Cell stiffness under small and large deformations measured by optical tweezers and atomic force microscopy: effects of actin disruptors CK-869 and jasplakinolide

Špela Zemljič Jokhadar$^{1,3,*}$, Jagoba Iturri$^{2,3,*}$, José Luis Toca-Herrera$^{2,3}$ and Jure Derganc$^{1,3}$

$^1$ Institute of Biophysics, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, SI-1000 Ljubljana, Slovenia
$^2$ Institute for Biophysics, Department of Nanobiotechnology, University of Natural Resources and Life Sciences Vienna (BOKU), Muthgasse 11/II, 1190 Vienna, Austria

E-mail: spela.zemljic-jokhadar@mf.uni-lj.si and jagoba.iturri@boku.ac.at

Received 20 August 2020, revised 12 November 2020
Accepted for publication 4 December 2020
Published 14 January 2021

Abstract

Cytoskeleton-disrupting drugs can have different effects on cell mechanics at different deformation scales. We therefore applied two complementary indentation techniques to study the effects of two actin-disrupting drugs on cellular stiffness of human umbilical vein endothelial cells. Optical tweezers were used to probe the cortical stiffness at small deformations, and atomic force microscopy was used to probe the bulk cell stiffness at larger deformations. The first drug studied was CK-869, which is an inhibitor of the actin branching complex Arp2/3, and has not been analysed yet in terms of mechanical effects. A significant decrease in cell stiffness upon treatment with CK-869 was measured with both techniques, which implies that actin branching is important for cell mechanics at small and large deformations. The second drug studied was jasplakinolide, for which ambiguous effects on cell mechanics have been reported. In line with previous studies, we found that jasplakinolide caused significant cell stiffening at large deformations but slight cell softening under small deformations. This result implies that jasplakinolide has different effects on different levels of actin organization.

Supplementary material for this article is available online

Keywords: atomic force microscopy, optical tweezers, Arp2/3, actin cortex, cell mechanics

(Some figures may appear in colour only in the online journal)
Abbreviations

AFM  atomic force microscopy
OT  optical tweezers
HUVEC  human umbilical vein endothelial cells
Arp2/3  actin-related protein 2/3
Cyto-D  cytochalasin-D
SMIFH2  Formin FH2 Domain Inhibitor
PBST  PBS with added Tween 20
WGA  wheat germ agglutinin

1. Introduction

The cell cytoskeleton is an intracellular network that provides cell mechanical integrity and plays an important role in cell signalling. One of the most prominent constitutive elements of the cytoskeleton are actin filaments, which are highly dynamic, reorganize rapidly, and support tissue homeostasis and processes that require cell remodelling, such as cell migration and cellular differentiation processes [1, 2]. Importantly, cell mechanics does not depend only on the properties of individual actin filaments but also on the properties of the higher-level structures they form [3, 4]. An example of a high-level structure is the actomyosin cortex, a thin network composed of actin filaments and associated proteins, that underlies the plasma membrane and modulates the properties at the cell surface [5].

The actin network comprises long and short filaments, which are formed by two nucleation pathways of actin monomers [6, 7]. Long filaments are polymerized by formin proteins, which associate with the fast-growing end, while short filaments are nucleated by filament-bound Arp2/3 complexes and branch from existing long filaments. Both types of nucleation complexes are also closely associated with focal adhesions, which link the cortex to the extracellular matrix [8]. The actin filament length distribution within the cortex is a parameter that, together with crosslink dynamics and fibre density, plays a fundamental role in cortical structural integrity and mechanics [9]. The short actin filament population that emerges due to Arp2/3-induced branching accounts for up to 80% of the total filamentous actin in some cell types [6, 7, 10], so one could expect that impaired actin branching or reduced formation of such short filaments should be reflected in the mechanical properties of cells.

The role of actin in providing cellular mechanical integrity have been extensively scrutinized by a systematic application of drugs that target specific aspects of actin organization, yet some of the details remain unclear. For example, it has been repeatedly demonstrated that treatment with Cyto-D, a potent inhibitor of actin polymerization and a disruptor of actin microfilaments, significantly reduces the cell elastic modulus, as tested for endothelial cells (HUVEC, GM7373), fibroblasts, chondrocytes and diverse cancerous lines (HT1080, DU145) [11–14]. Similar effects have been reported for SMIFH2, which is an inhibitor of formin Diaph 2 that initiates long actin filaments [12]. Marine toxins Latrunculin A and B were used as F-actin disruptors in mechanical studies on fibroblast and embryonic kidney HEK 293 cells [14, 15]. On the contrary, no significant impact on cell mechanics has been reported for epithelial DU145 and SKOV3 cells exposed to CK-666, which is a small molecular inhibitor that blocks the nucleation of actin filaments by Arp2/3 complex [10, 12, 16]. In addition, apparently contradictory data have been reported on the effects of jasplakinolide on MCF7 cells and fibroblasts (3TR, NRK, REFS2, NH3T3), showing that jasplakinolide can both stabilize [17–19] and destabilize the actin skeleton [20, 21]. Recently, a comprehensive multi-lab study indicated that these ambiguities could originate in the intrinsic differences among different experimental approaches that have been used to assess the cell mechanics, such as differences in length- and time-scales of applied deformations, or differences in probe geometry [22]. Thus, a simultaneous application of complementary experimental methods in the same experimental conditions is recommended for a thorough analysis of the effects of cytoskeleton disruptors on cell mechanics.

In this work, two complementary techniques were employed to assess the impact of actin disruptors on the mechanics of HUVEC at two different scales: AFM and OT. Both methods used probe indentation in order to determine the mechanical response of cells, yet, in our set-up, they differed significantly in the direction of the indentation and the exerted loading force (figures 1 and S1 (available online at stacks.iop.org/JPD/54/124001/mmedia)).

In the case of AFM, a pyramidal probe with a sharp tip (the tip radius was 22 nm) was used to indent the cell perpendicularly from the top, above the nucleus, with applied loads in the range from 1 to 2 nN. In turn, the probe employed in OT experiments was a microsphere with a 5 μm diameter that indented the cell from the lateral side, exerting loading forces in the range 70–100 pN. In both methods, the analysis of forces during probe retraction also provided information about the adhesion-related behaviour of the cell membrane. The later depends on the mechanical state of the cell. The simultaneous use of these two techniques has been already proven to effectively cover various aspects of cell mechanics [23].

Two actin filament disruptors with unclear effects on cell stiffness were studied. The first disruptor tested was CK-869, which is a small molecular inhibitor of Arp2/3 complex, and has not been examined yet in terms of its impact on cell mechanics. In contrast to CK-666, which acts as an allosteric effector stabilizing the inactive state of the complex, CK-869 directly disrupts key protein–protein interfaces in the short pitch of Arp2/3 dimer to destabilize the active state [24]. The end effect of both drugs, i.e. the inhibition of actin filament branching, appears to be similar [24]. The second drug employed was jasplakinolide [25], for which both stiffening and softening effect had been reported with different experimental techniques [17–19]. In addition, Cyto-D, a well-scrutinized disruptor of actin filaments, was utilized as a positive control to validate the experimental methods. Results obtained in this way allow an optimal comparison between the respective influence of these molecules on different mechanics-related factors (cell stiffness, adhesion force, etc.).
Figure 1. Schematic illustration of experimental protocols for atomic force microscopy (AFM, (a)–(c)) and optical tweezers (OT, (d)–(f)). (a) AFM probe is pushed into a cell from the top, and the force on the cantilever is monitored in real-time. (b) The cantilever is retracted when the force reaches 2 nN. (c) A typical force–distance curve. The cell Young modulus is determined by fitting a Sneddon extension of the Hertz model to the indentation curve. During the retraction, strong adhesion between the membrane and the probe was observed; the maximal adhesion force was determined ($F_{adh}$), and the subsequent rupture events were counted. The range of forces and displacements for AFM experiments is significantly larger than for OT experiments (a typical range accessible by OT experiments is illustrated by a small dotted box at the axes origin). (d) A silica microbead is trapped in a fixed optical trap 2 µm above the substrate at the trailing edge of the cell and the cell is pushed against the bead by a piezo-stage. (e) The bead displacement from the centre of the optical trap ($\Delta x$) is monitored in real-time. When the bead is displaced for 500 nm, the piezo-stage is retracted. (f) A typical force–deformation curve. The cell stiffness is determined as the slope of the linear part of the curve (red line), $k_{cell} = \frac{\Delta F}{\Delta \varepsilon}$, where $\varepsilon(t) = x_{stage}(t) - \Delta x(t)$. Occasionally, adhesion of the bead on the membrane was detected as a negative force during the retraction, and the maximal adhesion force was measured ($F_{adh}$).

2. Materials and methods

2.1. Cell lines and treatments

HUVEC cells were grown in serum-reduced minimum essential medium (Gibco) supplemented with 5% foetal bovine serum (Gibco) and antibiotics (streptomycin-penicillin) (Gibco). Before the experiments, the cells were seeded into a custom-built experimental chamber with a glass substrate. The cell density was sufficiently low (20,000 cells ml$^{-1}$), so that they did not form a confluent layer. To modify the actin cytoskeleton, the cells were treated in the following way: (a) 10 µM solution of Cyto-D for 20 min at 37 °C, (b) 0.5 µM solution of jasplakinolide for 30 min at 37 °C, and (c) 1 µM solution of CK-869 also for 30 min at 37 °C (all the substances, from Sigma-Aldrich). The choice of the times and concentrations used for the treatments was based on the literature data and our preliminary tests on HUVEC cells. The time and concentration range of jasplakinolide treatment for different cell lines found in the literature varies considerably (from 0.1 µM to 1 mM) [16–18]. For CK-869, concentrations of up to 200 µM were shown previously to impact actin organization without significantly affecting cell viability [26, 27]. The doses employed in our study were selected at the lower limits of the concentrations that started to induce changes of HUVEC cells in preliminary tests. For both OT and AFM experiments, the cells were seeded on clean, uncoated, plasma treated, glass substrates for 24 h before measurements took place.

2.2. Immunofluorescence

To assess the distribution of actin filaments, the cells were left either untreated (control) or treated as described above. Thereafter they were rinsed three times with PBST, which was used for all subsequent washings. The fixation was done in ice-cold acetone for β-actin labelling. After fixation HUVEC
cells were rehydrated in PBST three times, for 5 min each. The non-specific binding sites were blocked in PBST containing 1% BSA. Antibodies against β-actin (Sigma-Aldrich, A2228), were added to the blocking solution and left for 2 h at room temperature. After washing, cells were incubated with secondary antibodies marked with Alexa fluor 488 (Molecular Probes).

Adhered cell area and cell height of both control and treated cells were determined from confocal images after WGA, Alexa Fluor 488 conjugate (Thermo Fisher Scientific) was added to the cells. WGA, which is widely used for plasma membrane imaging, binds to sialic acid and N-acetylgalcosaminyl residues. The adhered cell area was determined manually with Nis-Elements AR 3.2 software (Nikon) from the boundary of individual cells in the basal confocal section \( n = 42 \) for each treatment. The cell height was determined as the height difference between the lowermost and uppermost image from the confocal stack, from three independent samples.

2.3. Atomic force microscopy

Measurements were performed on a JPK Nanowizard III (JPK Instruments, Germany) with a Cell Hesion module mounted on an inverted optical microscope (Axio Observer Z1, Zeiss) at 37°C (figure S1(a)). Triangular, silicon nitride cantilevers with four-sided pyramidal tips and nominal spring constant of 0.12 N m \(^{-1}\) (DNP-S, Bruker) were used. The choice of a sharp tip was related to the application of local deformations and, additionally, to higher measurement versatility under these conditions, which mainly affects the retraction segment (evaluation of rupture events). Tips remained uncoated after the usual cleaning protocol, so measurements proceeded under non-specific forces. Spring constant calibration using the thermal noise method was performed.

The cells were approached perpendicularly at a loading rate of 5 \( \mu m\ s^{-1} \) and indented up to a maximum load (set-point) of 2 nN (figures 1(a)–(c)), which induced an indentation between 1 and 2.5 \( \mu m \). The choice of a mid-high approach rate derives from the minimization of the biological responses caused by indentation [28]. The tip was then held at a constant, fixed height above the cell for 5 s. After that, the tip was retracted at 5 \( \mu m\ s^{-1} \), and its motion was recorded for a range of 50 \( \mu m \). Each cell was indented at least five times at approximately 1 min intervals. At least ten cells were probed per experiment. The substrate was probed to ensure the tip cleanliness between indentations of individual cells. Cells were indented above the nucleus region to reduce the variability and the substrate artefacts. The starting number of seeded cells was kept low, to ensure indentation on individual cells, and in order to minimize the influence of neighbouring ones.

In force (spectroscopy) measurements, the force between tip and sample is directly proportional to the cantilever displacement times the cantilever stiffness (spring model). AFM data were pre-processed by using the JPKSPM software. Force measurements, i.e. force–distance plot (F vs. d), allow real-time monitoring of the tip-sample forces along its downward–upwards motion (figure 1(c)). This plot consists of three segments: approach, contact, and retraction, where each of them delivers information about one particular type of forces [29]. The study focused on the analysis of approach and retraction segments in order to extract information about sample elasticity (Young’s modulus) and adhesion (\( F_{\text{adh}} \) and subsequent rupture events), respectively.

For calculation of the modulus of Young (\( E \)), the Sneddon extension of the Hertz model for pyramidal indenters as seen in equation (1) was used:

\[
F = \frac{E}{1 - \nu^2} \beta^{\frac{1}{2}} \delta^{\frac{3}{2}},
\]

where \( \nu \) is the Poisson’s ratio (0.5), \( \alpha \) is the face angle of the pyramid (22°), and \( \delta \) is the indentation employed for the fitting. The latter was conducted for the initial 500 nm of indentation, which stays in the optimal range for Hertz–Sneddon model application. In addition, the obtained results can have a good consistency with those from previous works [30].

The adhesion force (\( F_{\text{adh}} \)) correlates to the maximum force required to break the tip-cell contact, and its value corresponds to the minimum of the peak. Rupture event formation is featured by the stepwise recovery of the force, until full contact splitting or zero force. These events can be studied based on their number as well as on their distribution (appearance distance) and rupture force (step height), to determine the membrane accessibility upon pulling.

2.4. Optical tweezers

Cell deformation experiments were performed on an Eclipse Ti inverted microscope (Nikon) equipped with laser tweezers (Tweez 250si, Aresis, Ljubljana, Slovenia). The OT were set on constant optimal power so that no evidence of cell damage or bleb formation was noticed (the laser wavelength was 1064 nm). The laser beam was focused through a water immersion objective (60×, NA 1.00, Nikon) into a sample chamber. The sample heater was mounted on the microscope and the objective to ensure a constant temperature 37°C (figure S1(b)).

The experimental chamber with cells was mounted on the microscope and silica beads with diameter 5.06 \( \mu m \) (CS01N, Bangs Labs, Fishers, IN) were added into the sample. A bead was trapped by OT and positioned near the side of the cell without lamellipodia, where the cell membrane is relatively vertical. The piezo microscope stage (Nano-LPS-200, Mad City Labs, USA) was then raised until the glass slide touched the bead and then immediately retracted for 2 \( \mu m \), so that the bead centre was positioned approximately 5 \( \mu m \) above the surface. The cell was then pushed into the bead by moving the piezo stage with a constant velocity of 1 \( \mu m\ s^{-1} \), and the bead position was monitored in real-time with a digital camera (Zyla 5.5, Andor, Ireland) at 50 fps and by using a custom-written Matlab program (Mathworks, USA). After the bead...
was pushed for 0.5 µm from the centre of the optical trap, the piezo stage was automatically retracted (figures 1(d) and (e)). The force was calculated from $F(t) = \kappa \Delta x(t)$, where $\kappa$ is the stiffness of the optical trap. The deformation is calculated from the known stage position and the bead displacement: $\varepsilon(t) = x_{\text{stage}}(t) - \Delta x(t)$. In the case of OT, fitting of the Hertz–Sneddon model was unreliable because the point of contact was often hard to determine accurately, possibly due to the presence of glycocalyx. The cell stiffness was therefore determined as the slope of the linear part of the force–deformation curve. The value of this stiffness cannot be directly compared to the value of the Young modulus measured with AFM, but it is nevertheless a robust way to compare the cortical stiffness of different cell lines and to compare and complement relative effects of cell treatments [31]. For each treatment, the measurements were repeated in at least seven independent samples. For each sample, measurements on untreated (control) cells were also performed.

2.5. Statistics

For comparison of the median cell stiffness between treated and control cells, the Mann–Whitney–Wilcoxon test (Mathematica, Wolfram Research) was used to calculate the $p$-values, with a $p$-value < 0.05 deemed significant.

3. Results

3.1. Impact of treatments on actin filaments distribution and cell size

To ensure that the treatments with CK-869 and jasplakinolide did not induce extensive cell damage, the treated cells were first visually analysed. The distribution of actin filaments was examined by confocal microscopy of fluorescently labelled β-actin (figure 2(a)) under the same conditions (exposure, gain) for all the samples. The cells were labelled with anti-β-actin rather than with phalloidin because the latter shares the binding site with jasplakinolide [32], and was thus not appropriate for our study. As expected, we found that the reference treatment with Cyto-D profoundly disrupted the actin filaments: the filament-like structures were absent, and actin tended to agglomerate in the form of massive clumps all over the cell. For jasplakinolide-treated cells, the actin filaments were not as distinct as in control cells. However, an increase in the signal of β-actin, especially at the cell periphery, was observed. Finally, the actin distribution in cells treated by CK-869 was similar to the control.

The impact of treatment on the cell size was analysed by confocal microscopy of cells labelled with WGA-Alexa Fluor 488 (figure 2(b) and supporting information figure S2). This stain binds to sugars at the cell surface and clearly delineates
the cell boundary. While CK-869 did not have a significant effect, treatment with jasplakinolide significantly reduced the adhered cell area and increased the cell height, much like the treatment with Cyto-D.

3.2. Impact of treatments on cell stiffness

In the main part of our study, the effects of the applied treatments on the cell stiffness were measured by two complementary methods. AFM was used to measure the stiffness in the cell interior by means of perpendicular indentation in the 1–2.5 µm range (figures 1(a) and (c)). In turn, OT probed stiffness by lateral indentations in the range below 200 nm (figures 1(d)–(f)). In the case of AFM, the cell stiffness is described in terms of Young modulus, which was obtained by fitting the Hertz–Sneddon model to the initial 500 nm of the force–deformation curve (equation (1), section 2). In the case of OT, the cell stiffness was characterized as the slope of the linear part of the force–deformation curve.

The resulting plots of cell stiffness measurements are presented in figure 3. In order to simplify the comparison of the results gathered by the two methods used, the results appear normalized with respect to the median values of their respective controls (the median Young’s modulus for control cells was 2.9 kPa, and the median stiffness of control cells in OT experiments was 140 pN µm⁻¹). Furthermore, the use of relative variations for AFM helps to minimize the potential heterogeneity obtained when using sharp tips [22]. In line with the published data [11, 14], the median cell stiffness of Cyto-D-treated cells was significantly reduced for both AFM (a 90% decrease in stiffness, \( p < 0.001 \)) and OT (a 40% decrease, \( p < 0.001 \)). The cells treated with CK-869 were also found to be significantly softer in both methods (a 60% decrease for AFM, \( p < 0.001 \), and a 30% decrease for OT, \( p < 0.001 \)). On the other hand, the cells treated with jasplakinolide were found significantly stiffer with AFM (a 30% increase, \( p = 0.02 \)), but slightly softer with OT (a 20% decrease, \( p = 0.34 \)). The AFM measurements on jasplakinolide-treated cells showed a much larger variation than the rest.

3.3. Impact of treatments on the adhesion of the probe on the cell surface

The retraction part of the force–deformation curves often showed a negative bump (see figures 1(c) and (f)), which was a clear indication of the adhesion between the probe and the cell surface. In AFM experiments, the probe adhered to the cell surface in nearly all cases, while in OT measurements, the microbead adhered to the cell surface in only approximately 25% of cases. In the latter case, the Cyto-D-treated cells exhibited the most frequent adhesion and CK-869-treated cells the least frequent adhesion (figure 4(a)). If adhesion occurred, the maximal adhesion force was also measured (figures 4(b) and (c)). Here, the most striking difference was observed with AFM on Cyto-D-treated cells, where the adhesion force was more than five times larger than in the control.

A detailed inspection of the AFM force–deformation curve also revealed a significant number of rupture events during probe retraction and before the tip-cell contact is fully split apart (figures 1 and 5). Unfortunately, the resolution of OT experiments was not sufficient to detect individual rupture events. The median number of these rupture events was 6 for control, 4 for CK-869 treatment, and 8 for jasplakinolide. For Cyto-D (figure 5(b)), this value increased up to almost 50. Such a large increase results from the appearance of multiple rupture events in addition to regular tethers observed in control cells. These new ruptures occur in much less ordered fashion than in those cases where the cytoskeleton maintained certain integrity. Thus, Cyto-D again produced a much larger deviation from the control than other treatments.

Further analyses revealed the distribution of these rupture events with respect to the Z-displacement of the cantilever and to the rupture force measured. The respective plots are depicted in figures 5(c) and (d). In the case of control cells and jasplakinolide-treated cells, the majority of the rupture events...
Figure 4. Frequency of cell-probe adhesion in OT experiments (a) and the corresponding maximal adhesion forces for both methods (b), (c). In AFM experiments, the frequency was practically 100% for all treatments. The values are rescaled relative to the controls. In OT controls, the adhesion to the bead occurred in 24% of measurements. Boxes span over 50% of the force measurements, the white horizontal lines represent the medians, and the dots represent the outliers. For each treatment, the median value is indicated in the box, and the $p$-value describing the difference relative to the control is indicated at the top. The median adhesion force for AFM controls was 0.35 nN, and the median adhesion force for OT controls was 15 pN.

4. Discussion and conclusions

Actin filaments are an essential structural and functional element of living cells but how they contribute to cellular mechanics at different deformation scales remains an open question. In this work, we employed two complementary techniques, AFM and OT, to analyse the effects of CK-869 (a disruptor of branching of short actin filaments), and jasplakinolide (a drug that interferes with actin organization and has a range of reported effects) on mechanical properties of HUVEC cells. Both methods were based on measuring the force–deformation curve during cell indentation by an external mechanical probe. However, the applied forces and the corresponding deformations were very different and thus affected different layers of cellular structures.

In AFM experiments, the probe was a sharp nanometric tip (radius ca. 20 nm) shaped as a quadratic pyramid (face-to-angle of 22), which was pushed from above into the cell centre with indentation depths ranging from 1 to 2.5 µm. Thus, the measured cell stiffness resulted from the combined properties of the cortex, the deeper-lying cytoskeleton and cytosol, including microtubules and intermediate filaments, as well as the nucleus. While glycocalyx of HUVEC in vitro after the cells were one day in culture is relatively thin and patchy [33] and may not contribute to the cell stiffness, this regime is influenced by the viscous nature of the cell interior [23]. In OT experiments, the indenter was a microbead (diameter 5 µm) and the indentation depth reached approximately 200 nm. In this case, the membrane and the underlying cortex were the main contributors to the cellular mechanical response [23]. While in previous OT-based studies, the cells were typically indented from the top [23], here, the cells were indented laterally from the side opposite to the leading edge, with extended lamellipodia, where the cell membrane is relatively vertical (figure S2). To minimize the influence of the cell curvature on indentation experiments, the contact points were chosen to be at relatively even parts of the surface (above the nucleus for AFM, and at the most vertical cellular side for OT). In addition, these effects will depend on the contact area between the indenting probe and the sample. In AFM measurements, the sharp probe together with the indenting force applied (1.5 nN) permits a highly localized contact, which is almost curvature-independent. Although the diameter of OT probe falls in the micrometric range, the contact required for reaching 100 pN is very subtle and depends more on the local roughness of cell surface rather than on its macroscopic curvature.
Figure 5. Rupture events during retraction of the AFM probe. (a) A schematic representation of rupture events. For each rupture, its Z-displacement and the rupture force were recorded. (b) Number of rupture events per probe retraction. Boxes span over 50% of the measurements, the white horizontal lines represent the medians, and the dots represent the outliers. The median value is indicated in the box. (c) Scatterplots of rupture force vs. Z-displacement for each individual rupture event. (d) Distributions of Z-displacements of individual rupture events (bin size = 2 μm).

As described by Wu et al [22], the absolute values of cell stiffness obtained by different methods on different length scales may not be directly comparable to each other. This was also true in our study. With AFM, the cell stiffness was quantified as the Young modulus, which was obtained by the standard fitting the Hertz–Sneddon model to the force–deformation curve. With OT, fitting the Hertz–Sneddon model proved to be unreliable because the exact point of contact between the microbead and the cell was hard to determine. In OT experiments, the cell stiffness was therefore quantified as the slope of the force–deformation curve in the linear regime. Importantly, since the experimental details can influence the measurement of mechanical properties [22], both techniques applied in the present study were implemented under the same experimental conditions (at 37 °C, on the same glass substrate using the same culturing medium etc.).

The techniques were first validated on Cyto-D-treated cells. In agreement with previous reports [12, 13, 17, 34], Cyto-D caused a complete breakdown of actin filaments (figure 2(a)), the cell area decreased and consequently, as the adhesion sites were disrupted and the cells were not firmly attached, the cell height increased (figure 2(b)). In line with previous studies, both techniques detected a significant drop of cell stiffness upon Cyto-D treatment (figure 3). As a side note, the validation and comparison of different methods could be further enhanced if the methods could be calibrated against a well-characterized synthetic sample with a known structure mimicking the cell.

The main focus of the study was on the first measurements of the impact of CK-869 on the cell mechanical properties. Although the applied concentration of CK-869 was sufficiently low not to significantly disrupt the actin cytoskeleton and cell geometry (figure 2), a significant decrease of the cell stiffness was recorded with both methods (figure 3). This indicates that Arp2/3-mediated branching of short actin filaments plays an important role in mechanical properties of HUVEC cells both at large and small deformations.

We also analysed the impact of jasplakinolide, a drug that disrupts actin organization and has ambiguous concentration- and time-dependent effects on cell mechanics [17, 18]. The experimental conditions were selected so that jasplakinolide did not induce a noticeable cell damage. Still, treatment with jasplakinolide induced some actin condensation in the periphery of the cell (figure 2(a)), which is in line with an increased density of the cortical meshwork reported in previous studies [13]. Thus, an increase in stiffness was expected with
both experimental approaches. Indeed, the AFM experiments revealed that jasplakinolide treatment increased the Young modulus for 30% (p = 0.022, figure 3(a)). On the other hand, the stiffness measured with OT was 20% lower; however, the difference was not significant (p = 0.35, figure 3(b)). While these results are seemingly ambiguous, they are in line with previous reports that have also demonstrated cell softening at small deformations and a more solid-like behaviour at larger deformations [18, 19]. In addition, the softening effects were reported in other OT studies on NIH3T3 fibroblasts [21] and on neurons [35]. These opposing results can be explained by the fact that jasplakinolide completely stops the activity of the cortical actomyosin [36], which contributes essentially to the cortical stiffness but not to the stiffness in the cell interior [37]. In turn, the apparent stiffening under larger deformations can be related to reorganization of the actin filaments that eventually leads to the emergence of actin aggregates in the cell interior [19, 21, 38]. This reorganization could also lead to a very high variability of the Young modulus measured by AFM (figure 3(a)). These results show that the diverse effects of jasplakinolide on actin organization cannot be attributed solely to varying experimental conditions and different cell lines used in the past studies, but rather to its complex mode of action, which apparently affects different levels of cytoskeleton organization in different ways, e.g. it has been already noted that jasplakinolide affects actin filaments but does not disassemble stress fibres [17]. Further studies are needed to fully elucidate the effects of jasplakinolide on cells and consequently to clarify the role of different levels of actin organization in cell mechanics.

Finally, we analysed the retraction part of the AFM indentation curves to gain additional insight into adhesion-related parameters of cells (figures 4(b) and 5; the adhesion in the case of OT was too weak to provide reliable measurements). Many factors may contribute to the retraction part of the force–deformation curve: the (nonspecific) adhesion of the probe to the plasma membrane, the adhesion of the probe to membrane proteins connected to the cortex (e.g. integrins), the adhesion (interaction) between the plasma membrane and the cortex, the deformation of the cortex, and also membrane detachment from the cortex. The latter can lead to formation of thin membrane tubules (tethers), a process that depends on membrane tension, membrane availability and viscosity [39–41]. The adhesion force itself depends on the contact area, the applied rate, and the residence time the probe stays in contact with the sample measured, as shown for diverse biopolymers [42, 43]; cells and hydrogels (Weber et al, Microscopy Research Technique, under revision).

During the retraction of the AFM probe, we observed distinctive rupture events (figure 5(a)). The force–deformation curve did not provide the information about the exact point at which the cortex-membrane-probe chain ruptured, but a detailed analysis of adhesion events largely corroborated the findings of the stiffness measurements. In the case of control and jasplakinolide-treated cells, the rupture events started immediately at zero distance, indicating that the cell is stiff enough to resist any outward deformation during probe retraction. In contrast, for CK-869 and Cyto-D, the first rupture events were recorded at a larger distance (2–4 μm) from the cell, showing that the retracting probe deformed the softened cell in the outward direction before the onset of ruptures. Cyto-D represents the extreme case, in which the cells seemingly comprise of a flaccid membrane wrapped on a severely disrupted cortex, which resulted in very strong adhesion between the membrane and the probe and consequently the largest adhesion forces (figure 4(b)) and the most numerous subsequent rupture events (figure 5(b)). For Cyto-D and jasplakinolide treated cells, the rupture events extended much further away than for control and CK-869-treated cells. Whether this is a consequence of an impaired membrane–cortex interactions or a large excess membrane surface area due to a changed cell shape (figure 2) remains to be explored.

In general, different layers of cellular structures will deform hierarchically and to various extents as the load on the cell increases. A small load will only bend the plasma membrane and the underlying actomyosin cortex. As the load increases, the membrane and the cortex will start to stretch and, eventually, the cytoskeleton comprising intermediate filaments, actin filaments and microtubules will begin to deform as well. During the probe retraction, the reverse is true and the membrane will remain the sole actor during the final rupture events. Cellular response to large deformations applied by AFM therefore necessarily reflects a combined response of all these different structures. On the other hand, the drugs that were used in the present study also influence all these structural layers simultaneously. Thus, unravelling the response of each layer individually is a challenging task, one that is beyond the scope of this study. In addition, a thorough analysis will have to consider also the cellular and membrane viscosity, as they play an important role in the mechanical response. For example, it has been shown that both the intrinsic membrane cholesterol content (which related to membrane fluidity) and F-actin have an important role in the mechanical response at the cell surface [15, 44].

In summary, the experiments with AFM and OT showed that CK-869, which is the inhibitor of branching caused by Arp2/3, significantly softened HUVEC cells under large and small deformations. This softening effect was evident even at the concentration that did not affect cell integrity, indicating that actin branching is important for mechanical resistance at all deformation scales. On the other hand, jasplakinolide stiffened the cells under large deformations, but slightly softened the cells under small deformations. This was in agreement with previous studies [18, 19] and indicates that jasplakinolide has different effects on different scales of actin organization. Finally, the study supports the claim made by Wu et al [22], that simultaneous use of complementary indentation techniques can tackle differences in cell mechanics that could be otherwise attributed to varying experimental conditions among different studies.
Acknowledgments

JI and J-L T-H want to thank Amsatou Andorfer-Sarr for the technical support with the cell culture and Andreas Weber for his support with statistical analysis.

Funding

They are also thankful to Austrian Science Funds (FWF, Project Number 29562-N62) for funding. S Z J and J D would like to thank the Slovenian Research Agency Grant P1-0055 for founding.

Conflict of interest

None.

ORCID iD

Špela Zemljic Jokhadar https://orcid.org/0000-0001-8820-9869

References

[1] Chalut K J and Paluch E K 2016 The actin cortex: a bridge between cell shape and function Dev. Cell 38 571–3
[2] Svitkina T 2018 The actin cytoskeleton and actin-based motility Cold Spring Harbor Perspect. Biol. 10 1–22
[3] Moeendarbary E and Harris A R 2014 Cell mechanics: principles, practices, and prospects Wiley Interdiscip. Rev. Syst. Biol. Med. 6 371–88
[4] Svitkina T M 2018 Ultrastructure of the actin cytoskeleton Curr. Opin. Cell Biol. 54 1–8
[5] Chugh P and Paluch E K 2018 The actin cortex at a glance J. Cell. Sci. 131 14
[6] Fritzsche M, Lewalle A, Duke T, Kruse K and Charras G 2013 Analysis of turnover dynamics of the submembranous actin cortex Mol. Biol. Cell 24 757–67
[7] Fritzsche M, Erlenkamper C, Moeendarbary E, Charras G and Kruse K 2016 Actin kinetics shapes cortical network structure and mechanics Sci. Adv. 2 e1501337
[8] Chorev D S, Moscovitz O, Geiger B and Sharon M 2014 Regulation of focal adhesion formation by a vinculin-Arp2/3 hybrid complex Nat. Commun. 5 3758
[9] Kasza K E et al 2010 Actin filament length tunes elasticity of flexibly cross-linked actin networks Biophys. J. 99 1091–100
[10] Fritzsche M, Li D, Colin-York H, Chang V T, Moeendarbary E, Felce J H, Sezgin E, Charras G, Betzig E and Eggeling C 2017 Self-organizing actin patterns shape membrane architecture but not cell mechanics Nat. Commun. 8 14347
[11] Grady M E, Compoto R J and Eckmann D M 2016 Cell elasticity with altered cytoskeletal architectures across multiple cell types J. Mech. Behav. Biomater. 61 197–207
[12] Efremov Y M, Dokrunova A A, Efremenko A V, Kirpichnikov M P, Shaitan K V and Sokolova O S 2015 Distinct impact of targeted actin cytoskeleton reorganization on mechanical properties of normal and malignant cells Biochim. Biophys. Acta 1853 3117–25
[13] Kronlage C, Schafer-Herte M, Boning D, Oberleithner H and Fels J 2015 Feeling for filaments: quantification of the cortical actin web in live vascular endothelium Biophys. J. 109 687–98
[14] Wakatsuki T, Schwab B, Thompson N C and Elson E L 2001 Effects of cytochalasin D and latrunculin B on mechanical properties of cells J. Cell. Sci. 114 1025–36
[15] Khatibzadeh N, Spector A A, Brownell W E and Anvari B 2013 Effects of plasma membrane cholesterol level and cytoskeleton F-actin on cell proliferation mechanics PloS One 8 e57147
[16] Nolen B J et al 2009 Characterization of two classes of small molecule inhibitors of Arp2/3 complex Nature 460 1031–4
[17] Rotsch C and Radmacher M 2000 Drug-induced changes of cytoskeletal structure and mechanics in fibroblasts: an atomic force microscopy study Biophys. J. 78 520–35
[18] Kubitschke H, Schnauss J, Netau K D, Warmt E, Stange R and Kaes J 2017 Actin and microtubule networks contribute differently to cell response for small and large strains New J. Phys. 19 093003
[19] Bruckner B R, Noding H, Skamrahl M and Janshoff A 2019 Mechanical and morphological response of confluent epithelial cell layers to reinforcement and dissolution of the F-actin cytoskeleton Prog. Biophys. Mol. Biol. 144 77–90
[20] Bubb M R, Spector I, Beyer B B and Fosen K M 2000 Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations J. Biol. Chem. 275 5163–70
[21] Ayala Y A et al 2017 Effects of cytoskeletal drugs on actin cortex elasticity Exp. Cell Res. 351 173–81
[22] Wu P H et al 2018 A comparison of methods to assess cell mechanical properties Nat. Methods 15 491–8
[23] Nawaz S, Sanchez P, Bodensieck K, Li S, Simons M and Schaap I A 2012 Cell visco-elasticity measured with AFM and optical trapping at sub-micrometer deformations PloS One 7 e45297
[24] Hetrick B, Han M S, Helgeson A L and Nolen B J 2013 Small molecules CK-666 and CK-869 inhibit actin-related protein 2/3 complex by blocking an activating conformational change Chem. Biol. 20 701–12
[25] Holzinger A 2009 Jasplakinolide: an actin-specific reagent that promotes actin polymerization Methods Mol. Biol. 586 71–87
[26] Ilatovskaya D V et al 2013 Arp2/3 complex inhibitors adversely affect actin cytoskeleton remodeling in the cultured murine kidney collecting duct M-1 cells Cell Tissue Res. 354 783–92
[27] Beckham Y, Vasquez R J, Stricker J, Sayegh K, Campillo C and Gardel M L 2014 Arp2/3 inhibition induces amoeboid-like protrusions in MCF10A epithelial cells by reduced cytoskeletal-membrane coupling and focal adhesion assembly PloS One 9 e100943
[28] Guz N, Dokukin M, Kalaparthi V and Sokolov I 2014 If cell mechanics can be described by elastic modulus: study of different models and probes used in indentation experiments Biophys. J. 107 564–75
[29] Iturri J and Toca-Herrera J L 2017 Characterization of cell scaffolds by atomic force Microscopy Polymers (Basel) 9 383
[30] Weber A, Iturri J, Benitez R, Zemljic-Jokhadar S and Toca-Herrera J L 2019 Microtubule disruption changes endothelial cell mechanics and adhesion Sci. Rep. 9 14903
[31] Salbreu G, Charras G and Paluch E 2012 Actin cortex mechanics and cellular morphogenesis Trends Cell Biol. 22 536–45
[32] Bubb M R, Senderowicz A M, Sausville E A, Duncan K L and Korn E D 1994 Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phallolidin to F-actin J. Biol. Chem. 269 14869–71
[33] Bai K and Wang W 2012 Spatio-temporal development of the endothelial glyocalyx layer and its mechanical property in vitro J. R. Soc. Interface 9 2290–8
[34] Borin D, Puzzi L, Martinelli V, Cibinel M, Lapasin R and Sbaizero O 2017 An engineering insight into the relationship of selective cytoskeletal impairment and biomechanics of HeLa cells Micron 102 88–96

[35] Amin L, Ercolini E, Shahapure R, Migliorini E and Torre V 2012 The role of membrane stiffness and actin turnover on the force exerted by DRG lamellipodia Biophys. J. 102 2451–60

[36] Colin-York H et al 2019 Cytoskeletal control of antigen-dependent T cell activation Cell Rep. 26 3369–79

[37] Van Citters K M, Hoffman B D, Massiera G and Crocker J C 2006 The role of F-actin and myosin in epithelial cell rheology Biophys. J. 91 3946–56

[38] Zhang X et al 2012 Actin stabilization by jasplakinolide affects the function of bone marrow-derived late endothelial progenitor cells PloS One 7 e50899

[39] Sheetz M P and Dai J 1996 Modulation of membrane dynamics and cell motility by membrane tension Trends Cell Biol. 6 85–89

[40] Raucher D and Sheetz M P 1999 Characteristics of a membrane reservoir buffering membrane tension Biophys. J. 77 1992–2002

[41] Smolyakov G et al 2016 Elasticity, adhesion, and tether extrusion on breast cancer cells provide a signature of their invasive potential ACS Appl. Mater. Interfaces 8 27426–31

[42] Sumarokova M et al 2018 Influencing the adhesion properties and wettability of mucin protein films by variation of the environmental pH Sci. Rep. 8 9660

[43] Sumarokova M, Iturri J and Toca-Herrera J L 2018 Adhesion, unfolding forces, and molecular elasticity of fibronectin coatings: an atomic force microscopy study Microsc. Res. Tech. 81 38–45

[44] Khatibzadeh N, Gupta S, Farrell B, Brownell W E and Anvari B 2012 Effects of cholesterol on nano-mechanical properties of the living cell plasma membrane Soft Matter 8 8350–60