Cell-Based Cytotoxicity and Immunocytotoxicity Assays for Colchicine and Ovine Anti-Colchicine Sera and Fab Fragments

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Keywords: Colchicine; Colchicine antidote; Cytotoxicity; Immunotherapy; Poisonings; Overdoses; Immunocytotoxicity; Immunoglobulins

Abstract

In vitro cell-based assays were developed, optimized and validated to assess the cytotoxicity of colchicine, and the ability of specific ovine polyclonal antibodies and their Fab fragments to neutralize and, possibly, reverse these cytotoxic effects. The assays were based on measuring the effects of colchicine on Vero cells in a monolayer culture, as assessed by the visual appearance of cell rounding, and by a loss of electrical impedance as determined by real-time electronic sensing. Colchicine did not impair the ability of Vero cells to take up neutral red.

The cytotoxicity of colchicine was time and dose-dependent. Maximum cell rounding of up to 90% of Vero cells occurred after 16-24 h at a concentration of 1000 µg/L. The lethal cytotoxic dose (LC50) that results in 50% cell rounding was 220 µg/L. Equimolar amounts of polyclonal anti-colchicine immunoglobulins and a Fab-based immunotherapeutic product, ColchiBind, completely inhibited the cytotoxic effects of colchicine when added simultaneously or up to 2 h after the addition of colchicine to the Vero cultures. Considerable protection was also provided when the addition of antibodies or ColchiBind was delayed for 4 h, but a further delay was associated with progressive, irreversible effects and by 8 h the protective effect was negligible.

Materials and Methods

Reagents

Colchicine, cell culture media and supplements were purchased from Sigma (Sigma-Aldrich Company Ltd, Dorset SP8 4XT, UK). Foetal bovine serum (Origin: EU Approved, South America) was obtained from Invitrogen (Life Technologies Ltd, Paisley, PA4 9RE, UK).
Colchicine-Specific Antisera and Fab Fragments (Colchibind): The 4-formylcolchicine - (O-carboxymethyl) oxime was prepared and conjugated to keyhole limpet haemocyanin (KLH) following the method described by Pontikis and colleagues [22] (Figure 1).

![Figure 1: Structure of colchicine (a), 4-formylcolchicine - (O-carboxymethyl) oxime derivative (b) and 4-formylcolchicine - (O-carboxymethyl) oxime-KLH (c) Immunogen](image)

This conjugate was used to immunize a group of 10 sheep following MicroPharm’s standard immunization procedures at a dose of 33 µg per sheep per immunization. The antisera were collected and stored at -20°C until used. Fab fragments were prepared by papain digestion and formulated at a final concentration of 50 g/L in 20 mM sodium acetate buffer (pH 4.0) containing 153 mM sodium chloride as described elsewhere [18].

Aliquots of 10 mL were sterile-filled to produce a Fab-based product referred to as ColchiBind.

Cell culture

African green monkey kidney (Vero) cells were obtained from Public Health England and grown in a 75 cm² culture flask at 37°C in a 5% CO₂ humidified atmosphere using Dulbecco’s Modified Eagles medium supplemented with 10% heat-inactivated foetal bovine serum and 2 mM L-glutamine without antibiotics. This was subdivided to give a number of vials (the master bank) which were stored at -130°C in the vapour phase over liquid nitrogen. At intervals, one of the master bank vials was used to prepare up to 20 further vials (the working bank) which were also stored at -130°C. When required, a vial from the working bank culture was used for a maximum of 10 passages, after which a fresh culture was prepared from the working bank.

Cytotoxicity assay based on cell rounding

The cytotoxicity assay was based on the rounding of Vero cells grown in a monolayer culture when exposed to colchicine. Working solutions of colchicine were prepared freshly by appropriate dilution in culture medium containing HEPES and sterilized by passing through a 0.2 µm filter. Vero cells were prepared by trypsinisation, using 0.25% trypsin and 0.1% EDTA and seeded in 96 well culture plates at a density of 2 × 10⁴ cells/well (200 µL) and grown for 16-24 h at 37°C in a 5% CO₂ humidified atmosphere. A 100 µL volume of culture medium was then removed from each well and replaced with an equal volume of fresh medium containing colchicine at varying concentrations. Control wells received fresh medium only.

![Figure 2: Time course of colchicine effect on Vero cells at a concentration of 1000 µg/L as measured microscopically.](image)

![Figure 3: A dose-response curve based on the degree of effects on Vero cells as measured microscopically (visually) and by cell impedance 16 h after colchicine addition at a concentration of 1000 µg/L.](image)
Figure 4: Real-time monitoring of cytotoxic effect of colchicine on Vero cells as measured by changes in cell impedance using an electronic sensing device (the xCELLigence). For the first 16 h Vero cells were left to grow normally in culture media with a steady increase of cell impedance. After 16 h colchicine was added over a range of concentrations (39 µg/L-2500 µg/L). There was no difference in the continuing increase in cell impedance shown by cells exposed to the lowest concentration of colchicine (39 µg/L) as compared to controls. At higher concentrations, there was a progressive dose-related decrease in cell impedance as the drug prevented cell mitosis and disrupted cellular attachment – the initial colchicine phase.

Each concentration was tested in duplicate. Treated and control wells were compared after 24 h using an inverted microscope and the cells were scored morphologically for cytotoxic effects, taking the control cells as 100% survival (negative effect). The lethal cytotoxic concentration (LC50) was estimated, by curve interpolation, as the colchicine dose resulting in 50% cell rounding (colchicine-induced mitotic arrest) after plotting the mean percentage of rounded cells against the concentration of colchicine solution. A colorimetric endpoint was also assessed based on the Vero cell uptake of neutral red and carried out according to a standard procedure [19,20].

Cytotoxicity assay based on cell impedance

The real-time cell electronic sensing system (RT-CES, or xCELLigence from Roche Applied Science, Indianapolis, IN) was employed to monitor the dynamic response of Vero cells to colchicine via measurement of cell impedance (CI).

A 16-well microelectrode-embedded micro plate (E-plate) was seeded with Vero cells (2 × 10^4/well) before being placed on the RT-CES device. Cells were grown overnight before the addition of colchicine in the absence or presence of anti-colchicine antibody, or mixed with these reagents directly before being added into the E-plates. The dynamic change in impedance as a result of changes in cell attachment, size and number was recorded using the parameter CI.

Immunocytotoxicity assay

Vero cells were seeded into each well of a 96-well plate at a seeding rate of 2 x 10^4 cells/well (200 µL) and allowed to grow over a 16-24 hour period, as previously described.

Figure 5: The antimitotic effect of colchicine on the multiplication rate of Vero cells. The number of cells in the control wells continued to increase at the expected rate while there was no rise in the cell-count of colchicine treated cells.

The neutralising capacity of the antisera pool and the Fab product were assessed and quantified by adding serial two-fold dilutions of the test sample preparations to medium containing colchicine at a dose that induces a rounding effect of 80-90% of the cells. After incubating the colchicine and antibody together at 37°C for 60 minutes, aliquots of the colchicine/antibody mixture were added to the prepared Vero cell monolayer cultures and cell rounding was assessed after a further 24 h. Controls included colchicine incubated with normal (non-immunised) sheep serum. Results were expressed as the minimum neutralising effective dose (ED50), defined as the lowest dilution (titre) of sample required to prevent rounding of 50% of the Vero cells. The specific antibody concentration can be calculated by assuming that the molecular weight of colchicine is 399 Da while that of IgG and Fab are 160,000 Da and 60,000 Da respectively, with two antibody binding sites for specific IgG and one to Fab:

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\text{Antibody concentration (g/L)} = \frac{\text{CCD (µg/L)} \times \text{LC50 (µg/L)}}{\text{Mw Ab/Mw Colchicine/BS}} \times 10^{-6}\text{ED50}
\]

Where CCD: Colchicine Challenge Dose; Mw: Molecular Weight; BS: Number of Binding Sites.
Cytotoxicity assay based on cell rounding

This cytotoxicity assay was based on measuring the effect of colchicine on Vero cells in a monolayer culture. Under the conditions described earlier, the cytotoxic effects of colchicine are both time- and dose-dependent. Addition of an excess of colchicine of 1000 µg/L (2.5 µmol/L) or more, led to virtually complete mitotic arrest with a maximum of up to 90% of cell rounding by 24 h (Figure 2) and a dose-response curve could be established with a range of 50 µg/L to 500 µg/L (Figure 3). The minimum detectable level, defined as that resulting in 5% cell rounding, was 80 µg/L (0.2 µmol/L). The average lethal cytotoxic concentration (LC50) that induces 50% cell rounding was 214 µg/L (0.51 µmol/L) and the within and between coefficients of variation for 4 replicates were 6.6% and 13%, respectively. A dose of 500 µg/L (1.25 µmol/L) that produced approximately 80% cell rounding was chosen for the immunocytotoxicity studies. In an attempt to further improve the accuracy of this assay, studies were undertaken based on employing the uptake of neutral red by the Vero cells as an end-point. Both control and colchicine treated cells took up the dye equally (data not shown), so that this approach could not be used.

Cytotoxicity assay based on cell impedance

An electronic sensing device (the xCELLigence) was employed for the real-time detection of colchicine cytotoxicity as measured by changes in cell impedance. Thus impedance to the passing of a small electronic current corresponds to the number of cells attaching to and spreading over the bottom of an E-plate. As seen in Figure 4 the patterns of changes in impedance are complex. For the first 16 h Vero cells were left to grow normally in culture media and the degree of impedance was found to increase steadily-the pre-colchicine phase. After 16 h colchicine was added over a range of concentrations. There was no difference in the continuing increase in cell impedance shown by cells exposed to the lowest concentration of colchicine (39 µg/L) as compared to controls not exposed to colchicine. However at higher concentrations, after a short delay there was a progressive dose-related decrease in impedance as the drug prevented cell mitosis and disrupted cellular attachment - the initial colchicine phase. A dose-response curve could be established using data obtained at any point during this phase and Figure 3 shows such a curve based on the degree of cell impedance 16 h after colchicine addition. It shows that this approach is slightly more sensitive than that based on the visual microscopic estimation of cell rounding with a LC50 (as discussed earlier) of 180 µg/L.

Unexpectedly some 24 h after colchicine addition and following the initial reduction in cell impedance, values started to increase progressively once again for ~10 h – the mid-colchicine phase. Addition of trypan blue was used to determine the number of cells present in each well following addition of colchicine for comparison with the number in the control wells. The results show (Figure 5) that while the number of cells in the control wells continued to increase at the expected rate there was no rise in the cell-count of colchicine treated cells. Thus the increase in cell impedance seen during the mid-colchicine phase (from about 20 to 40 h) is not due to a rise in cell number but, probably, reflects an increase in cell size. Subsequent changes possibly relate to cell death.

Immunocytotoxicity assay

A cytotoxicity assay based on the visual assessment of cell rounding was used to determine the concentration of the immunoglobulin present in the pool of ovine antisera and of Fab in ColchiBind that specifically bound to and neutralized 500 µg/L (1.25 µmol/L) of colchicine. Figure 6 shows that serum from non-immunized sheep had no effect while both a pool of antisera from immunized sheep and ColchiBind were highly effective in preventing colchicine-induced mitotic arrest. The neutralising effective dilutions (ED50) of the antisera and Fab-based products were 1:180 and 1:70 respectively and the coefficient of variation for 4 replicates for within and between assays were 10% and 15% respectively. Based on their ED50 it can be calculated that the levels of specific immunoglobulin in the antisera pool and of specific Fab in ColchiBind are 9.0 g/L and 2.2 g/L, respectively.

The effect of delaying the addition of antibodies in preventing and reversing the anti-mitotic effects of colchicine was also assessed. All cells were exposed to colchicine at a concentration of 500 µg/L and 0, 2, 4, 6 and 8 h later duplicate sets were exposed to serial dilutions of the ovine antisera pool. One set of duplicates was exposed to a similar concentration of colchicine that had been premixed with serial dilutions of the pool for 1 hour at 37°C. As shown in Figure 7a the addition of excess antibodies at 1 and 2 h completely prevented cell rounding when the Vero cell cultures were assayed after 24 h while there was ~10%, ~45% and ~75% of cell rounding when antibody addition was delayed for 4, 6 and 8 h respectively. When reassessed after 48 h (Figure 7b) many of the cells appear to have recovered with the percentage of cell rounding falling to ~5%, ~10% and ~20% in the 4, 6 and 8 hour delay groups.

Discussion

Several high affinity polyclonal and monoclonal antibodies directed against colchicine have been prepared and used to develop specific, sensitive immunoassays [21-25]. However the fact that an antibody or its fragments bind to a toxin does not necessarily mean that it will prevent its toxic effects. Thus it was considered essential to develop an *in-vitro* cytotoxicity assay to determine the neutralising efficacy of antisera from sheep immunized with a colchicine-KLH conjugate and of a Fab-based product, ColchiBind, prepared from those antisera. The availability of such an assay also reduces reliance on animal experimentation.

In 1990 Rouan et al. [26] reported studies of the cytotoxicity of colchicine using Chinese hamster ovary (CMO) cells and of the ability of their murine monoclonal antibody to prevent and reverse its cytotoxic effects. Their findings are similar to our own. Thus colchicine caused the complete cessation of cellular multiplication and an increase in cell size with a doubling of cellular DNA content. Prolonged exposure led to polyploidy and severe chromosomal damage. They noted “when colchicine and equimolar anti-colchicine antibody are added simultaneously to cultures of CHO cells, the cellular effects of colchicine are completely inhibited” and “Colchicine-induced changes were reversible when equimolar antibody was added up to 6 h after colchicine. When cells were exposed to colchicine for 16 h before antibody addition some cells recovered but 60% were polyploid, indicating incomplete reversal”.

The Vero cell line derived from the kidney of the African green monkey was selected for all the present experimental procedures because of its robustness, suitability for microscopic analysis and rapid
cell division (doubling time of about 24 h under optimal conditions). Numerous endpoints have been employed in such assays including the ability of viable cells to take up neutral red. However, this was shown to be inappropriate in the present context since both control and colchicine-treated cells continued to take up the dye. This reflects the fact that the cell membrane, lysozymes and mitochondria are not colchicine's main target. Thus its primary effect is to bind to tubulin with a high affinity constant (of $1 \times 10^8$ to $2 \times 10^9$) and, thereby, prevent cell mitosis [27,28] (Bhattacharya and Wolff 1976; Garland 1978). As a result cell numbers cease to rise and, since DNA and protein synthesis continues, the cells increase in size and become rounded.

Vero cell counting and rounding in combination with an electronic sensing device were used in developing sensitive cytotoxicity and immunocytotoxicity assays. Colchicine disrupts cell attachment of Vero cells in monolayer culture thereby lowering cell impedance. These effects were both time- and dose-dependent and the standard curves obtained were reproducible with between and within coefficients of variation of less than 15%. The minimum detection limit was 80 µg/L while a colchicine concentration of 1000 µg/ml caused a maximum effect with up to 90% of cells becoming rounded. In studies of the time course of colchicine toxicity it was shown that even after only 2 h exposure there is a significant reduction in cell numbers 16 h later as compared with control cells not exposed to the drugs.

A colchicine-specific polyclonal antibody with an affinity constant significantly higher for colchicine than that of its tubulin receptor would be expected to prevent and reverse the drug's toxic effects. When sufficient colchicine to cause about 80% cell rounding (500 µg/L) together with an excess of anti-colchicine immunoglobulin's were added simultaneously to Vero cell monolayers, the toxic effects of the drug were neutralised completely while sera from non-immunised sheep had no protective effect. Based on this phenomenon we developed an Immunocytotoxicity assay with which to determine the variation of less than 15%.

There appear to be two significant problems regarding the use of immunotherapy for patients with colchicine overdose, namely the relatively large amounts of ColchiBind required and the need to initiate such treatment quickly. The first problem reflects the fact that only about 10% of the total Fab is specific to colchicine (since an affinity chromatographic step is not included in its manufacture) and the large differences in molecular weight between Fab (~50,000 Da) and colchicine (~400 Da). The need to begin treatment sufficiently early may pose more of a problem. Thus Rouan et al. (1990) noted "these studies have uncovered a potentially serious problem for the treatment of colchicine poisoning; a time dependent increase in the irreversibility of colchicine effects." The present study supports this view with significant protection of Vero cells requiring the addition of an excess of neutralising antibodies within a few h of their exposure to colchicine. We have experienced similar results in an in vivo piglet model in which recovery from an intravenously administered lethal dose of colchicine requires the administration of ColchiBind within about 5 h.

Fortunately colchicine is available only for oral administration and not all the ingested colchicine will be absorbed. In addition the drug is partially cleared by hepatic first pass metabolism [2]. There will also be a significant delay before it reaches the tissue fluid compartment and metabolically active cells throughout the body. The amount of drug absorbed may be further decreased by its effect on the epithelial cells lining the intestine and this may further delay absorption. Some support for this is provided by a recent study in which colchicine was administered orally followed by ColchiBind (formerly ColchiFab) by intraperitoneal injection 2 h later. Thus the ovine Fab greatly increased the urinary excretion of the drug and reversed the colchicine-induced diarrhoea [29]. Nonetheless it would be essential to administer ColchiBind as soon as an overdose is suspected and, to this end,
hospitals that care for such patients will need to keep a small stockpile available.

References


