the effects of clinical diagnostic ultrasound in combination with ultrasound contrast agents on cell stress: Single cell analysis of intracellular phospho-signaling pathways in blood cancer cells and normal blood leukocytes

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Abstract—Clinical diagnostic ultrasound has been known as one of the safest imaging modalities available, yet very little is known about the cellular response to such acoustic conditions. With the increased interest in therapeutic ultrasound it is becoming ever more important to understand the effects of ultrasound on cells.

In our work here we investigate the effect of clinical diagnostic ultrasound on several cell signalling proteins (p38 p-Thyr180/p-Thyr182, ERK 1/2 p-Thyr202/p-Thyr204 and p53 ac-Lys382) on leukaemia cells (MOLM-13) and monocytes.

Our results show that leukaemia cells and monocytes react differently to ultrasound and microbubbles. A relatively small increase in p38 signalling was seen in the leukemic cells, and only at higher intensities in combination with microbubbles. In contrast the monocytes showed an increase in p38 signalling at all acoustic intensities with microbubbles and at the high acoustic intensity without microbubbles.

Furthermore, the leukaemic cells showed an overall increase in ERK 1/2 p-Thyr202/p-Thyr204 signalling whereas the monocytes showed a decrease. These results indicate that the leukaemia cells are less sensitive to stress induced by ultrasound and microbubbles when compared to normal monocytes.

In conclusion, our results show that clinical diagnostic ultrasound does have a measurable effect on intracellular signalling but may differ drastically between different cell types. This may affect the conditions necessary for therapeutic ultrasound.

I. INTRODUCTION

Diagnostic ultrasound is widely used in the clinic and is considered a safe imaging modality with little to no side effects. Currently, the only guidelines for the use of ultrasound are based on the thermal and cavitation effects of ultrasound with or without an ultrasound contrast agent (UCA) [1]. Current state-of-the-art research is looking into the bio-effects

possible due to ultrasound and microbubbles. In the past years, it has been made clear that at higher acoustic intensities microbubbles may form pores in cell membranes, aiding in drug delivery, or even resulting in DNA double-strand breaks [2], [3]. Furthermore, it has been speculated that the cells themselves may be acoustically active, resulting in cell stress due to sonication. Primarily, ultrasound-microbubble research has been done on adherent cells, and focussed on increasing drug uptake, but little to no research has focussed on floating cells and blood cancer (leukaemia) cells.

In our work here we evaluate the effect of ultrasound and microbubbles on three specific stress activated intracellular signalling proteins: p38, ERK 1/2 and acetylated p53. The signalling pathway p38 is known to respond to stress and inflammation, and regulate proliferation, differentiation and survival [4]. ERK 1/2 is linked to the regulation of cell growth and differentiation [5]. The p53 protein acts as a tumour suppressor and transcription factor activated by DNA damage and other cellular stress signals leading to cell-cycle arrest, apoptosis, and DNA repair [6].

II. MATERIALS AND METHODS

A. Cell types

The acute myeloid leukemia cell line, MOLM-13 (DSMZ, Braunschweig, Germany) and peripheral blood mononuclear cells (PBMCs) collected from healthy volunteers were used. PBMCs consist of multiple cell types where approximately 20% are monocytes and 80% are T cells; cells that play a critical role to the immune system [7].

The MOLM-13 cells were cultured as previously described [8]. The PBMCs were prepared using density gradient

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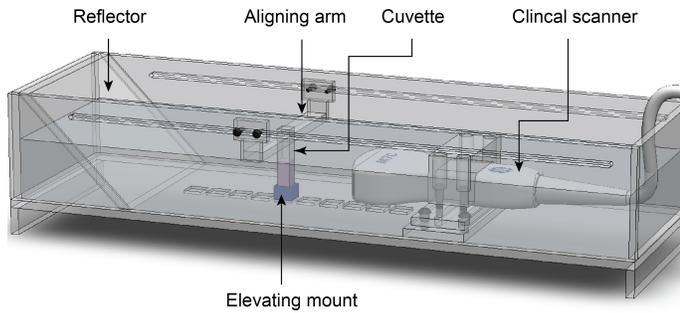


Fig. 1. Graphical rendering depicting ultrasound treatment method. An M12L clinical ultrasound probe is clamped in the water bath treating the cells placed in the cuvette. A polycarbonate plate, positioned at the back of the tank at 45° to reduce reflections.

TABLE I
ACOUSTIC CONDITIONS GENERATED FROM M12L DIAGNOSTIC
ULTRASOUND PROBE USED TO SONICATE CELLS.

	Frequency (MHz)	Focal Depth (cm)	MI	Intensity (mW/cm ²)	Peak-Negative Pressure (MPa)
Low	6.80	2.22	0.16	0.35	0.419
Medium	6.40	4.44	0.59	1.17	1.416
High	5.80	3.89	0.72	1.61	1.813

TABLE II
LIST OF MODIFICATION SPECIFIC ANTIBODIES USED TO ASSESS
INTRACELLULAR SIGNALING.

Antigen	Epitope	Clone	Conjugate
p38	p-Thr180 / p-Tyr182	36/p38	Alexa Fluor® 488
ERK 1/2	p-Thr202 / p-Tyr204	20A	Alexa Fluor® 647 or Per-CP-Cy5.5
p53	p-Ser37	J159-641.79	Alexa Fluor® 647
p53	p-Ser15	16G8	Alexa Fluor® 488
p53	ac-Lys382	L82-51	Alexa Fluor® 647
NFκB	p-Ser529	K10-895.12.50	PE or Alexa Fluor® 488
CD3	CD3	SK7	PE-Cy7
CD5	CD5	L17F12	PE-Cy7
CD20	CD20	H1(FB1)	PerCP-Cy5.5
CD163	CD163	Mac2-158	PE

65 separation (Ficoll-Hypaque, Nycomed, Oslo, Norway). Cells
66 were re-suspended in RPMI-1640 + 10% FBS, diluted to a
67 concentration of 1×10^6 cells/ml, medium and incubated for
68 1 hour at 37°C.

69 Flow cytometric labelling with CD163 was used to
70 distinguish monocytes from T-cells (*c.f.*, Table II).

71 B. Ultrasound & microbubble treatment

72 A custom made setup incorporating a clinical diagnostic
73 ultrasound scanner and disposable cuvette was used to sonicate
74 MOLM-13 and PBMC cells (Fig.1). An M12L ultrasound
75 probe was used as the ultrasound source and driven by
76 an unmodified Logiq 9 clinical ultrasound scanner (GE
77 Healthcare, Buckinghamshire, United Kingdom). A standard
78 abdominal imaging mode was used, and the focal depth
79 was adjusted to vary the acoustic intensity. The sound field
80 was calibrated in-situ using a 0.2-mm PVDF hydrophone
81 connected to a 3-axis, manual, micrometer stage. The acoustic
82 conditions used are shown in Table I.

83 During treatment 2.5 ml cells, *i.e.*, 2.5×10^6 cells were
84 placed in the cuvette for treatment. SonoVue® ultrasound
85 contrast agent was prepared according to manufacturers
86 recommendation. A total of 40-μL was mixed into the
87 2.5 ml cell containing cuvette and continuously homogenized
88 using a magnetic stirrer. The total treatment time for each
89 sample was 10 min. Following treatment the cells were
90 placed in an incubator for 0 – 24 hours. After the required
91 incubation time the reactions were stopped by fixation in
92 1.5% paraformaldehyde for 20 min at room temperature and
93 subsequently stored at -80°C until further analysis by flow
94 cytometry. The control sample consisted of cells of the same
95 culture that had not been treated with neither ultrasound or
96 microbubbles.

97 C. Flow cytometry

98 Flow cytometry is a well-established technique that allows
99 single cell analysis and characterisation. Cell samples were
100 labelled with different concentrations of succinimidyl esters
101 of Pacific Blue (Invitrogen, Carlsbad, Ca, USA) and Pacific
102 Orange (Invitrogen, Carlsbad, Ca, USA) in which each sample

can be identified by the specific barcode of fluorescence
intensity and emission wavelength. Barcoded cell samples
were permeabilized with ice cold methanol, washed and
treated with human IgG (Octagam, Octapharma AG, Lachen
, Switzerland). Barcoding allows high throughput analysis,
reduces antibody consumption – and importantly, reduces
analytical variation [9].

Following treatment, approximately 0.5×10^6 cells were
stained with a combination of fluorochrome-conjugated
antibodies. These anti-bodies stain the cells when a specific
cell signalling pathway has been activated. The flow cytometer
measured the mean fluorescence intensity (MFI) of specific
wavelengths correlating to the signalling pathway. Increased
intensity indicates a more active pathway, *e.g.*, a five-fold
increase in MFI indicates the path was substantially more
active compared to the control sample.

The intracellular signalling was evaluated at multiple time
points ranging from 0 minutes to 24 hours after ultrasound
exposure. This allowed us to evaluate the time-dependant
change in intracellular signalling in due to ultrasound and
ultrasound and microbubbles. Results are presented as a heat
map, allowing for faster comparison in large datasets and a
linear graph.

D. Cell death and proliferation assays

To investigate if the acoustic conditions had any direct
effect on cell death, live/dead cells were discriminated by
Trypan blue staining. Trypan blue (Invitrogen, Carlsbad, CA,) is used as a dead/alive discriminator; 10 μl of Trypan blue were added to 10 μl of cell suspension. The dead and alive cells were counted using optical microscopy and a Bürker haemocytometer. Apoptosis was evaluated using DNA specific

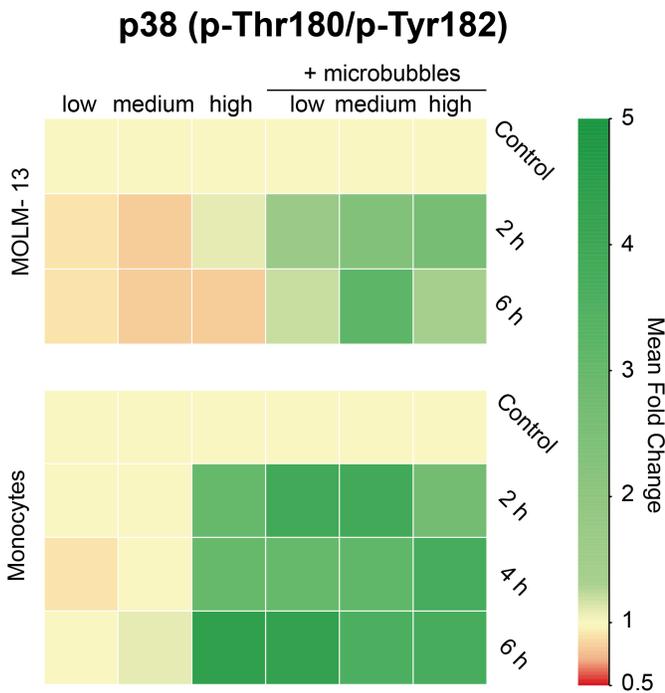


Fig. 2. Heatmaps showing modulation of the p38 signalling over time following ultrasound stimulation for leukemic cancer cells (MOLM-13) and normal monocytic (monocytes) cells. In both cell lines an increase in signalling can be seen with the addition of microbubbles.

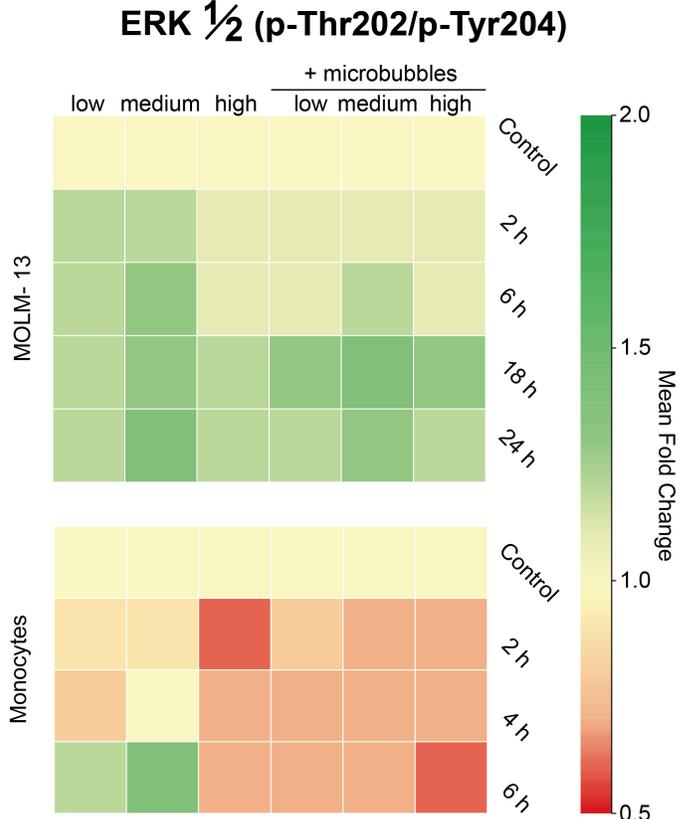


Fig. 3. Heatmaps showing modulation of the ERK 1/2 signalling over time following ultrasound stimulation for leukemic cancer cells (MOLM-13) and normal monocytic (monocytes) cells. In both cell lines an increase in signalling can be seen with the addition of microbubbles.

134 staining with Hoechst 33342 (10 $\mu\text{g/ml}$) (Invitrogen) followed
 135 by optical counting of normal and fragmented/condensed cell
 136 nuclei using an inverted fluorescence microscope (Leica IRB,
 137 Leica Miceosystems GmbH, Wetzlar, Germany). To evaluate if
 138 the ultrasound had an effect on the proliferation, immediately
 139 after ultrasound exposure, 2×10^3 cells were re-suspended
 140 in 100 μl cell medium and mixed with 400 μl MethoCult
 141 GF H4434 (StemCell Technologies, Vancouver, BC, Canada)
 142 and cultured for 5 days. The number of colonies formed
 143 in cell culture were counted in a bright field microscope
 144 (10 \times /0.30 objective, Nikon Eclipse Ti, Nikon Corporation,
 145 Tokyo, Japan).

146 III. RESULTS AND DISCUSSION

147 A. Cell signalling

148 Our results showed that both the monocytic leukemia cells,
 149 and the monocytes had a reaction to ultrasound, in terms
 150 of phosphorylation of cells. Depending on the pathway and
 151 cell type, a different response was elicited at the various
 152 stimulation conditions.

153 In the normal blood monocytes, phosphorylation of p38 (*c.f.*
 154 Fig. 2) was only increased when the cells were exposed to high
 155 intensity ultrasound with no microbubbles, and at all intensities
 156 when combining with microbubbles with ultrasound. The
 157 monocytic leukemic cell line, MOLM-13, showed a smaller
 158 fold increase in terms of p38 pathway activity when compared
 159 to the monocytes, yet a similar trend was seen. In both cell

160 types the addition of microbubble contrast agent resulted in an
 161 increase of p38 stress signalling at medium and low ultrasound
 162 intensities. The minor differences were that in the leukemic
 163 cell line, ultrasound alone reduced the signalling significantly
 164 more when compared to the monocytes. These results indicate
 165 that the monocytes were more susceptible to external stresses
 166 than the leukemic cell line.

167 ERK 1/2 signalling (*c.f.* Fig. 3) showed a similar response
 168 to that seen in the p38 pathway. In the monocytes we see
 169 a decrease in signalling at the same conditions we saw an
 170 increase in p38 signalling. This may be explained by the
 171 fact that increased activity in the p38 pathway can cause
 172 inactivation of the ERK 1/2 pathway in normal cells [4].

173 This effect is not observed in the leukemic cell line. In the
 174 leukemic cells ERK 1/2 phosphorylation is slightly increased
 175 after 6 hours, and a further increase is seen at 18–24 hours;
 176 both with and without microbubbles. ERK 1/2 activation may
 177 contribute to cell cycle arrest or apoptosis in response to
 178 different levels of DNA damage [11], which would be a
 179 wanted effect in cancer cells. In the current experiment no
 180 significant change proliferation and cell viability was observed
 181 (*c.f.*, Fig. 5), and further studies are needed to determine the
 182 downstream effects of ultrasound and microbubble induced

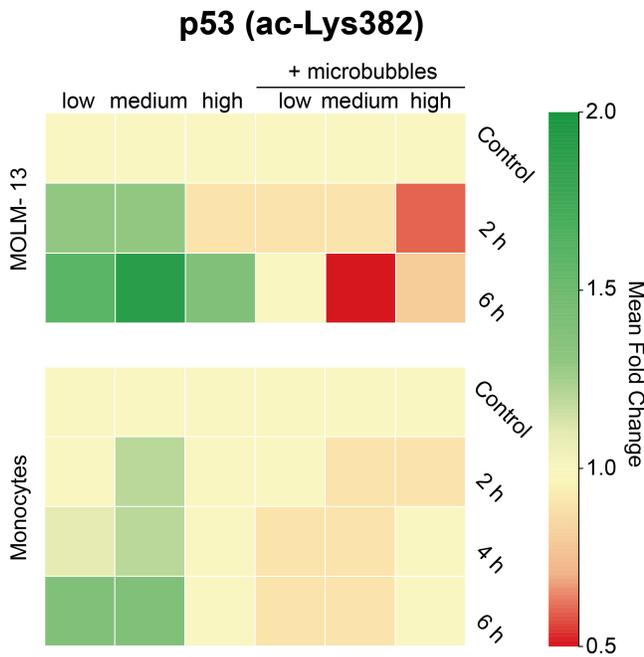


Fig. 4. Heatmaps showing modulation of p53 acetylation over time following ultrasound stimulation for leukemic cancer cells (MOLM-13) and normal monocytic (monocytes) cells. In both cell lines an increase in signalling can be seen with the addition of microbubbles.

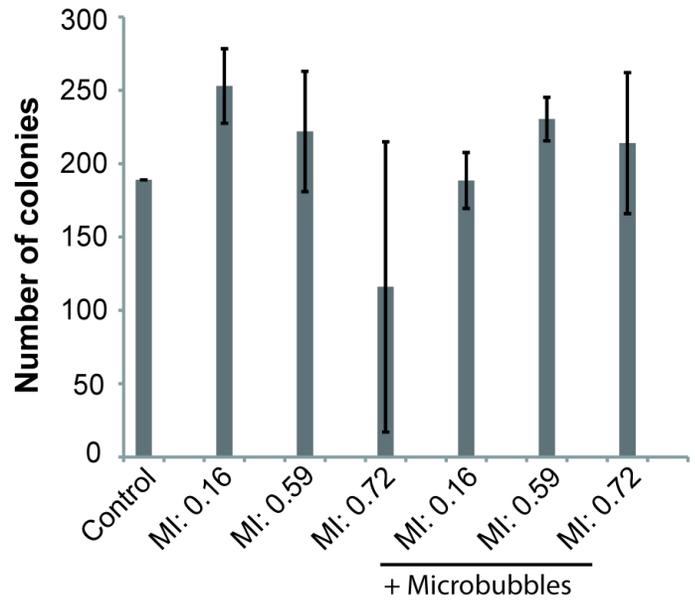


Fig. 5. Results of colony-forming assay used to assess proliferation in MOLM-13 cells. The number of colonies was measured in sample cultured for 5 days after sonication. No statistical difference was seen between all samples other than a greater variance at higher MI. Error bars indicate standard error of mean.

183 ERK 1/2 signalling.

184 Figure 4 shows the effect of ultrasound, and ultrasound and
 185 microbubbles on Lys384 acetylation of the tumor suppressor
 186 p53. Ultrasound alone, showed an increase in both monocytes
 187 and MOLM-13 cells. In contrast, when adding microbubbles
 188 p53 signalling was reduced. However, the effects were not very
 189 pronounced. Acetylation has been found to be important for
 190 activation of p53 and regulating downstream processes [12].

191 Negligible change was seen in phosphorylation of p53
 192 (Ser15 and Ser37) (Data not shown) also implying no DNA
 193 damage occurred at any acoustic conditions [13]. This was in
 194 agreement with the results shown in Fig. 5.

195 It is currently not known exactly how ultrasound and
 196 microbubble ultrasound contrast agents modulate signal
 197 transduction in the cells. Cells respond to their surrounding
 198 environment through receptors on the cell surface which initiate
 199 responses in within the cells. Different kinds of receptors may
 200 for instance be implicated in activating the ERK pathway, in
 201 response to either ultrasound or fluid shear stress [14], [15],
 202 [16]. In addition to the large intracellular signaling proteins,
 203 small intracellular signaling molecules like Ca^{2+} bind to and
 204 alter the behavior of signaling proteins or target proteins.
 205 In a study by Kumon et al ultrasound and microbubble
 206 induced sonoporation was shown to cause transient changes
 207 in Ca^{2+} concentration in cells [17], and this may further
 208 modulate intracellular signaling. Studies have shown Ca^{2+} to
 209 be involved in activation of both p38 and ERK 1/2 proteins
 210 [18], [19].

B. Cell viability and clonogenicity

211

212 Cell viability and nuclear morphology remained unaltered
 213 after sonication with and without microbubbles. Figure 5
 214 shows the results of the colony forming assay for the
 215 MOLM-13 assay under the various conditions. Whilst a larger
 216 standard deviation was seen at the higher acoustic intensities,
 217 no statistical significant difference was seen between any of
 218 the samples.

IV. CONCLUSION

219

220 Our results indicate that both the cancer cell line and normal
 221 blood cells responded 2 to 6 hours after ultrasound exposure
 222 with changed phosphorylation of p38 (p-Thr180/p-Tyr182)
 223 and ERK 1/2 (p-Thr202/p-Tyr204). Activation was ultrasound
 224 contrast agent and acoustic pressure dependent. These
 225 signalling proteins are shear stress activated indicating that
 226 higher acoustic pressures result in higher shear stresses.
 227 Both leukemic and normal cells showed a decrease in p53
 228 (ac-Lys382) activation at the higher acoustic pressures or when
 229 microbubbles were added. In all experimental conditions, the
 230 cell viability remained unaltered, consistent with the safety
 231 regulations for clinical diagnostic ultrasound.

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238 [1] F. Piscaglia, C. Nolsoe, C. F. Dietrich, D. O. Cosgrove, O. H.
 239 Gilja, M. Bachmann Nielsen, T. Albrecht, L. Barozzi, M. Bertolotto,
 240 O. Catalano, M. Claudon, D. A. Clevert, J. M. Correas, M. D'Onofrio,
 241 F. M. Drudi, J. Eyding, M. Giovannini, M. Hocke, A. Ignee, E. M. Jung,
 242 A. S. Klauser, N. Lassau, E. Leen, G. Mathis, A. Saftoiu, G. Seidel,
 243 P. S. Sidhu, G. ter Haar, D. Timmerman, and H. P. Weskott, "The
 244 EFSUMB Guidelines and Recommendations on the Clinical Practice
 245 of Contrast Enhanced Ultrasound (CEUS): update 2011 on non-hepatic
 246 applications," *Ultraschall Med.*, vol. 33, no. 1, pp. 33–59, 2012.

247 [2] Y. Furusawa, Y. Fujiwara, P. Campbell, Q. L. Zhao, R. Ogawa, M. A.
 248 Hassan, Y. Tabuchi, I. Takasaki, A. Takahashi, and T. Kondo, "DNA
 249 double-strand breaks induced by cavitation mechanical effects of
 250 ultrasound in cancer cell lines," *PLoS One*, vol. 7, no. 1, p. e29012,
 251 2012.

252 [3] M. M. Forbes, R. L. Steinberg, and W. D. O'Brien, "Examination of
 253 Inertial Cavitation of Optison in Producing Sonoporation of Chinese
 254 Hamster Ovary Cells," *Ultrasound Med. Biol.*, vol. 34, no. 12, pp.
 255 2009–2018, 2008.

256 [4] A. Cuadrado and A. R. Nebreda, "Mechanisms and functions of p38
 257 MAPK signalling," *Biochem. J.*, vol. 429, no. 3, pp. 403–417, 2010.

258 [5] J.-C. Chambard, R. Lefloch, J. Pouyssegur, and P. Lenormand, "ERK
 259 implication in cell cycle regulation," *Biochim. Biophys. Acta - Mol. Cell
 260 Res.*, vol. 1773, no. 8, pp. 1299–1310, 2007.

261 [6] M. V. Blagosklonny, "P53: An ubiquitous target of anticancer drugs,"
 262 *Int. J. Cancer*, vol. 98, no. 2, pp. 161–166, 2002.

263 [7] I. M. Roitt, *Essential immunology*, 2nd ed. Oxford: Blackwell Scientific
 264 Publications, 1971.

265 [8] T. Bredholt, E. A. Dimba, H. R. Hagland, L. Wergeland, J. r. Skavland,
 266 K. O. Fossan, K. J. Tronstad, A. C. Johannessen, O. K. Vintermyr, and
 267 B. r. T. Gjertsen, "Camptothecin and khat (*Catha edulis* Forsk.) induced
 268 distinct cell death phenotypes involving modulation of c-FLIPL, Mcl-1,
 269 procaspase-8 and mitochondrial function in acute myeloid leukemia cell
 270 lines." *Mol. Cancer*, vol. 8, p. 101, 2009.

271 [9] P. O. Krutzik and G. P. Nolan, "Fluorescent cell barcoding in
 272 flow cytometry allows high-throughput drug screening and signaling
 273 profiling," *Nat Meth*, vol. 3, no. 5, pp. 361–368, 2006.

274 [10] N. Doan, P. Reher, S. Meghji, and M. Harris, "In vitro effects of
 275 therapeutic ultrasound on cell proliferation, protein synthesis, and
 276 cytokine production by human fibroblasts, osteoblasts, and monocytes,"
 277 *J. Oral Maxillofac. Surg.*, vol. 57, no. 4, pp. 409–419, 1999.

278 [11] D. Tang, D. Wu, A. Hirao, J. M. Lahti, L. Liu, B. Mazza, V. J. Kidd,
 279 T. W. Mak, and A. J. Ingram, "ERK activation mediates cell cycle arrest
 280 and apoptosis after DNA damage independently of p53." *J. Biol. Chem.*,
 281 vol. 277, pp. 12710–12717, 2002.

282 [12] Y. Tang, W. Zhao, Y. Chen, Y. Zhao, and W. Gu, "Acetylation Is
 283 Indispensable for p53 Activation," *Cell*, vol. 133, pp. 612–626, 2008.

284 [13] M. Ashcroft, Y. Taya, and K. H. Vousden, "Stress Signals
 285 Utilize Multiple Pathways To Stabilize p53," *Mol. Cell. Biol.*,
 286 vol. 20, no. 9, pp. 3224–3233, May 2000. [Online]. Available:
 287 <http://mcb.asm.org/content/20/9/3224.short>

288 [14] N. P. Whitney, A. C. Lamb, T. M. Louw, and A. Subramanian,
 289 "Integrin-Mediated Mechanotransduction Pathway of Low-Intensity
 290 Continuous Ultrasound in Human Chondrocytes," *Ultrasound Med.
 291 Biol.*, vol. 38, pp. 1734–1743, 2012.

292 [15] A. Makino, E. R. Prossnitz, M. Bünemann, J. M. Wang, W. Yao,
 293 and G. W. Schmid-Schönbein, "G protein-coupled receptors serve as
 294 mechanosensors for fluid shear stress in neutrophils." *Am. J. Physiol.
 295 Cell Physiol.*, vol. 290, pp. C1633–C1639, 2006.

296 [16] M. J. Rane, S. L. Carrithers, J. M. Arthur, J. B. Klein, and
 297 K. R. McLeish, "Formyl peptide receptors are coupled to multiple
 298 mitogen-activated protein kinase cascades by distinct signal transduction
 299 pathways: role in activation of reduced nicotinamide adenine
 300 dinucleotide oxidase." *J. Immunol.*, vol. 159, pp. 5070–5078, 1997.

301 [17] R. E. Kumon, M. Aehle, D. Sabens, P. Parikh, Y. W. Han, D. Kourennyi,
 302 and C. X. Deng, "Spatiotemporal Effects of Sonoporation Measured
 303 by Real-Time Calcium Imaging," *Ultrasound Med. Biol.*, vol. 35, pp.
 304 494–506, 2009.

305 [18] T. S. Mombberger, J. R. Levick, and R. M. Mason, "Mechanosensitive
 306 synovocytes: A Ca²⁺-PKCa-MAP kinase pathway contributes to
 307 stretch-induced hyaluronan synthesis in vitro," *Matrix Biol.*, vol. 25,
 308 pp. 306–316, 2006.

[19] J. Lotem, R. Kama, and L. Sachs, "Suppression or induction of apoptosis
 by opposing pathways downstream from calcium-activated calcineurin." *Proc. Natl. Acad. Sci. U. S. A.*, vol. 96, pp. 12016–12020, 1999. 309
 310
 311