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Highlights

- A direct effect of imiquimod is demonstrated on hemangioma cells in vitro.
- Imiquimod impairs motility and disorganize actin cytoskeleton on hemangioma cells.
- Hemangioma but not normal endothelium undergo apoptosis upon imiquimod treatment.

Abstract

Infantile hemangiomas are the most common benign tumors of infancy, characterized by unregulated angiogenesis and endothelial cells with high mitotic rate. Although spontaneous regression occurs, sometimes treatment is required and alternatives to corticosteroids should be considered to reduce side effects. Imiquimod is an imidazoquinoline, approved for some skin pathologies other than hemangioma. It is proposed that the effectiveness of imiquimod comes from the activation of immune cells at tumor microenvironment. However, the possibility to selectively kill different cell types and to directly impede angiogenesis has been scarcely explored *in vitro* for endothelial cells. In this work we showed a dramatic cytotoxicity on hemangioma cell, with a significant lower IC50 value in hemangioma compared to normal endothelial cells and melanoma (employed as a non-endothelial tumour cell line). Nuclear morphometric and flow-cytometry assays revealed

imiquimod-induced apoptosis on hemangioma and melanoma cells but a small percentage of senescence on normal endothelial cells. At sub-lethal conditions, cell migration, a key step in angiogenesis turned out to be inhibited in a tumor-selective manner along with actin cytoskeleton disorganization on hemangioma cells. Altogether, these findings pointed out the selective cytotoxic effects of imiquimod on transformed endothelial cells, evidencing the potential for imiquimod to be a therapeutic alternative to reduce extensive superficial hemangioma lesions.

Keywords: Imiquimod; Hemangioma; Endothelium; Cytotoxicity.

1. Introduction.

Infantile hemangiomas (IH) are benign tumours of the vascular endothelium constituted by endothelial cells with high mitotic rate and stromal components such as fibroblasts, mast cells and pericytes (1). IH are characterized by a rapid postnatal growth (2, 3) and in most cases, these lesions do not need treatment as they resolve spontaneously within the first years of life without major sequellae (4). When involution does not occur or specific location in vital structures compromise functional impairment, treatment include topical, intralesional and systemic corticosteroids, cryosurgery, interferon, vincristine, radiation or laser therapy (5-10). Propranolol is now considered first-line therapy for complicated and cosmetically challenging IH (11, 12), however side effects can be life threatening or produce recurrence (13,14). Since 2002 several American and European reports have suggested the efficacy and safety of the topical application of imiquimod (IQ) for IH treatment (15-18). IQ is a small molecule also known as S-26308 or R-837, included into an immune response modifier agents group (19, 20). Topical IQ has been reported to be effective in the treatment of lentigo maligna (21, 22), melanoma metastases (23), cutaneous anogenital warts (24), actinic keratosis (AK) (25, 26) and superficial basal cell carcinoma (BCC) (27-29). Even though it has not been approved for IH by US Food and Drug Administration (FDA) yet, some clinical trials were designed and safety has been proven for superficial IH (28, 29).

IQ activates TLR7 and this activation is MyD88 dependent, leading to the induction of the transcription factor NF-kB which positively regulates the activity of both the innate and acquired immune responses (32, 33). By inducing IFN-α and other cytokines, IQ is able to enhance T-helper cells (Th1) response by indirectly stimulating the IFN-ɣ release through IFN-α former liberation from the innate immune cells (34, 35). Little is known about the direct activity of IQ and especially on hemangioma. Therefore, it is particularly intriguing whether IQ directly affects tumour cells triggering other mechanisms than immune stimulation. Even more interesting is the idea of IQ contributing to an antineoplastic effect by distinguishing between normal and tumor

cells. Herein, we studied the cytotoxic effects of IQ on hemangioma cells and compared differential effects on melanoma, a non-endothelial model of tumor cells, and normal endothelial cells, assessing viability and relevant morphological cell aspects such as actin cytoskeleton distribution and nuclear structure.

2. Materials and Methods.

2.1 Cells Culture Conditions and Reagents

Three established murine cell lines were used in this study: H5V as hemangioma model (36), 1G11 as normal endothelium (37) and the melanoma cell line B16-F1 (ATCC® CRL-6323™). Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Saint Louis, USA) supplemented with 10% fetal bovine serum (FBS, Natocor, Córdoba, Argentina), 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. 1G11 medium was supplemented with 2 mM sodium pyruvate and 1 % non-essential amino acid. Cells were incubated in 5% CO_2 atmosphere at 37°C. Media was changed after reseeding 2-3 times a week. Cell detachment was performed employing trypsin-EDTA solution. Cultures were routinely tested to be mycoplasma-free. IQ (kindly provided by Cassará Laboratories) was prepared as 1 mg/mL stock solution of IQ in pyrogen-free distilled water (pH=2) by heating-homogenization cycles. Cell culture confluency was approximately 80% confluency at the time of treatment. Results obtained after treating cells with vehicle were strictly comparable to those obtained with cells untreated.

2.2 Cell Viability Assay and IC_{50} Estimation

All cell lines ($3x10^4$ cells/well) were plated onto 96 flat-bottom multiwell and incubated with increasing concentrations of IQ (0-100 µg/mL) in 5% FBS-supplemented DMEM for 24 hs. Supernatants were discarded and cells fixed with 4% paraformaldehyde (PFA) for 15 minutes. After washing once with PBS, cells were stained using 0.1% crystal violet dye for 30 minutes. Stained cells were washed twice with distilled water and the dye was solubilized with water:methanol:acetic acid (85:10:5) solution. Optical density (OD) at 595 nm was measured using a FLUOstar OPTIMA Microplate Reader (BMG LABTECH GmbH, Ortenberg, Germany) to calculate viability percentage (%V) as:

$$\%\mathbf{Viability} = \frac{\mathbf{OD\ treated\ cells}}{\mathbf{OD\ untreated\ cells}}\ \mathbf{x\ 100} \quad \text{(Equation 1)}$$

Relative inhibitory concentration 50% (IC_{50}) values for IQ were calculated with a 4-parameter logistic model using OriginPro 9.1 software (OriginLab Corporation) software (38). The four parameters shown in Figure 1 were calculated with the Origin Pro 9.1 software as the best-fit values in the model by an iterative procedure.

2.3 Cell Migration and Scratch Assay

Confluent monolayers were carefully wounded using a sterile 1000 µL tip, making three scratches horizontally and three vertically. After washing several times with fresh culture medium to remove the cell debris, cultures were incubated for 12 hs either with 5% FBS-supplemented DMEM as control or the same medium supplemented with increasing concentrations of IQ (1-10 µg/mL). After fixation with 4% PFA for 15 minutes, ≥ 50 pictures were taken along the wounds avoiding wound intersections. The free-cell area (FCA) was quantified with DP2-BSW Olympus software and the percentage of migration (%M) was calculated as indicated in Equation 2

$$\%\mathbf{M} = \left(\frac{\textbf{FCA of treated cells}}{\textbf{FCA of untreated cells}}\right)^{-1} \mathbf{x\ 100} \text{ (Equation 2)}$$

2.4 Phalloidin-TRITC Staining for Stress Fibres

Endothelial H5V, 1G11 and melanoma B16-F1 cells (1x10^4 cells/well) were seeded overnight onto 1% collagen I coated glass coverslips. Subsequently, cells were cultured for 12 hs in the absence or presence (1-10 µg/mL) of IQ. To allow staining, after fixation with 4% PFA for 15 minutes, cells were permeabilized with 0.2% Triton™ X-100 (Sigma-Aldrich, Saint Louis, USA) for 15 minutes, washed three times with a 0.05% Triton™ X-100/PBS solution and incubated for 1 hour with a solution containing 1 µg/mL phalloidin-TRITC and 1 µg/mL Höescht 33458. Cell images were taken using an Olympus BX40F-3 fluorescence microscope and analyzed using Image J Pro Plus 6.0 software (IPP6-Media Cybernetics, Bethesda, MD, USA).

2.5 Flow Cytometry and Nuclear changes detection through Nuclear Morphometric Assay (NMA)

Cells were treated for 24 hs with increasing concentrations of IQ (0-10 µg/mL). For each condition, adherent and detached cells were collected and resuspended in binding buffer (1x10^6 cells/mL) to detect apoptotic and necrotic cells following the FITC Annexin V Apoptosis detection kit instructions (BD Biosciences, CA, USA). The cells were acquired in a Flow cytometer (BD FACSAria™ II, BD Biosciences, Becton, Dickinson and Company) and the results analyzed using the FlowJo vX.0.7 software (Tree Star, Inc). For NMA cells were fixed and stained with a 10 µg/mL Höescht 33458 in PBS for 1 hour. Images were taken using an Olympus BX40 fluorescence microscope with an original magnification of 400X. The analysis was performed with ImageJ Pro Plus 6.0 tool Nuclear Irregularity Index (NII) as described (39). It included as many random pictures as to compile 500 nuclei for each experimental condition.

2.6 Statistical Analysis

Results were expressed as mean ± S.E.M of three independent experiments. Data were analyzed by one-way ANOVA followed by Tukey HSD post-test by using Origin Pro 9.1 software. Prior to ANOVA, data were tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene's tests, respectively by using Origin Pro 9.1 software as well. Pairwise comparisons between IC_{50} were done using Student t-test.

3. Results.

3.1 IQ affects cell viability of transformed cells in a tumor-selective manner.

We assessed whether IQ was capable to exert a selective cytotoxic effect *in vitro* in the absence of immune cells. For this purpose, H5V transformed endothelial cells as hemangioma model and normal microvascular 1G11 cells were compared. B16F-1 melanoma cells were included as a model of a non-related skin tumor, which could also be responsive to IQ. When assayed in the presence of increasing concentrations of IQ, ranging 0-100 µg/mL, a steep drop in cell viability was consistently observed for both H5V and B16 cells in a concentration-dependent manner. Each IC_{50} was significantly different from the other (Table I). H5V hemangioma cells presented a relative IC_{50} four-folds lower than for 1G11, indicative that H5V exhibited a higher susceptibility (Figure 1B). After 24 hs treatment, H5V cytotoxicity reached 85-90% compared with untreated cells, a result also obtained for melanoma cells (Figure 1B). Therefore, we were able to demonstrate not only the selective effect of IQ but also its direct *in vitro* effect on different endothelial cells.

3.2 IQ induces apoptosis on hemangioma cells

We further characterized cell death through flow cytometry to elucidate whether IQ triggered an apoptotic or necrotic cell death. After IQ treatment of H5V cells for 24 hours, a striking dose-dependent (0-10 µg/mL) increase was observed in the Annexin V-FITC/PI positive cell population (Figure 2 A-D), reaching a maximum of 60% of the cells for the highest concentration (Figure 2E). A similar behavior was observed for melanoma cells (data not shown). When 1G11 cells were cultured in the presence of IQ, a slight dose-dependent (0-10 µg/mL) death was appreciated (Figure 2 F-I), reaching a maximum of 10%-15% of the cells which is within the basal cell death found in routine cultures (Figure 2J). Consequently, hemangioma but not normal endothelial cells would undergo an apoptotic death, supporting the idea of a selective cytotoxic effect on transformed cells for IQ.

In order to gain further insight about nuclear morphology changes to complement the results obtained by flow cytometry, NMA was performed. Alterations in nuclear morphology are expected to occur during physiologic processes, like mitosis or throughout cell death such as nuclear constriction and fragmentation observed in

apoptosis (39, 40). According to NMA assay we were able to distribute nuclei in four separate populations: normal (N), apoptotic (small regular, SR), senescent (large regular, LR) or irregular (I).

In absence of IQ, both H5V and 1G11 showed 80-85 % N nuclei with round-shaped and well-defined regular surfaces (Figure 3 A, F, E and J). It is worth mentioning that shapes parameters and average area of nuclei from both lines are different. In fact, 1G11 nuclei are about 30% bigger than H5V in normal culture conditions and they have a similar NII (S1, supplementary material).

When increasing IQ concentrations (5-10 µg/mL) hemangioma cells exhibited a significantly abundant population (65-75%) of shrunken and pyknotic regular (SR) nuclei, consistent with an apoptotic morphology (Figure 3 C, D and E). On the other hand, IQ-treated 1G11 completely lacked of SR nuclei population. However, there was a significant increase (20-25%) of LR nuclear morphology compared to untreated cells, evidence consistent with a senescent nuclear phenotype (Figure 3 H, I and J). These findings strongly support the idea of IQ triggering an apoptotic program on hemangioma cells but not in normal endothelial 1G11. Moreover, these results revealed a new population of senescent cells that were disregarded at the flow cytometry analysis and could be part of the double negative population in cytometric determinations. The presence of senescent cells under IQ treatment was exclusively confirmed in IQ-treated 1G11 cultures by detection of senescence-associated beta-galactosidase (SA-β-gal) (data not shown).

3.3 Cell migration is inhibited on IQ-treated melanoma and hemangioma cells.

Hemangiomas are highly vascularized tumors. A key step during angiogenesis is cell migration, process also significant for tumor cells to invade towards surrounding and distant tissue. Due to its relevance, we decided to investigate whether IQ is able to interfere in normal cell behavior under sub-lethal time conditions. To assess the possibility of IQ having an inhibitory role in cell migration, we performed scratch assays on both endothelial and melanoma cells. In the absence of IQ and 12 hs after wounding, there was a substantial influx of migrating cells into the FCA of the wound (Figure 4 A, D and G) and taken as the maximum migration. On the contrary, when IQ was added to the cultures (1-10 µg/mL), no wound closure was evident for H5V or B16F-1 cells after 12 hs treatment (Figure 4 C, E and F), representing a 40% drop in migration for H5V cells and 60% for melanoma cells (Figure 4J). Nonetheless, IQ treatment had no significant effect on normal endothelial cell migration with respect to control cultures for all assessed concentrations of IQ (Figure 4 G, H, I and J). Of note, the absence of migration on IQ treated cells was not due to cell death or detachment from the plate (Figure 4). Consequently, the inhibitory effect of IQ on migration was more important on H5V and B16F-1 cells.

3.4 IQ selectively induces cell depolarization and stress fibres loss on hemangioma cells.

Cell polarization and actin stress fibers formation play a key role in the migratory phenotype orchestrated by the cytoskeleton (CSK). In order to explain whether IQ effects on cell migration were attributable to changes in actin CSK, we performed phalloidin-TRITC staining on IQ-treated cells (0-10 µg/mL) for 12 hours. Surprisingly, the presence of IQ specifically affected H5V hemangioma cells actin CSK but neither 1G11 CSK nor B16F-1 melanoma cells CSK were altered (Figure 5 A). In the absence of IQ (first column, Figure 5 A), the vast majority of the cells were polarized and showed actin stress fibres at the longitudinal axis, extending a broad lamellipodium at the leading edge (green arrows) and a narrow trailing edge at the rear (white arrows). When cells were treated with IQ, H5V depolarized and became rounded along with increasing concentrations of the drug (Figure 5 A, first row). Quantification of cell with non-polarized morphology indicated a significant increase of cells with rounded shape and absence of actin stress fibres associated to IQ concentrations ≥ 5 µg/mL (Figure 5B). On the contrary, B16F-1 and 1G11 cells exhibited no changes in its actin arrangements and displayed a migratory, fibroblast-like morphology similar to their control counterparts (Figure 5 A, second and third rows respectively). These results could explain migratory defects on hemangioma cell when treated with IQ and highlights the selective effect of the compound.

4. Discussion.

The use of IQ on hemangiomas is an off-label use and its potential benefit in the treatment of this vascular pathology has been only shown in some case reports that involved a reduced number of patients (15-18). This drug is known as a TLR7 agonist capable to stimulate Th1 response (19, 20). Here, we demonstrated that in the absence of such activated immune response, IQ was able to reduce cell viability in a tumor-selective fashion on hemangioma and melanoma cells. This cytotoxic effect of IQ is a relevant finding since scarce research works have shown such antitumor effect at lower concentrations than 50 µg/mL (42, 43).

In consonance with the Nomenclature Committee on Cell Death (NCCD), in absence of clear biochemical biomarkers a cell must be consider dead if: (a) the cell is able to incorporate vital dyes such as propidium iodide (PI) *in vitro*, indicating permeability loss; (b) both the cell and its nucleus have become fragmented into discrete bodies (apoptotic bodies); (c) apoptotic bodies can be recognized through phosphatidylserine exposure (41). Our results showed an augmented PI-positive cells population upon IQ treatment, which could be indicative of late apoptosis/necrosis. However, NMA did not show the same proportion of swelling nuclei. Consequently, the PI-positive fraction of IQ-treated H5V was understood as a late apoptotic population. More interestingly, our data revealed that the treatment induced apoptosis preferentially on transformed endothelial cells but not on its normal counterpart where only a small fraction was senescent. This selective induction on transformed endothelial cells is an encouraging result, which may contribute to control non-involuted IH. These original findings for endothelial lineage should be confirmed by in vivo assays but are in agreement with

results obtained from biopsy of patients with BCC treated with topical IQ. Biopsy images of these patients showed complete remission of the tumor tissue while the surrounding normal tissue remained healthy (27, 28). Interestingly, under sublethal treatment conditions, we found that IQ was capable of inhibiting tumor cell migration of hemangioma and melanoma cells. According to our results, cell motility inhibition was related to actin cytoskeleton disorganization in hemangioma but not in melanoma cells. Thereby, IQ would inhibit migration affecting different components of motile cell cycle in different tumor cell types, an aspect that should be further investigated.

Altogether, these data which were not previously described *in vitro* on hemangioma cells encourage us to perform *in vivo* studies and open an interesting possibility to consider the use of topical IQ to control superficial IH or to contribute to reduce the time of treatment of conventional therapies.

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Legends to Figures

Figure 1. IQ affected cell viability of transformed cells. (A) Structural formula of Imiquimod (1-(2-Methylpropyl)-1H-imidazole [4, 5-c] quinoline-4-amine). **(B)** Effect of increasing concentrations of IQ on cell types viability. Values are shown as Mean ± SEM, N= 3. All cell types showed a cell viability drop in a concentration-dependent manner (H5V $p<0.0001$; B16F-1 $p<0.0001$; 1G11 $p<0.0001$). Curves are fits to the logistic equation with A1 being the maximum percentage of viability; A2 the minimal percentage of viability and "p" the steepness of the dose–response curve. IC50 values are detailed.

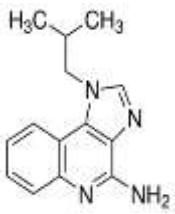
Figure 2. **IQ-induced apoptosis on hemangioma cells.** One of two representative flow cytometry plots of Annexin V-FITC/IP staining performed on H5V cells **(A-E)** and 1G11 cells **(F-I)**. A concentration-dependent apoptosis appeared in hemangioma cells after IQ treatment but minor percentages were observed for normal endothelial cells (**A, F** = 0 µg/mL; **B, G**= 1 µg/mL; **C, H**= 5 µg/mL and **D, I**: 10 µg/mL). (**E** and **J**) Bar graphics showing the percentage (%) of cells in each quadrant for each IQ concentration as mean ± SEM from two independent experiments.

Figure 3. **IQ induced apoptosis on hemangioma and senescence in normal endothelial cells.** Representative images of stained nuclei with Höescht 33458 for hemangioma (H5V, **A-E**) and normal endothelium (1G11, **F-I**) cells. A significantly augmented population of SR nuclei for IQ-treated hemangioma cells (**C-D**) but a reduced population of LR for IQ-treated normal endothelial (**H-I**) cells with respect to untreated cells (**E, J**; *, $p<0.01$). **A, F: 0** µg/mL; **B, G**: 1 µg/mL; **C, H**: 5 µg/mL and **D, I**: 10 µg/mL. Original magnification 400X. (**E** and **J**) Bar graphics showing the percentage (%) of nuclei in each population for each concentration of IQ as mean ± SEM from three independent experiments. N: normal nuclei.

Figure 4. Inhibition of transformed cell migration upon IQ treatment. Phase contrast images after 12 h of IQ treatment. Original magnification 100X. H5V and B16F-1 migration was significantly reduced at 5 µg/mL IQ (*$p<0.05$) and (**$p<0.0015$), respectively. H5V: 0 µg/mL **(A)**, 1 µg/mL **(B)** and 5 µg/mL **(C)**. B16F-1: 0 µg/mL **(D)**, 5 µg/mL **(E)** and 10 µg/mL **(F)**. 1G11: 0 µg/mL **(G)**, 5 µg/mL **(H)** and 10 µg/mL **(I)**. H5V treatment with 10 µg/mL was not recorded since cell death was evident at 12 hs for this concentration. Dotted lines mark the edges at the start of the experiment. (**J**) Migration percentage (%M, according to equation 2) versus IQ concentrations. Data plotted are mean ± SEM of 3 independent experiments.

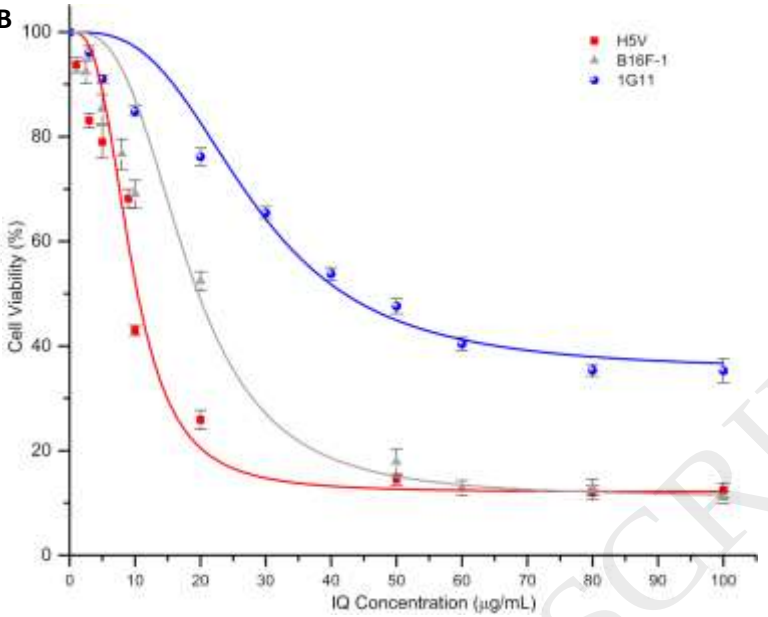
Figure 5. IQ selectively induces cell depolarization and stress fibres lost on hemangioma cells. Melanoma (B16F-1) and endothelial 1G11 cells displayed migratory, fibroblast-like, morphologies with a leading (**A**, green arrows) and trailing edge (**A**, white arrows). No changes in actin stress fibres organization or content is shown. Stress fibres were stained with phalloidin-TRITC (red) and nuclei with Höescht 33458 (blue). Original magnification 400X. (**B**) Cell count (%) for each IQ concentration (µg/mL) showing significant increasing number of hemangioma (H5V) cells (*$p<0,005$) with loss of migratory morphology. Bars represent the mean ± SEM of 3 independent experiments. CSK=cytoskeleton.

A

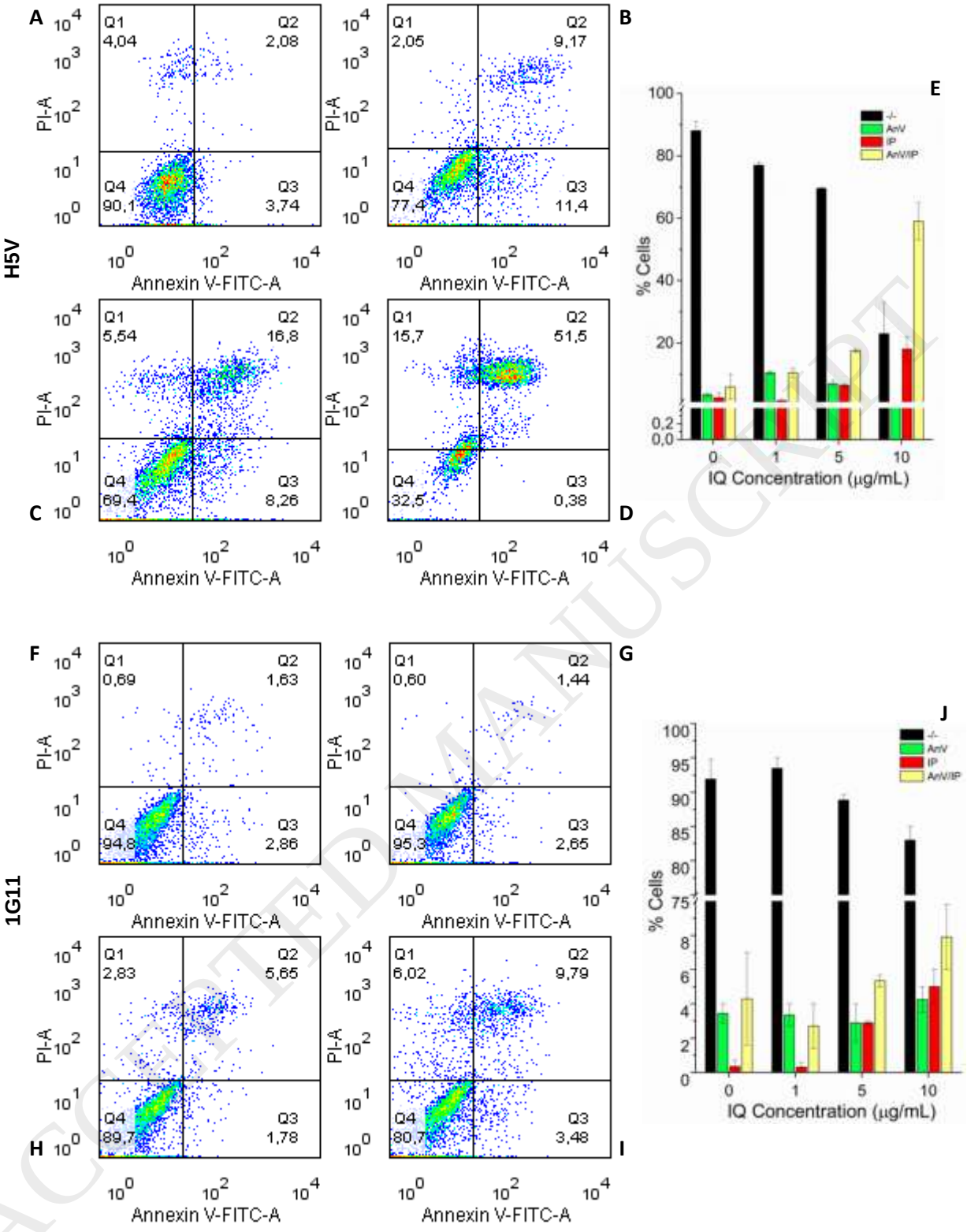


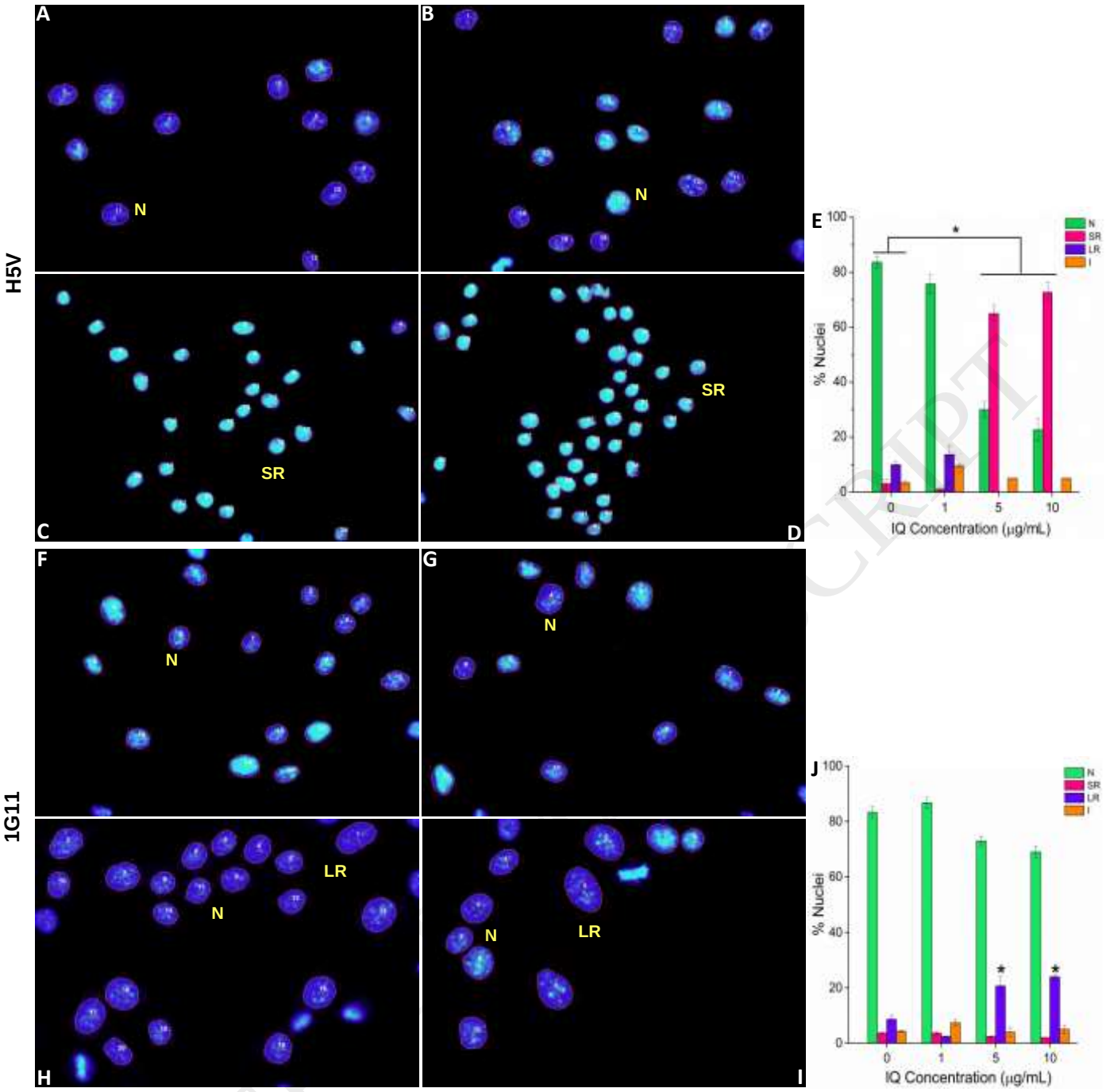
Imiquimod
($C_{14}H_{16}N_4$)

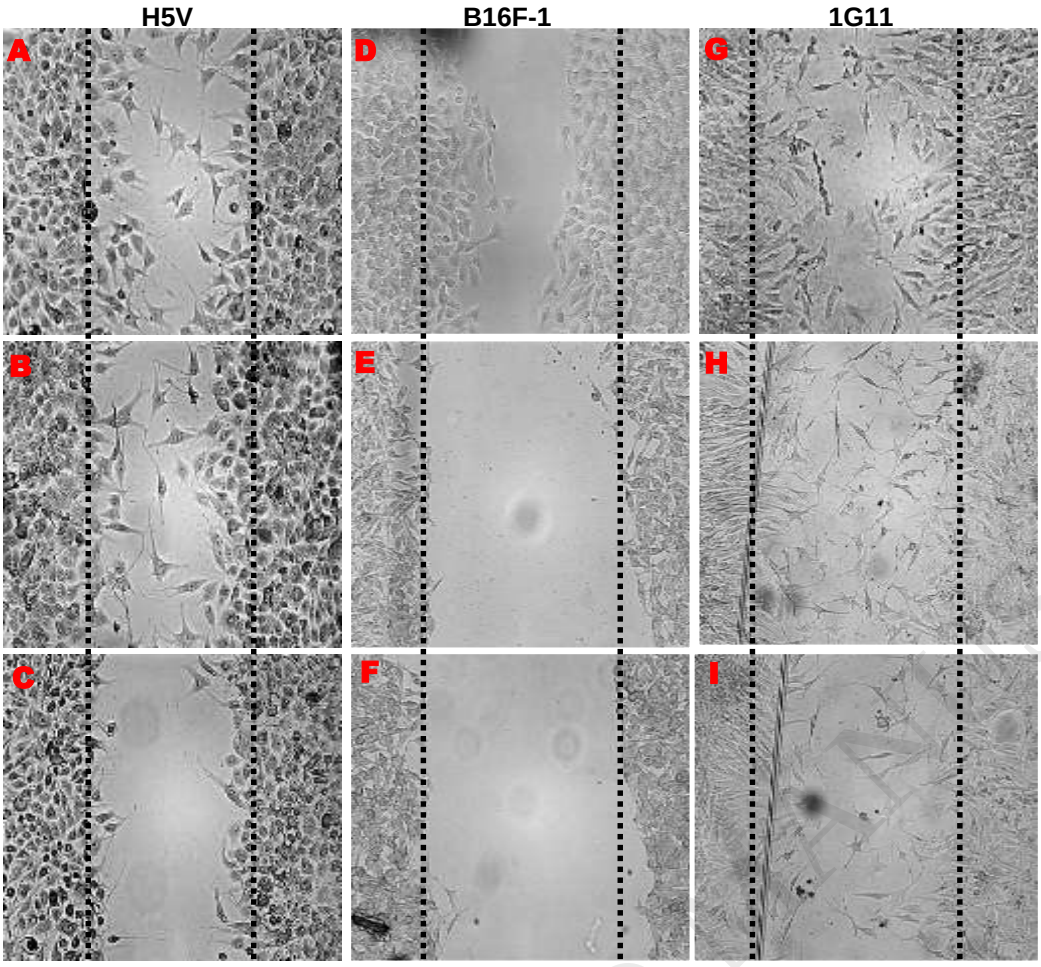
B

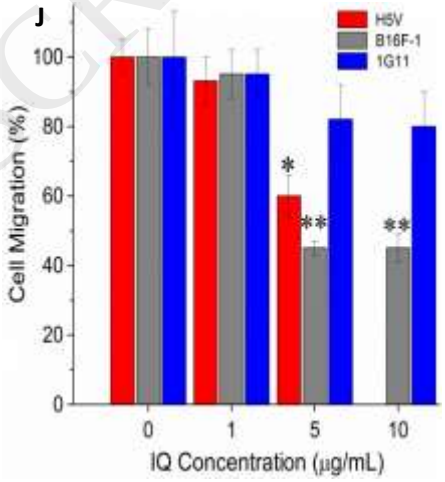


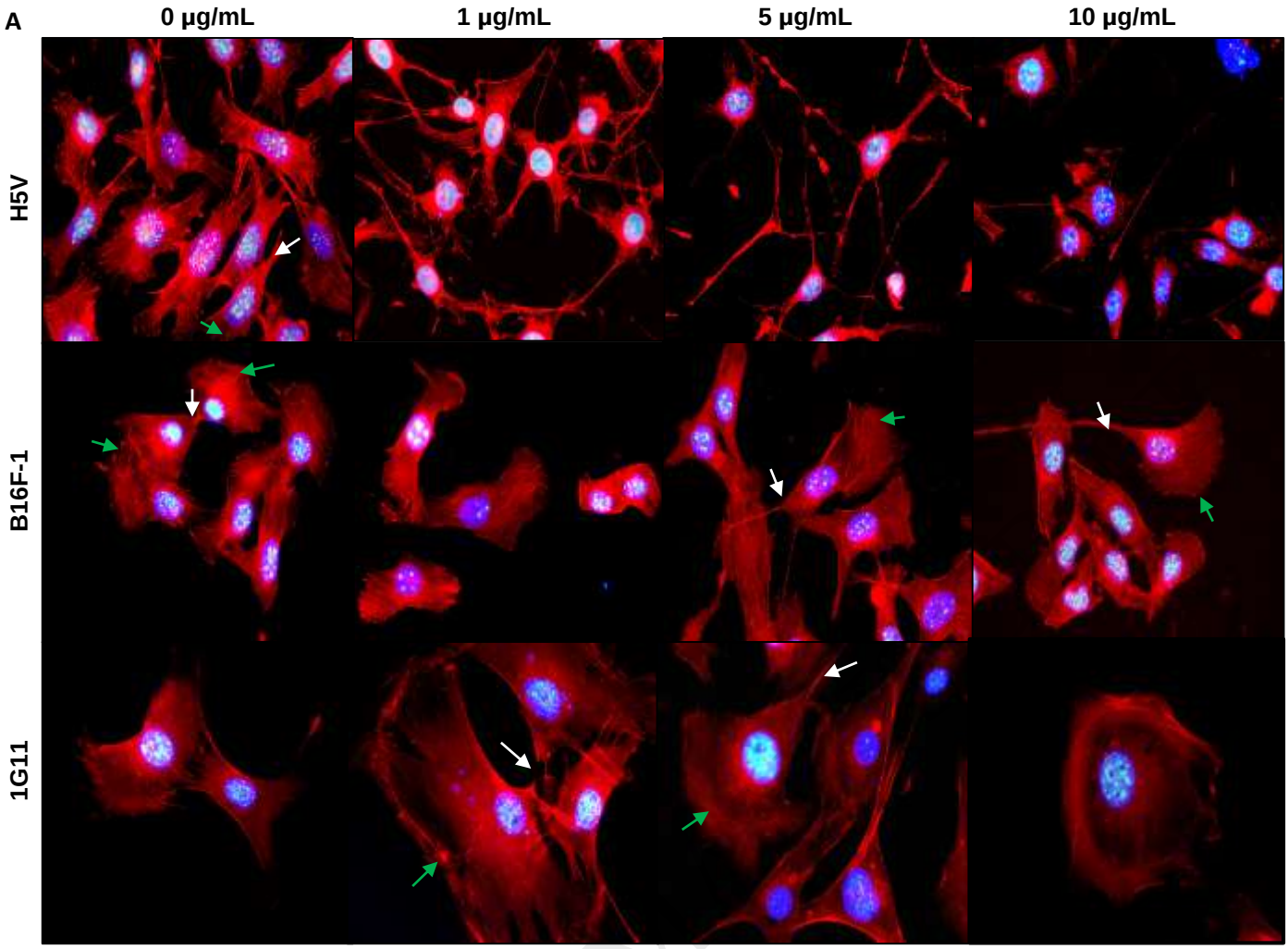
Cell line	A_1	A_2	p	R^2	IC_{50} (µg/mL)	IC_{50} (µM)
H5V	100.0 ± 0.0	12.0 ± 0.0	1.6 ± 0.3	0.9627	09.5 ± 1,4	39.5 ± 05.8
B16F-1	100.0 ± 0.0	11.0 ± 0.0	1.4 ± 0.2	0.9935	20.0 ± 3.0	83.2 ± 12.5
1G11	100.0 ± 0.0	35.0 ± 0.0	3.0 ± 0.6	0.9571	37.2 ± 2.9	155.0 ± 12.0











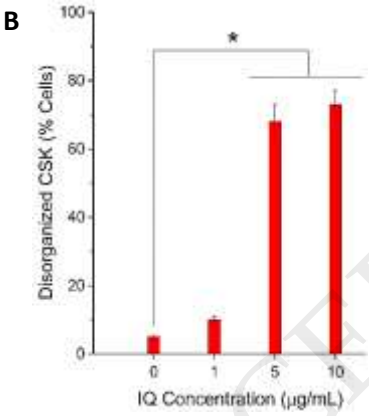


Table I. P-values of the pairwise comparison of IC_{50} values between the three cell lines assayed.

IC50 contrasts	p-value
H5V vs. 1G11	p<0.01
H5V vs. B16F-1	p<0.01
B16F-1 vs. 1G11	p<0.01