DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

Overcoming P-glycoprotein mediated drug resistance with noscapine derivatives[a]

Divya Muthiah, Georgia K. Henshaw, Aaron J. DeBono, Ben Capuano, Peter J. Scammells & Richard Callaghan

Division of Biomedical Science and Biochemistry, Research School of Biology and Medical School, Australian National University, Canberra, ACT 0200, Australia (DM, GKH, RC)

Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia (AJD, BC, PJS)

Running Title: Inhibition of P-glycoprotein by noscapine derivatives

Author to whom correspondence should be addressed:

Assoc Prof Richard Callaghan

Research School of Biology, Building 134, Linnaeus Way, Australian National University,

Acton, Canberra, ACT 2601 Australia

Telephone: +61 2 6125 0824

E-mail:richard.callaghan@anu.edu.au

Manuscript details:

Text pages: 44

Tables: 4

Figures: 5

References: 57

Abstract word count: 188

Introduction word count: 745

Discussion word count: 1499

Abbreviations:

ABC ATP Binding Cassette

BBB blood-brain barrier

CI combination index

DDM dodecyl-β-maltoside

DMSO dimethyl-sulfoxide

HBSS Hanks balanced salt solution

IMAC immobilised metal affinity chromatography

MTT 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide

NBD nucleotide binding domain

P-gp P-glycoprotein

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

The antitussive agent noscapine has previously been shown to inhibit the proliferation of cancer cells by disruption of tubulin dynamics. However, the efficacy of several anticancer drugs that inhibit tublin dynamics (vinca alkaloids and taxanes) is reduced by the multidrug resistance phenotype. In particular, these compounds are substrates for P-glycoprotein (P-gp) mediated extrusion from cells. Consequently, the anti-proliferative activity of noscapine, and a series of derivatives, was measured in drug sensitive and drug resistant cells that over-express P-gp. None of the noscapine derivatives displayed lower potency in cells over-expressing P-gp; thereby suggesting a lack of interaction with this pump. However, the cellular efflux of a fluorescent substrate by P-gp was potently inhibited by noscapine and the majority of derivatives. Further investigation with purified, reconstituted P-gp demonstrated that inhibition of P-gp function was due to direct interaction of noscapine derivatives with the transporter. Moreover, co-administration of vinblastine with two of the noscapine derivatives displayed synergistic inhibition of proliferation, even in P-gp expressing resistant cell lines. Therefore, noscapine derivatives offer a dual benefit of overcoming the significant impact of P-gp in conferring multidrug resistance and synergy with tubulin-disrupting anticancer drugs.

INTRODUCTION

The alkaloid noscapine is found in the opium poppy (*Papaver somniferum*) and belongs to the chemical class of phthalideisoquinolines. Noscapine is classified as an opiate alongside morphine, thebaine, codeine and papaverine. However, noscapine does not produce respiratory depression, sedation or analgesia and its main therapeutic use is as an antitussive agent (Empey et al., 1979; Dahlstrom et al., 1982; Mahmoudian and Rahimi-Moghaddam, 2009). Noscapine has a low toxicity profile and is sufficiently water to soluble to be administered orally.

Based on the chemical similarity of noscapine to colchicine and the podophyllotoxins, the possibility of an interaction with tubulin was investigated (Ye et al., 1998). Noscapine was demonstrated to bind to tubulin, alter tubulin dynamics and arrest mitosis at the G_2/M-phase (Ye et al., 1998; Mahmoudian and Rahimi-Moghaddam, 2009); properties that are common to several anticancer agents including vinca alkaloids and taxanes. The anticancer activity of noscapine was confirmed by its ability to inhibit the proliferation of cancer cells and the growth of solid tumours *in vivo* (Ye et al., 1998). Despite the comparatively low toxicity profile of noscapine, its anticancer activity lacked potency, with growth inhibitory effects on cultured cells in the micromolar range. As a result, medicinal chemistry programmes have generated a number of derivatives with the intention of generating higher potency.

A series of analogues were generated with halo-substitutions at the 9'-position of noscapine (see Figure 1) with the goal of improving the potency of cell cycle arrest (Aneja et al., 2006b). The 7'-position of noscapine was also targeted with the synthesis of carbamate or urea-analogues (Anderson et al., 2005a; Anderson et al., 2005b) and a series of more bulky substitutions (Mishra et al., 2011). The N-demethylation of noscapine to yield nor-noscapine provided a template for

introduction of multiple substitutions at the 6'-position (DeBono et al., 2012). Finally, a more recent investigation generated noscapine derivatives with multiple substitutions at the 6', 7' and 9'-positions (Debono et al., 2014). The series of compounds were divided into lactones (**2-5**) and cyclic ethers (**6-11**), with carbamate and urea derivatives and halogen substitutions. These combined medicinal chemistry efforts provided considerable information on the structure-activity relationship for noscapine. The new derivatives displayed improved affinity of interaction with tubulin, greater potency to inhibit cell proliferation and increased cell cycle arrest at G_2/M-phase.

The ability of noscapine to interfere with tubulin dynamics raises the possibility of synergism with anticancer drugs that also target tubulin dynamics; for example, vinblastine and paclitaxel. However, it is noteworthy that the efficacy of both of these drugs is markedly reduced by the resistance modifier P-glycoprotein (P-gp) (Dumontet et al., 1996; Martin et al., 1999). P-gp is a member of the ATP-Binding Cassette (ABC) superfamily of transport proteins and mediates the active extrusion of xenobiotics from cells. P-gp is characterised by a high promiscuity towards substrates and is over-expressed in many cancer types. The overexpression in cancer cells confers a resistant phenotype, which is a negative prognostic indicator for chemotherapy (Modok et al., 2006). The prevalence of P-gp mediated resistance and the breadth of drugs involved has engendered considerable research efforts into developing inhibitors of the pump. An astonishing number of potent inhibitors have been developed; however, none have reached clinical use to date. Consequently, the spectre of P-gp mediated resistance remains a major consideration for drug development in oncology. Moreover, the protein is expressed in healthy tissues, particularly those involved in secretory roles or in the formation of barriers for sanctuary sites. In fact, the FDA and the International Transporter Consortium have published guidelines on the methods

and importance of establishing possible interactions of new molecular entities with P-gp (Agarwal et al., 2013).

Despite considerable efforts, the pharmacophore of interaction with P-gp remains unknown although substrates and inhibitors typically contain a planar hydrophobic ring system and a cationic nitrogen group. It is not possible to accurately predict whether a compound is likely to interact with P-gp. Therefore, the susceptibility of noscapine and its derivatives to P-gp mediated efflux from cancer cells is unknown. The aim of this investigation was to ascertain whether the potency of a series of multi-functionalised noscapine derivatives to inhibit proliferation is altered in drug resistant cancer cells. Modified drug potency may indicate an interaction with P-gp since this protein confers a resistant phenotype in these cells. Consequently, the interaction between noscapine derivatives and P-gp was investigated using a series of direct functional assays. Finally, the impact of P-gp expression on synergy between noscapine derivatives and the tubulin disruptor vinblastine were described.

MATERIAL & METHODS

Cell culture

The drug-sensitive human breast adenocarcinoma cell line MCF7 (ATCC® HTB-22™) was purchased from the American Type Culture Collection (Rockville, MD). The doxorubicin-selected P-gp-expressing variant NCI/Adr^{Res} cell line was obtained from Dr K Cowan (National Cancer Institute, Bethesda, MD) and has been verified as a derivative of the MCF7^{WT} line (Mehta et al., 2002). All cell lines were grown at 37°C with 5% CO_2 as monolayer cultures in DMEM medium supplemented with 10% FCS, penicillin (10000 U/mL) and streptomycin (10000 mg/mL). Every third passage of NCI/Adr^{Res} was supplemented with doxorubicin (3μM) to maintain selection pressure for resistance. All cells were grown to a maximum of 20 passages of sub-culture. DNA fingerprint analysis has verified that both cell lines originated from breast cancer tissue and that the NCI/Adr^{RES} cells are derived from the MCF7 line. The NCI/Adr^{RES} cells did, however, contain a minor contaminant of A2780 ovarian cancer cells. Despite the supplementation of medium with doxorubicin, there was no detectable expression of the multidrug transporters ABCG2 or ABCC1 in the NCI/ADR^{Res} cell line as previously reported (Rivers et al., 2008).

Compound synthesis

The compounds used in the study were synthesised as described in DeBono *et al.* (DeBono et al., 2012).

Cell growth assay

Cell growth assays were done as previously described (Muthiah and Callaghan, 2017). Noscapine derivatives, anticancer drugs and P-gp inhibitors were added from a 2X stock solution in medium (100µL) to produce a final concentration range of 10^{-12} to 10^{-4} M. The solvent (DMSO) concentration in the wells was kept to 0.2 % (v/v). Cell viability following drug treatment was assessed using the MTT assay (Mosmann, 1983) after six days, at which point the control wells (i.e. DMSO treated) had reached confluence. The absorbance in the control wells was assigned a value of 100% and the potency (IC_{50}) of each compound to inhibit growth was derived. The absence of selection agent (namely doxorubicin) during the six day period did not alter the expression of P-gp in the NCI/Adr^{RES} cells (*Supplemental Figure 1*).

For combination cytotoxicity assays, cells were incubated with vinblastine as described above either in the presence of absence of Noscapine and its derivatives. Drugs and cells were incubated at 37ºC with 5% CO_2 for 6 days. The nature of interaction between drugs co-administered in cytotoxicity assays was evaluated using Combination Indices (CI) that were derived from the *Lowe's Additivity Model* (Chou, 2006).

Calcein-AM transport in whole cells

The calcein-AM assay to measure the transport function of ABCB1 was based on a modified version (Muthiah and Callaghan, 2017) of a previously published method (Homolya et al., 1993). MCF7^{WT} and NCI/Adr^{RES} cells were seeded into 96-well tissue culture plates at a density of 2 x 10^3 cells per well in 100µl and allowed to adhere for 2 days at 37°C with 5% CO_2. Test compounds (100µl) were added from a 2X final concentration in HBSS from an original stock

solution of 50mM in DMSO. Calcein-AM was added to a concentration of 1μM and the fluorescence measured 37°C using an excitation of λ=488nm and an emission of λ=515nm.

Purification and reconstitution of P-gp

P-gp was expressed in *Trichoplusia ni* (High-Five) cells following infection with recombinant baculovirus as previously described (Taylor et al., 2001). Crude membranes were prepared from the cells using differential ultracentrifugation following cell disruption by nitrogen cavitation. P-gp was purified using immobilised metal affinity chromatography (IMAC) according to previously published methods with some modifications (Taylor et al., 2001; Crowley et al., 2009). The primary difference was the solubilisation of membranes in 2% (w/v) dodecyl-β-D-maltoside (DDM) supplemented with a 0.4% (w/v) lipid mixture comprising a 4:1 ratio of *E coli* extract:cholesterol. Reconstitution was achieved by detergent adsorption to SM-2 BioBeads as described (Crowley et al., 2009).

ATP hydrolysis activity of P-gp

The ATPase activity of P-gp was measured in proteoliposomes based on the rate of inorganic phosphate liberation using a modified colorimetric assay (Chifflet et al., 1988; Crowley et al., 2009). In all cases the activity was expressed as μmoles of P_i liberated per minute per mg of pure protein. To determine the Michaelis-Menten parameters, proteoliposomes (0.1-0.5μg protein per point) were incubated with ATP (0-1.75mM) either in the presence of 10μM nicardipine (i.e. stimulated activity) or with the solvent DMSO (i.e. basal activity). To investigate the potency of

drugs to stimulate ATP hydrolysis, activity was measured as a function of added drug concentration (10^{-9} to 10^{-4} M) in the presence of 2mM ATP.

Data analysis

Curve-fitting was done using non-linear, or linear, least squares regression and an F-test was used for comparison of multiple curves. The Student's t test was used for statistical analyses of two groups and for comparison of three or more groups, a one-way analysis of variance (ANOVA) using the Dunnett's post-hoc test. P-values of <0.05 were considered statistically significant. All curve-fitting and statistical analyses of data were carried out using GraphPad Prism® Software v6.

RESULTS

Growth inhibition of sensitive and resistant cancer cell lines by noscapine derivatives

Vinblastine produces growth arrest in cells by disrupting microtubule and this is the bass for its anticancer activity. In the MCF7 breast cancer cell line the potency of vinblastine to produce growth arrest was IC_{50} = 0.31±0.05nM as shown in Figure 2a. In the NCI/Adr^{RES} cells, the potency of vinblastine to reduce growth was significantly lower ($P<0.01$) and characterised by an IC_{50} = 159±15nM. This 512-fold reduction in potency is indicative of the resistant phenotype produced by over-expression of P-gp and the associated efflux of vinblastine from cells. Identical analysis was undertaken for noscapine and the 10 chemically modified derivatives shown in Figure 1. Modifications were made to four positions of noscapine, namely the 1, 7, 6' and 9'-positions, as such modification have previously been shown to have a major influence on cytotoxic activity. More specifically, compounds in which the lactone present in the isobenzofuranone ring system was reduced to corresponding cyclic ether (compounds **6**-**11**), the *N*-methyl in the 6'-position was converted to a carbamate (**2** and **6**) or urea moiety (**3**-**5**, **7**-**11**), the 7-methoxy was demethylated to give the corresponding phenol (**5**) and a halogen or amino group was installed in the 9'-position (**4**, **5**, **8**-**11**) were studied. The calculated partition coefficient (cLogP) values are provided for noscapine and its derivatives in Figure 1. With the exception of compound (**11**), all derivatives displayed higher partition coefficients (cLogP 3.39-4.53) than the parent noscapine (cLogP 2.81).

The growth inhibitory effects of noscapine and two of the derivatives **7** and **3** on MCF7 and NCI/Adr^{RES} cells are shown in Figure 2 (b-d). Noscapine produced growth inhibition in both cell lines, although the potency did not differ between the MCF7 (IC_{50} = 45.4±6.5μM) and

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

NCI/Adr^{RES} cells (IC_{50} = 32.1±7.0μM) (Table 1). In addition, the potency of noscapine was considerably lower than the cytotoxic anticancer drug vinblastine. Compound **7** was modified at the 1- and 6'-positions of the noscapine core (Figure 1) and this produced a modest increase ($P<0.01$) in potency for the derivative (Figure 2c). However, like noscapine, the growth inhibition produced by **7** was similar between the control and P-gp expressing cell lines.

The derivative **3** was not able to produce growth inhibition of either cell line at concentrations up to 100μM (Figure 2d). Compound **3** retained the carbonyl group at the R^1 position of noscapine, but contained a 6'-ethylaminocarbonyl substituent as per compound **7** (Figure 1). Similarly, compound **2** failed to elicit any growth inhibition at concentrations up to 100μM in either cell line (Table 1) and also contained a carbonyl substitution at position R^1. This substitution did not preclude growth inhibition as evidenced by the potent activity of **4** and **5** (Table 1). These two derivatives differed from the inactive compounds **2** and **3** at the 7-position (-OCH_3 vs –OH) and by the presence of a halide at position $R^{9'}$.

Table 1 provides the potency of a further four noscapine derivatives and all of them display significantly higher ($P<0.01$) potency than the parent compound. Another consistent feature was the lack of any difference in potency between the sensitive and resistant cell lines. This suggests that the expression of P-gp does not affect the growth inhibition produced by this series of noscapine derivatives. This is desirable given the ability of this transporter to confound conventional chemotherapy and suggests that the compounds are not substrates for transport by P-gp.

Effects of noscapine derivatives on the transport activity of P-gp in whole cells

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

P-gp is known to interact with over 300 distinct compounds, but only a proportion of these are substrates for efflux and a large number of compounds directly inhibit its transport activity. Therefore, the ability of noscapine derivatives to inhibit the transport of a known P-gp substrate, calcein-AM, was investigated.

Once calcein-AM enters cells it is rapidly cleaved by esterases to yield the highly fluorescent compound calcein. Calcein appearance, detected as an increase in fluorescence is shown for representative examples of both cell lines in Figure 3a. In MCF7 cells the rate of calcein appearance was 102±2 r.f.u/min, whereas the rate in NCI/Adr^{RES} cells was markedly lower at 0.17±0.26 r.f.u/min. The reduced rate of appearance in NCI/Adr^{RES} cells reflects the resistant phenotype attributed to over-expression of P-gp. Addition of the P-gp inhibitor nicardipine caused a dose-dependent increase in the rate of fluorescence in NCI/Adr^{RES} cells (Figure 3b). In contrast, nicardipine did not alter the rate of calcein accumulation in the drug sensitive MCF7 cells (*data not shown*). The accumulation rate of calcein in NCI/Adr^{RES} cells was plotted as a function of nicardipine concentration to generate the secondary plot displayed as Figure 3c. Two parameters were derived from the hyperbolic plot; the potency of nicardipine to increase the rate of calcein accumulation (MP_{50}) and the maximal rate of calcein accumulation in the presence of modulator (A_{MAX}). In the presence of nicardipine, the rate of calcein accumulation was increased to a maximum value of A_{MAX} = 110±19 r.f.u/min and the potency of nicardipine to increase the rate was MP_{50} = 2.5±0.6 μM (Figure 3a, Table 2). The A_{MAX} value derived for nicardipine was similar to the rate of calcein appearance in MCF7 cells and suggests complete inhibition of P-gp activity. Noscapine was also capable of increasing the rate of calcein accumulation specifically in NCI/Adr^{RES} cells; although the maximal rate observed was significantly lower at A_{MAX} = 24.6±6.7 r.f.u/min ($P<0.05$) (Table 2).

The chemical derivatives of noscapine were also investigated for their ability to modulate the rate of calcein accumulation in NCI/Adr^{RES} cells with the MP_{50} and A_{MAX} values listed in Table 2. Neither **4** nor **7** were able to modulate the rate of calcein appearance in NCI/Adr^{RES} cells at concentrations up to 30μM. Compound **6** increased the rate of calcein appearance and the value of A_{MAX} = 35.1±4.0 r.f.u/min was not significantly different to noscapine; however, the potency of this derivative to inhibit P-gp activity was significantly greater (Table 2). Compound **4** displayed potency that was indistinguishable from noscapine, whereas it produced the greatest increase in the A_{MAX} parameter of calcein appearance.

Compounds **9**, **10** and **11** produced a marked increase in the rate of calcein appearance and **8** generated a significant increase ($P<0.05$) in A_{MAX} compared to noscapine (Table 2). Compounds **9** and **10** displayed similar potencies to noscapine and contained halide substitutions at the $R^{9'}$ position (Figure 1). Compound **8** also contained a halide at position $R^{9'}$, but its potency was significantly higher ($P<0.05$) than that of noscapine. In contrast, **11** produced a similar extent of increase in A_{MAX}; however, its potency was the lowest. This compound contained an amino substituent at 9'-position rather than a halide, but identical moieties at the 1-, 7- and 6'-positions.

Overall, the majority of noscapine derivatives were capable of modulating the transport activity of P-gp. Compound **8** was optimal compound from this series for inhibition of P-gp since it markedly increased the rate of calcein appearance and did so with high potency.

Do noscapine derivatives interact directly with purified, reconstituted P-gp?

The noscapine derivatives may affect the cellular transport activity of P-gp by acting as specific inhibitors or through an indirect mechanism. To discriminate between these possibilities, P-gp

was purified and reconstituted into liposomes to enable direct investigation of the interaction of noscapine derivatives with the protein. P-gp uses the energy of ATP hydrolysis to drive vectorial transport and both substrates and inhibitors have been demonstrated to dramatically stimulate the rate of catalysis.

The ATPase activity of P-gp was measured as a function of varying concentrations of ATP in the absence or presence of nicardipine (Figure 4). In the absence of nicardipine, the basal rate of ATP hydrolysis was characterised by a maximal activity of V_{MAX} = 213±19 nmol P_i/min/mg and an affinity constant for ATP of K_M = 1.31±0.14 mM. In the presence of nicardipine the rate of ATP hydrolysis was significantly ($P<0.01$) increased (V_{MAX} = 904±66 nmol P_i/min/mg), but with no significant change in the affinity constant (K_M = 0.71±0.06 mM). The data demonstrate that both the maximal rate of ATP hydrolysis and the degree of stimulation by nicardipine were similar to values published by our laboratories (Taylor et al., 2001; Mittra et al., 2017). Moreover, this confirms the integrity of the purified P-gp preparations and enables their use to characterise interaction with noscapine and its derivatives.

A modified ATPase assay was used to enable characterisation of drug interaction with purified P-gp. In particular, the rate of hydrolysis was measured in the presence of a range of drug concentrations to ascertain the potency of interaction with P-gp. Figure 4b shows the effect of noscapine on the rate of ATP hydrolysis. The ATPase activity was stimulated by noscapine from 163±71 nmol P_i/min/mg to a value of 744±149 nmol P_i/min/mg. The 3.4±0.4 fold stimulation of ATPase activity was described by a sigmoidal relationship with a potency of 44.6±9.1μM for noscapine. Identical analysis was undertaken for each of the noscapine derivatives and for nicardipine (Table 3).

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

Although the degree of stimulation by noscapine was not different to nicardipine, its potency was significantly lower ($P<0.01$). As detailed in Table 3, the majority of noscapine derivatives were able to stimulate ATP hydrolysis by purified P-gp and the extents of this stimulation (1.4 to 4.4 fold) did not differ significantly from the parent compound noscapine or the P-gp modulator nicardipine. Only **10** and **11** were unable to affect the ATPase activity at the concentrations tested. However, the range of potencies was large with **3** (EC_{50} = 65.3±10.2μM) the most potent and **4** (EC_{50} = 0.95±0.28μM) the least.

The results demonstrate that the noscapine derivatives are able to interact directly with purified P-gp and this direct interaction with P-gp is responsible for the inhibition of calcein-AM transport. In addition, the equipotent growth inhibitory effects of the noscapine derivatives between the resistant and sensitive cell lines suggest that the compounds are not substrates for transport and thereby act as specific inhibitors of P-gp. As such, it is conceivable that the noscapine derivatives may potentiate the effects of cytotoxic drugs, particularly in P-gp expressing resistant cells.

Do noscapine derivatives improve the anticancer activity of vinblastine?

Noscapine was co-administered with the anticancer drug vinblastine to ascertain the potential for potentiated inhibition of cellular proliferation. The cytotoxicity profile of vinblastine in the absence or presence of noscapine is shown in Figure 5 for both drug resistant and sensitive cells. The addition of noscapine caused a reduction in the upper limit of the dose-response curve for vinblastine in MCF7 cells (Figure 5a). This reduction is presumably due to the growth inhibitory effects of noscapine *per se* (Figure 2b). In contrast, the potency of vinblastine was not

significantly altered. For example, in MCF7 cells, the potency of vinblastine alone was IC_{50} = 0.059±0.029nM and in the presence of the highest concentration of noscapine (30μM) it was unchanged at IC_{50} = 0.063±0.014nM.

The dose-response profiles in P-gp expressing NCI/Adr^{RES} cells displayed a reduction in the upper limit and a shift to higher potency (Figure 5b). The cytotoxic potency of vinblastine alone was IC_{50} = 188±65nM and the addition of 10μM noscapine caused no significant change to the potency (IC_{50} = 157±41nM). However, addition of 20μM noscapine increased the apparent vinblastine potency 9.6-fold to IC_{50} = 19±7nM. The reduction of the upper limit of the dose-response curves was broadly similar to the situation observed in MCF7 cells.

Isobologram analysis was undertaken to determine whether the alteration in the efficacy and potency of vinblastine produced by noscapine was additive or synergistic. The drug-drug interactions were analysed by determination of combination indices (CI) at each concentration of noscapine. The reduction in the upper limit of the dose-response relationships necessitated the use of an IC_{75} value since the co-addition of >10μM noscapine resulted in cell viability falling below 50%. The CI values (Table 4) were plotted for the combination of vinblastine and noscapine in both cell lines (Figure 5c-d).

In the MCF7 cells, the CI-values obtained for at each noscapine concentration were significantly different to 1.0 (Table 4) and were located in the sector below the line of additivity in the isobologram (Figure 5c); thus indicating a synergistic interaction. Similarly, in NCI/Adr^{RES} cells, the CI-values for the two highest concentrations of noscapine were also significantly different from 1.0 (Table 4) and were also located below the isobole in Figure 5d. Therefore, the

synergistic relationship between noscapine and vinblastine was retained in the P-gp expressing NCI/Adr^{RES} cells.

The CI-analysis was also undertaken for two derivatives of noscapine; namely **6** and **8** (Table 4). These two derivatives were chosen based on their high potency and efficacy to cause growth inhibition of the cell lines and to modulate the transport activity of P-gp. In the case of **6**, only the highest concentration tested (3μM) demonstrated synergy with vinblastine in the MCF7 cells. However, there was a greater propensity of **6** to generate a synergistic effect with vinblastine in NCI/Adr^{RES} cells.

Compound **8** produced a dramatic reduction in the upper limit of dose-response relationships and a CI could not be determined for concentrations above 0.3μM in the MCF7 cells and above 1 μM in NCI/Adr^{RES} cells (Table 4). There was no synergy between **8** and vinblastine in the MCF7 cells, albeit with a narrow range of concentrations. In contrast, a 1μM concentration of compound **8** was able to produce a synergistic effect on cell proliferation with vinblastine in the drug resistant NCI/Adr^{RES} cell line.

In summary, noscapine and the two derivatives examined were able to inhibit cell proliferation in a synergistic manner with vinblastine. Moreover, there was a higher predisposition for a synergistic interaction in the P-gp expressing, drug resistant cells.

DISCUSSION

Noscapine, and several derivatives, are able to modulate efflux of drugs by P-gp in resistant cancer cells. The modulation is by a direct interaction of the compounds with P-gp given their ability to alter the activity of purified protein. However, the expression of P-gp in cells did not alter the potency of noscapine and its derivatives to inhibit proliferation. This suggests that the compounds are not substrates for transport by P-gp and act as inhibitors of the protein. These properties of noscapine derivatives enabled synergy with vinblastine to inhibit proliferation of P-gp expressing resistant cancer cells. The inherent anti-proliferative activity and the ability to "evade" P-gp render the noscapine derivatives potentially innovative tools in chemotherapy regimes.

Substrates for transport by P-gp show reduced accumulation in cells expressing the protein and in the case of anticancer drugs this resulted in impaired inhibition of proliferation (Bellamy et al., 1988; Mazzanti et al., 1992; Martin et al., 1999). Inhibition of P-gp mediated transport should increase the intracellular accumulation of anticancer drugs and restore chemotherapy efficacy. This strategy has been used to identify an arsenal of P-gp inhibitors beginning with verapamil, through to the most potent compounds Tariquidar (XR9576) and Elacridar (GF120918) (Tsuruo et al., 1981; Hyafil et al., 1993; Martin et al., 1999).

Unlike transported substrates of P-gp, noscapine derivatives were equipotent between the resistant and sensitive cell lines. The simplest explanation is that noscapine derivatives do not interact with P-gp. However, the derivatives inhibited the calcein-AM transport by P-gp in whole cells. Moreover, the compounds altered the ATPase activity of purifiedP-gp, which

demonstrates that they modulate function by a direct interaction. Should the noscapine derivatives therefore be classified as non-transported inhibitors of P-gp?

The transport mechanism of P-gp requires multiple conformational transitions and the involvement of two distinct domains (Callaghan et al., 2006; Callaghan et al., 2012). Specifically, substrate translocation involves transition of the binding site from a high affinity and *inward facing*, to a low affinity and *outward facing* configuration (Martin et al., 2001; Rosenberg et al., 2001; Al-Shawi et al., 2003; Omote et al., 2004). Efficient transmembrane translocation requires a sufficiently large differential between the binding affinities of a substrate for the two configurations. Moreover, the conformational transitions are intimately linked (i.e. *coupled*) to the discrete stages of the ATP hydrolytic process in the nucleotide binding domains (NBDs).

In general, substrates for transport tend to stimulate the ATPase activity of P-gp and display tight coupling between binding sites and NBDs (Muller et al., 1996; Orlowski et al., 1996). Inhibitors, or modulators, have been demonstrated to bind to P-gp and either stimulate (nicardipine) or inhibit (Tariquidar) the overall rate of ATP hydrolysis (Martin et al., 1999; Mittra et al., 2017). Whole cell transport assays have demonstrated that [^{3}H]-Tariquidar is a non-transported substrate for P-gp. Trans-epithelial transport and permeability assays indicated that nicardipine is also unlikely to be a substrate for transport by P-gp (Lentz et al., 2000). It is therefore difficult to ascertain whether a compound is a substrate or a "classical" inhibitor of P-gp by its effects on ATPase activity. In fact, whether the ATPase activity of P-gp is stimulated or inhibited by a compound is dependent on the kinetics of (a) conformational transitions or (b) dissociation from the outward facing configuration (Martin et al., 2001; Al-Shawi et al., 2003).

The effects of noscapine derivatives on the proliferation of resistant cells and the activity of P-gp support their classification as non-transported inhibitors. Further evidence has been provided by the pharmacokinetics of noscapine *in vivo* (Landen et al., 2004). The oral administration of noscapine in mice produced growth inhibition of glioblastoma and did so following rapid passage across the blood-brain barrier (BBB). It is well-established that P-gp is expressed and active in endothelial cells of the BBB where it regulates the entry, or exit, of compounds to the CNS (Jette et al., 1993; Agarwal et al., 2011; Auvity et al., 2018). In particular, substrates for P-gp are prevented from entering the CNS and the ability of noscapine to cross the BBB indicates that it is not subject to efflux by P-gp.

It may also be possible for the noscapine derivatives to "by-pass" the actions of P-gp if their permeability is sufficiently high, resulting in rapid and extensive intracellular accumulation. Diffusional permeability of cells to drugs is in part dictated by their partition coefficients into the membrane. However, the cLogP values are broadly similar to that of vinblastine and paclitaxel, both substrates of the transporter. This observation does not to support a model whereby noscapine and derivatives bypass P-gp through kinetic factors. The compounds potentially act in a similar manner to that described for Tariquidar; namely as a non-transported P-gp modulator (Martin et al., 1999).

Synergism between anticancer drugs has long been viewed as a promising strategy to improve inhibition of tumour growth (Merlin, 1994). Noscapine and vinblastine bind to distinct sites on tubulin and synergy of activity is a distinct possibility. The present investigation has demonstrated synergism between noscapine, and two derivatives, with vinblastine. Another vinca alkaloid (vinorelbine) also displays synergy with paclitaxel, another tubulin disrupting drug that interacts at a distinct site (Photiou et al., 1997). In addition, noscapine also synergises

with paclitaxel (Altinoz et al., 2006) and two tubulin binding compounds with anticancer activity *in vitro*; peloruside and laulimalide (Gajewski et al., 2012). Similarly, a noscapine derivative, 9'-bromonoscapine, in combination with docetaxol produced a apoptotic response, mitotic arrest and inhibition of tumour growth in a synergistic manner (Pannu et al., 2011). However, synergistic behaviour of noscapine and its derivatives is not limited to tubulin disrupting compounds. For example, noscapine was also demonstrated to potentiate the activity of cisplatin (DNA cross-linking) and the alkylating agents Temozolamide and bis-chloroethylnitrosurea (BCNU) in glioblastoma cells and in xenograft models (Qi et al., 2013). Therefore, the noscapine derivatives provide the potential to reduce the dose of conventional genotoxic anticancer drugs and maintain significant tumour growth inhibition. Moreover, the inherently low toxicity of noscapine and a reduced dose of genotoxic drugs may diminish the limiting side-effects of chemotherapy. The synergy with approved anticancer drugs will require extensive pharmacokinetic investigation to fully validate this potential of noscapine in chemotherapy.

Drug interaction with tubulin has been extensively characterised for a number of antiproliferative agents. For example, it has been demonstrated using structural modelling that colchicine and noscapine bind to the same, or an overlapping, site on tubulin (Naik et al., 2011). In contrast, vinblastine and colchicine are known to interact at distinct sites on tubulin (Luduena and Roach, 1981; Wolff et al., 1991). By inference, noscapine and vinblastine alter tubulin dynamics through interaction at distinct sites, which may result in synergistic effects on cell proliferation. The synergistic interaction between noscapine derivatives and vinblastine raises the issue of where the two compounds interact on P-gp. Despite the availability of several structures for P-gp (Aller et al., 2009; Alam et al., 2018; Kim and Chen, 2018), the location of drug binding sites remains elusive, although biochemical approaches are beginning to shed detailed information (Loo et al.,

2009; Mittra et al., 2017). Thus it is not yet possible to assign the respective sites for vinblastine and noscapine binding on P-gp, but the differing interactions with tubulin support distinct sites.

As discussed earlier, the search for compounds to inhibit P-gp activity and restore accumulation of anticancer drugs has been exhaustive. However, the search has not yielded any clinically used inhibitors due primarily to pharmacokinetic issues. Consequently, the need for novel strategies to overcome the actions of P-gp has been touted (Callaghan et al., 2014). The ability of noscapine to evade P-gp mediated efflux and to potentiate the activity of vinblastine in drug resistant cells provides a major benefit. A similar property is shared with the epothilones, which also alter microtubule dynamics and inhibit mitosis in cancer cells, including an equipotent effect in P-gp expressing cells (Alberti, 2013). Furthermore, a 9'-nitro-noscapine derivative has been shown to elicit apoptosis and mitotic arrest in ovarian and T-cell lymphoma cell lines known to express P-gp and displaying resistance to paclitaxel and vinblastine (Aneja et al., 2006a).

The noscapine derivatives appear to short-circuit the resistant phenotype conferred by P-gp; namely they are able to avoid efflux from cells by the pump, yet are capable of inhibiting its activity. Moreover, the ability to potentiate the efficacy of conventional anticancer drugs, presents a unique strategy to restore chemotherapy in drug resistant cancer. Noscapine has long been established to display low toxicity and is well tolerated in its therapeutic setting as an antitussive agent (Mahmoudian and Rahimi-Moghaddam, 2009). Furthermore, pharmacokinetic studies in healthy volunteers indicate that low micromolar plasma concentrations may be attained via both oral and intravenous administration of noscapine. (Dahlstrom et al., 1982). Moreover, noscapine displayed the lowest potency of the derivatives tested in this manuscript. and the latter also displayed improved partition coefficients. The improved potencies and high partition

coefficients of the noscapine derivatives raises the possibility of them reaching sufficient plasma levels to facilitate restoration of chemotherapeutic success and diminish the actions of P-gp.

ACKNOWLEDGEMENTS

AUTHORSHIP CONTRIBUTIONS:

Research design: Callaghan, Scammells, Muthiah

Experimental: Muthiah, Henshaw, DeBono

Provision of reagents: DeBono, Capuano, Scammells

Data analysis: Muhiah, Henshaw, Callaghan

Writing contribution: Callaghan, Muthiah, Capuano, Scammells

REFERENCES

Agarwal S, Arya V, and Zhang L (2013) Review of P-gp inhibition data in recently approved new drug applications: utility of the proposed [I(1)]/IC(50) and [I(2)]/IC(50) criteria in the P-gp decision tree. *J Clin Pharmacol* **53:**228-233.

Agarwal S, Hartz AM, Elmquist WF, and Bauer B (2011) Breast cancer resistance protein and P-glycoprotein in brain cancer: two gatekeepers team up. *Curr Pharm Des* **17:**2793-2802.

Al-Shawi MK, Polar MK, Omote H, and Figler RA (2003) Transition state analysis of the coupling of drug transport to ATP hydrolysis by P-glycoprotein. *J Biol Chem* **278:**52629-52640.

Alam A, Kung R, Kowal J, McLeod RA, Tremp N, Broude EV, Roninson IB, Stahlberg H, and Locher KP (2018) Structure of a zosuquidar and UIC2-bound human-mouse chimeric ABCB1. *Proc Natl Acad Sci U S A* **115:**E1973-E1982.

Alberti C (2013) Taxane- and epothilone-based chemotherapy: from molecule cargo cytoskeletal logistics to management of castration-resistant prostate carcinoma. *Eur Rev Med Pharmacol Sci* **17:**1658-1664.

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, and Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323:**1718-1722.

Altinoz MA, Bilir A, Del Maestro RF, Tuna S, Ozcan E, and Gedikoglu G (2006) Noscapine and diltiazem augment taxol and radiation-induced S-phase arrest and clonogenic death of C6 glioma in vitro. *Surg Neurol* **65:**478-484; discussion 485.

Anderson JT, Ting AE, Boozer S, Brunden KR, Crumrine C, Danzig J, Dent T, Faga L, Harrington JJ, Hodnick WF, Murphy SM, Pawlowski G, Perry R, Raber A, Rundlett SE,

Stricker-Krongrad A, Wang J, and Bennani YL (2005a) Identification of novel and improved antimitotic agents derived from noscapine. *J Med Chem* **48:**7096-7098.

Anderson JT, Ting AE, Boozer S, Brunden KR, Danzig J, Dent T, Harrington JJ, Murphy SM, Perry R, Raber A, Rundlett SE, Wang J, Wang N, and Bennani YL (2005b) Discovery of S-phase arresting agents derived from noscapine. *J Med Chem* **48:**2756-2758.

Aneja R, Vangapandu SN, Lopus M, Chandra R, Panda D, and Joshi HC (2006a) Development of a novel nitro-derivative of noscapine for the potential treatment of drug-resistant ovarian cancer and T-cell lymphoma. *Mol Pharmacol* **69:**1801-1809.

Aneja R, Vangapandu SN, Lopus M, Viswesarappa VG, Dhiman N, Verma A, Chandra R, Panda D, and Joshi HC (2006b) Synthesis of microtubule-interfering halogenated noscapine analogs that perturb mitosis in cancer cells followed by cell death. *Biochem Pharmacol* **72:**415-426.

Auvity S, Caille F, Marie S, Wimberley C, Bauer M, Langer O, Buvat I, Goutal S, and Tournier N (2018) P-glycoprotein (ABCB1) inhibits the influx and increases the efflux of (11)C-metoclopramide across the blood-brain barrier: a PET study on non-human primates. *J Nucl Med*.

Bellamy WT, Dalton WS, Kailey JM, Gleason MC, McCloskey TM, Dorr RT, and Alberts DS (1988) Verapamil reversal of doxorubicin resistance in multidrug-resistant human myeloma cells and association with drug accumulation and DNA damage. *Cancer Res* **48:**6365-6370.

Callaghan R, Ford RC, and Kerr ID (2006) The translocation mechanism of P-glycoprotein. *FEBS Lett* **580:**1056-1063.

Callaghan R, George AM, and Kerr ID (2012) 8.8 Molecular Aspects of the Translocation Process by ABC Proteins, in: *Comprehensive Biophysics* (Editor-in-Chief: Edward HE ed), pp 145-173, Elsevier, Amsterdam.

Callaghan R, Luk F, and Bebawy M (2014) Inhibition of the multidrug resistance P-glycoprotein: time for a change of strategy? *Drug Metab Dispos* **42:**623-631.

Chifflet S, Chiesa UTR, and Tolosa S (1988) A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases. *Anal Biochem* **168:**1-4.

Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev* **58:**621-681.

Crowley E, O'Mara ML, Reynolds C, Tieleman DP, Storm J, Kerr ID, and Callaghan R (2009) Transmembrane helix 12 modulates progression of the ATP catalytic cycle in ABCB1. *Biochemistry* **48:**6249-6258.

Dahlstrom B, Mellstrand T, Lofdahl CG, and Johansson M (1982) Pharmacokinetic properties of noscapine. *Eur J Clin Pharmacol* **22:**535-539.

Debono AJ, Mistry SJ, Xie J, Muthiah D, Phillips J, Ventura S, Callaghan R, Pouton CW, Capuano B, and Scammells PJ (2014) The synthesis and biological evaluation of multifunctionalised derivatives of noscapine as cytotoxic agents. *ChemMedChem* **9:**399-410.

DeBono AJ, Xie JH, Ventura S, Pouton CW, Capuano B, and Scammells PJ (2012) Synthesis and biological evaluation of N-substituted noscapine analogues. *ChemMedChem* **7:**2122-2133.

Dumontet C, Duran GE, Steger KA, Beketic-Oreskovic L, and Sikic BI (1996) Resistance mechanisms in human sarcoma mutants derived by single-step exposure to paclitaxel (Taxol). *Cancer Res* **56:**1091-1097.

Empey DW, Laitinen LA, Young GA, Bye CE, and Hughes DT (1979) Comparison of the antitussive effects of codeine phosphate 20 mg, dextromethorphan 30 mg and noscapine 30 mg using citric acid-induced cough in normal subjects. *Eur J Clin Pharmacol* **16:**393-397.

Gajewski MM, Alisaraie L, and Tuszynski JA (2012) Peloruside, laulimalide, and noscapine interactions with beta-tubulin. *Pharm Res* **29:**2985-2993.

Homolya L, Hollo Z, Germann UA, Pastan I, Gottesman MM, and Sarkadi B (1993) Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J Biol Chem* **268:**21493-21496.

Hyafil F, Vergely C, Du Vignaud P, and Grand-Perret T (1993) In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. *Cancer Res* **53:**4595-4602.

Jette L, Tetu B, and Beliveau R (1993) High levels of P-glycoprotein detected in isolated brain capillaries. *Biochim Biophys Acta* **1150:**147-154.

Kim Y and Chen J (2018) Molecular structure of human P-glycoprotein in the ATP-bound, outward-facing conformation. *Science*.

Landen JW, Hau V, Wang M, Davis T, Ciliax B, Wainer BH, Van Meir EG, Glass JD, Joshi HC, and Archer DR (2004) Noscapine Crosses the Blood-Brain Barrier and Inhibits Glioblastoma Growth. *Clinical Cancer Research* **10:**5187-5201.

Lentz KA, Polli JW, Wring SA, Humphreys JE, and Polli JE (2000) Influence of passive permeability on apparent P-glycoprotein kinetics. *Pharm Res* **17:**1456-1460.

Loo TW, Bartlett MC, and Clarke DM (2009) Identification of residues in the drug translocation pathway of the human multidrug resistance P-glycoprotein by arginine mutagenesis. *J Biol Chem* **284:**24074-24087.

Luduena RF and Roach MC (1981) Interaction of tubulin with drugs and alkylating agents. 2. Effects of colchicine, podophyllotoxin, and vinblastine on the alkylation of tubulin. *Biochemistry* **20:**4444-4450.

Mahmoudian M and Rahimi-Moghaddam P (2009) The Anti-Cancer Activity of Noscapine: A Review. *Recent Patents on Anti-Cancer Drug Discovery* **4:**92-97.

Martin C, Berridge G, Mistry P, Higgins C, Charlton P, and Callaghan R (1999) The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. *Br J Pharmacol* **128:**403-411.

Martin C, Higgins CF, and Callaghan R (2001) The vinblastine binding site adopts high- and low-affinity conformations during a transport cycle of P-glycoprotein. *Biochemistry* **40:**15733-15742.

Mazzanti R, Croop JM, Gatmaitan Z, Budding M, Steiglitz K, Arceci R, and Arias IM (1992) Benzquinamide inhibits P-glycoprotein mediated drug efflux and potentiates anticancer agent cytotoxicity in multidrug resistant cells. *Oncol Res* **4:**359-365.

Mehta K, Devarajan E, Chen J, Multani A, and Pathak S (2002) Multidrug-Resistant MCF-7 Cells: An Identity Crisis? *Journal of the National Cancer Institute* **94:**1652-1654.

Merlin JL (1994) Concepts of synergism and antagonism. *Anticancer Res* **14:**2315-2319.

Mishra RC, Karna P, Gundala SR, Pannu V, Stanton RA, Gupta KK, Robinson MH, Lopus M, Wilson L, Henary M, and Aneja R (2011) Second generation benzofuranone ring substituted noscapine analogs: synthesis and biological evaluation. *Biochem Pharmacol* **82:**110-121.

Mittra R, Pavy M, Subramanian N, George AM, O'Mara ML, Kerr ID, and Callaghan R (2017) Location of contact residues in pharmacologically distinct drug binding sites on P-glycoprotein. *Biochem Pharmacol* **123:**19-28.

Modok S, Mellor HR, and Callaghan R (2006) Modulation of multidrug resistance efflux pump activity to overcome chemoresistance in cancer. *Curr Opin Pharmacol* **6:**350-354.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:**55-63.

Muller M, Bakos E, Welker E, Varadi A, Germann UA, Gottesman MM, Morse BS, Roninson IB, and Sarkadi B (1996) Altered drug-stimulated ATPase activity in mutants of the human multidrug resistance protein. *J Biol Chem* **271:**1877-1883.

Muthiah D and Callaghan R (2017) Dual effects of the PI3K inhibitor ZSTK474 on multidrug efflux pumps in resistant cancer cells. *Eur J Pharmacol* **815:**127-137.

Naik PK, Santoshi S, Rai A, and Joshi HC (2011) Molecular modelling and competition binding study of Br-noscapine and colchicine provide insight into noscapinoid-tubulin binding site. *J Mol Graph Model* **29:**947-955.

Omote H, Figler RA, Polar MK, and Al-Shawi MK (2004) Improved energy coupling of human P-glycoprotein by the glycine 185 to valine mutation. *Biochemistry* **43:**3917-3928.

Orlowski S, Mir LM, Belehradek J, and Garrigos M (1996) Effects of steroids and verapamil on P-glycoprotein ATPase activity: progesterone, desoxycorticosterone and verapamil are mutually non-exclusive modulators. *Biochem J* **317:**515-522.

Pannu V, Karna P, Sajja HK, Shukla D, and Aneja R (2011) Synergistic antimicrotubule therapy for prostate cancer. *Biochem Pharmacol* **81:**478-487.

Photiou A, Shah P, Leong LK, Moss J, and Retsas S (1997) In vitro synergy of paclitaxel (Taxol) and vinorelbine (navelbine) against human melanoma cell lines. *Eur J Cancer* **33:**463-470.

Qi Q, Liu X, Li S, Joshi HC, and Ye K (2013) Synergistic suppression of noscapine and conventional chemotherapeutics on human glioblastoma cell growth. *Acta Pharmacol Sin* **34:**930-938.

Rivers F, O'Brien TJ, and Callaghan R (2008) Exploring the possible interaction between anti-epilepsy drugs and multidrug efflux pumps; in vitro observations. *Eur J Pharmacol* **598:**1-8.

Rosenberg MF, Velarde G, Ford RC, Martin C, Berridge G, Kerr ID, Callaghan R, Schmidlin A, Wooding C, Linton KJ, and Higgins CF (2001) Repacking of the transmembrane domains of P-glycoprotein during the transport ATPase cycle. *EMBO J* **20:**5615-5625.

Taylor AM, Storm J, Soceneantu L, Linton KJ, Gabriel M, Martin C, Woodhouse J, Blott E, Higgins CF, and Callaghan R (2001) Detailed characterization of cysteine-less P-glycoprotein reveals subtle pharmacological differences in function from wild-type protein. *Br J Pharmacol* **134:**1609-1618.

Tsuruo T, Iida H, Tsukagoshi S, and Sakurai Y (1981) Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* **41:**1967-1972.

Wolff J, Knipling L, Cahnmann HJ, and Palumbo G (1991) Direct photoaffinity labeling of tubulin with colchicine. *Proc Natl Acad Sci U S A* **88:**2820-2824.

Ye K, Ke Y, Keshava N, Shanks J, Kapp JA, Tekmal RR, Petros J, and Joshi HC (1998) Opium alkaloid noscapine is an antitumor agent that arrests metaphase and induces apoptosis in dividing cells. *Proceedings of the National Academy of Sciences* **95:**1601-1606.

FOOTNOTE

[a] The work in this manuscript was generously supported by a project grant (#12-0008) from Worldwide Cancer Research.

FIGURE LEGENDS

Figure 1 Chemical structures of noscapine derivatives

A series of derivatives were generated using noscapine as a template. Chemical substituents were generated at the positions marked R^1, $R^{6'}$, R^7 and $R^{9'}$. The calculated permeability (cLogP) values were determined using the "*molinspiration*" property engine v2016.10.

Figure 2 Effects of vinblastine, noscapine and its derivatives on cell proliferation

Drug sensitive MCF7 (*empty symbols*) and the drug resistant NCI/Adr^{RES} cells (*filled symbols*) were incubated with drugs for a six days as described in the methods. The percentage of live cells remaining after exposure to (a) vinblastine, (b) noscapine, (c) compound (**7**) or (d) compound (**3**) were determined using the MTT assay. Sigmoidal dose-response curves were fitted using non-linear regression and the values correspond to mean±SEM obtained from at least three independent observations.

Figure 3 Accumulation of Calcein-AM in MCF7 and NCI/Adr^{RES} cells

The transport of calcein-AM (1μM) was measured using a fluorescence based assay in 96-well microplates over a 10 minute period. Liberation of the fluorescent calcein in cells was detected using an excitation λ=488nm and an emission λ=515nm.

(a) A representative fluorescence intensity profile measured in MCF7 (●) and NCI/Adr^{RES} (○) cells

(b) The fluorescence intensity was measured in NCI/Adr^{RES} cells in the presence of 1 (○), 3 (●) or 10μM (□) nicardipine. The rate of calcein accumulation was determined from the slope between 5-10 minutes

(c) The rate of calcein accumulation was plotted as a function of nicardipine and the data fitted with a hyperbolic relationship using non-linear regression. Values correspond to mean±SEM obtained from at least three independent observations

Figure 4 Drug stimulation of ATP hydrolysis by purified P-gp

The ATPase activity of P-gp was measured by the appearance phosphate using a colorimetric assay. Proteoliposomes (0.1-0.5μg protein) were incubated with ATP and drug as indicated below.

(a) ATP hydrolysis by purified P-gp was measured with a range of ATP concentrations (0-1.75mM) in the absence (○) or presence (●) of nicardipine (10μM) for 30 minutes. The Michaelis-Menten equation was fitted to the data using non-linear regression

(b) The ATPase activity of P-gp was measured in the presence of ATP (2mM) and a series of noscapine concentrations (10^{-7} to 10^{-3}M) for 30 minutes. A sigmoidal dose-response curve was fitted to the data using non-linear regression

The rate of ATP hydrolysis was normalised for both protein content and the assay time and values represent mean±SEM obtained from five independent observations.

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5 Effects of vinblastine and noscapine co-administration on cell proliferation

Cell proliferation was measured in MCF7 and NCI/Adr^{RES} cells in the presence of vinblastine and a series of noscapine concentrations. Cell viability was measured using the MTT assay following a six day incubation with drugs.

(a) The effects of vinblastine on the growth of MCF7 cells in the absence (●) or presence of 10 (●), 20 (●) or 30μM (●) noscapine. A sigmoidal dose-response relationship was fitted to the data by non-linear regression

(b) The effects of vinblastine on the growth of NCI/Adr^{RES} cells in the absence (■) or presence of 10 (■), 20 (■) or 30μM (■) noscapine. A sigmoidal dose-response relationship was fitted to the data by non-linear regression

(c) Isobologram for the interaction between vinblastine and noscapine in NCI/Adr^{RES} cells. Dose ratios are plotted for data obtained using 10, 20 and 30μM noscapine and the dotted line represents the isobole

(d) Isobologram for the interaction between vinblastine and noscapine in MCF7 cells. Dose ratios are plotted for data obtained using 10, 20 and 30μM noscapine and the dotted line represents the isobole

TABLES

COMPOUND	CYTOTOXIC POTENCY IC_{50} (μM)	
	MCF7	**NCI/Adr^{RES}**
Noscapine	45.4±6.5 *(13)*	32.1±7.0 *(12)*
(2)	N.E *(5)*	N.E *(4)*
(3)	N.E *(6)*	N.E *(4)*
(4)	5.3±0.9* *(6)*	3.3±0.41* *(5)*
(5)	1.6±0.4* *(11)*	6.4±2.4* *(4)*
(6)	2.5±0.4* *(11)*	3.0±0.6* *(6)*
(7)	14.6±2.5* *(8)*	12.6±2.8* *(7)*
(8)	2.5±0.5* *(11)*	2.1±0.5* *(6)*
(9)	2.3±0.7* *(15)*	2.1±0.5* *(7)*
(10)	4.3±0.7* *(7)*	2.4±0.2* *(7)*
(11)	21.5±7.3* *(6)*	18.3±2.8 *(3)*

Table 1 Potency of noscapine and several chemical derivatives to elicit cytotoxicity in drug sensitive and resistant breast cancer cell lines

The potencies of drugs to inhibit cell proliferation (IC_{50}) were determined using the MTT assay described in the methods. Values (mean±SEM) were obtained by non-linear regression of the general dose-response relationship. The values in parentheses correspond to the number of independent observations. * - indicates a statistically significant difference ($P<0.01$) compared to

the value obtained for noscapine in the specific cell line. N.E indicates that the compound did not generate a cytotoxic response.

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

COMPOUND	*(n)*	POTENCY MP_{50} (μM)	EXTENT A_{MAX} (*r.f.u*)
Nicardipine	*(11)*	2.5±0.6	110±19
Noscapine	*(9)*	8.5±2.1	24.6±6.7
(2)	-	*n.d*	*n.d*
(3)	-	*n.d*	*n.d*
(4)	*(4)*	10.1±2.7	145±33*
(5)	*(4)*	N.E	N.E
(6)	*(6)*	0.37±0.07*	35.1±4.0
(7)	*(4)*	N.E	N.E
(8)	*(5)*	1.2±0.2*	137±30*
(9)	*(8)*	6.9±2.0	67.6±26.0
(10)	*(7)*	3.8±1.0	76.3±28.7
(11)	*(4)*	20.9±5.3**	83.5±21.7

Table 2 The effects of nicardipine and several noscapine derivatives on calcein-AM accumulation in drug sensitive and resistant breast cancer cell lines

The accumulation of calcein in NCI/Adr^{RES} cells was determined from the rate of fluorescence change and plotted as a function of added drug concentration. The secondary plot was used to determine the potency of drugs (MP_{50}) to alter the accumulation of calcein and the maximal rate (A_{MAX}) for this accumulation. The parameters (mean±SEM) were obtained by non-linear regression of a hyperbolic equation. (n) – corresponds to the number of independent observations. * - indicates a statistically significant difference ($P<0.05$) compared to the value

for noscapine and ** indicates P<0.01. N.E indicates that the compound did not alter calcein-AM accumulation and *n.d*. indicates that no values were determined.

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

COMPOUND	*(n)*	POTENCY *EC_{50} (μM)*	STIMULATION *fold basal*
Nicardipine	*(6)*	2.9±0.5**	5.3±0.5
Noscapine	*(6)*	44.6±9.1	3.4±0.4
(2)	*(3)*	4.1±1.6**	2.5±0.3
(3)	*(2)*	65.3±10.2	4.4±1.3
(4)	*(3)*	0.95±0.28**	2.4±0.4
(5)	*(6)*	18.7±4.5**	3.1±0.3
(6)	*(4)*	1.0±0.4**	1.4±0.9
(7)	*(6)*	19.1±1.4**	2.7±0.7
(8)	*(4)*	10.1±5.3**	2.2±0.3
(9)	*(3)*	22.5±5.8*	2.8±0.6
(10)	*(4)*	N.E	N.E
(11)	*(3)*	N.E	N.E

Table 3 The effects of nicardipine and several noscapine derivatives on the rate of ATP hydrolysis by purified, reconstituted P-gp

The ATPase activity of purified P-gp was measured using a colorimetric assay and plotted as a function of added drug concentration. The general dose-response relationship was fitted to the data to determine the potency to stimulate hydrolysis (EC_{50}) and the extent of stimulation. Values correspond to mean±SEM and (n) corresponds to the number of independent observations. * indicates a statistically significant difference compared to noscapine ($P<0.05$) and ** refers to $P<0.01$. N.E indicates that the compound did not alter ATP hydrolysis by P-gp.

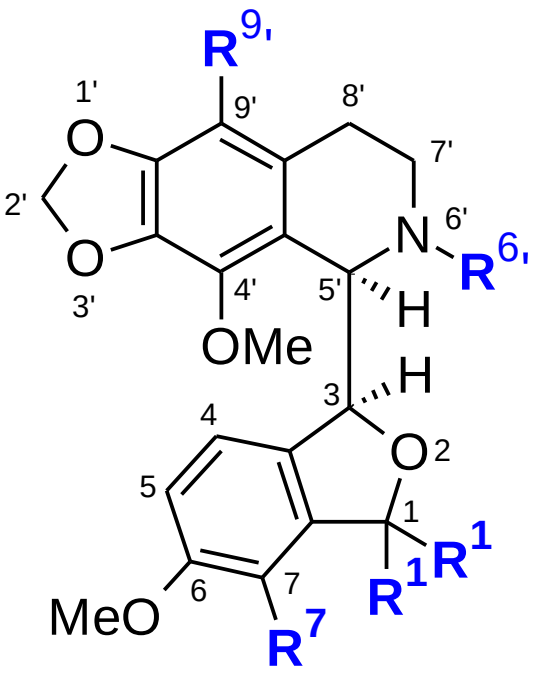
DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.

COMPOUND	Conc (μM)	COMBINATION INDEX MCF7	COMBINATION INDEX NCI/Adr^{RES}
Noscapine	10	0.69±0.02**	1.13±0.362
	20	0.26±0.05**	0.11±0.02*
	30	0.33±0.08**	0.19±0.06*
(**6**)	0.1	1.64±0.33	1.10±0.35
	0.3	1.04±0.46	0.49±0.25
	1	1.11±0.35	0.33±0.15*
	3	0.59±0.02**	0.24±0.04**
(**8**)	0.1	2.57±1.01	2.11±0.89
	0.3	0.62±0.36	0.75±0.22
	1	*n.d.*	0.52±0.27*
	3	*n.d.*	*n.d.*

Table 4 ***Combination index analysis of the cytotoxicity of noscapine derivatives co-administered with vinblastine with in drug sensitive and resistant breast cancer cell lines***

Combination indices (CI) were determined for the effects of co-administration of vinblastine and noscapine derivatives on cell proliferation. Complete dose-response relationships were determined for vinblastine in the absence or presence of the indicated concentrations of noscapine, (**6**) and (**8**). All values (mean±SEM) were obtained from three independent observations. * ($P<0.05$) and ** ($P<0.01$) indicate a statistically significant difference from the isobole, which is defined with CI=1. *n.d.* indicates that no values were determined.

DMD Fast Forward. Published on November 26, 2018 as DOI: 10.1124/dmd.118.083188
This article has not been copyedited and formatted. The final version may differ from this version.



Compounds	cLogP	R^1	R^7	$R^{6'}$	$R^{9'}$
Noscapine (1)	2.81	=O	OMe	Me	H
2	3.85	=O	OMe	**C(O)OtBu**	H
3	3.39	=O	OMe	**C(O)NHEt**	H
4	4.00	=O	OMe	**C(O)NHEt**	**Cl**
5	3.72	=O	**OH**	**C(O)NHEt**	**Cl**
6	4.16	**H,H**	OMe	**C(O)NHPr**	H
7	3.52	**H,H**	OMe	**C(O)NHEt**	H
8	4.12	**H,H**	OMe	**C(O)NHEt**	**Cl**
9	4.25	**H,H**	OMe	**C(O)NHEt**	**Br**
10	4.53	**H,H**	OMe	**C(O)NHEt**	**I**
11	2.93	**H,H**	OMe	**C(O)NHEt**	**NH_2**

Figure 1

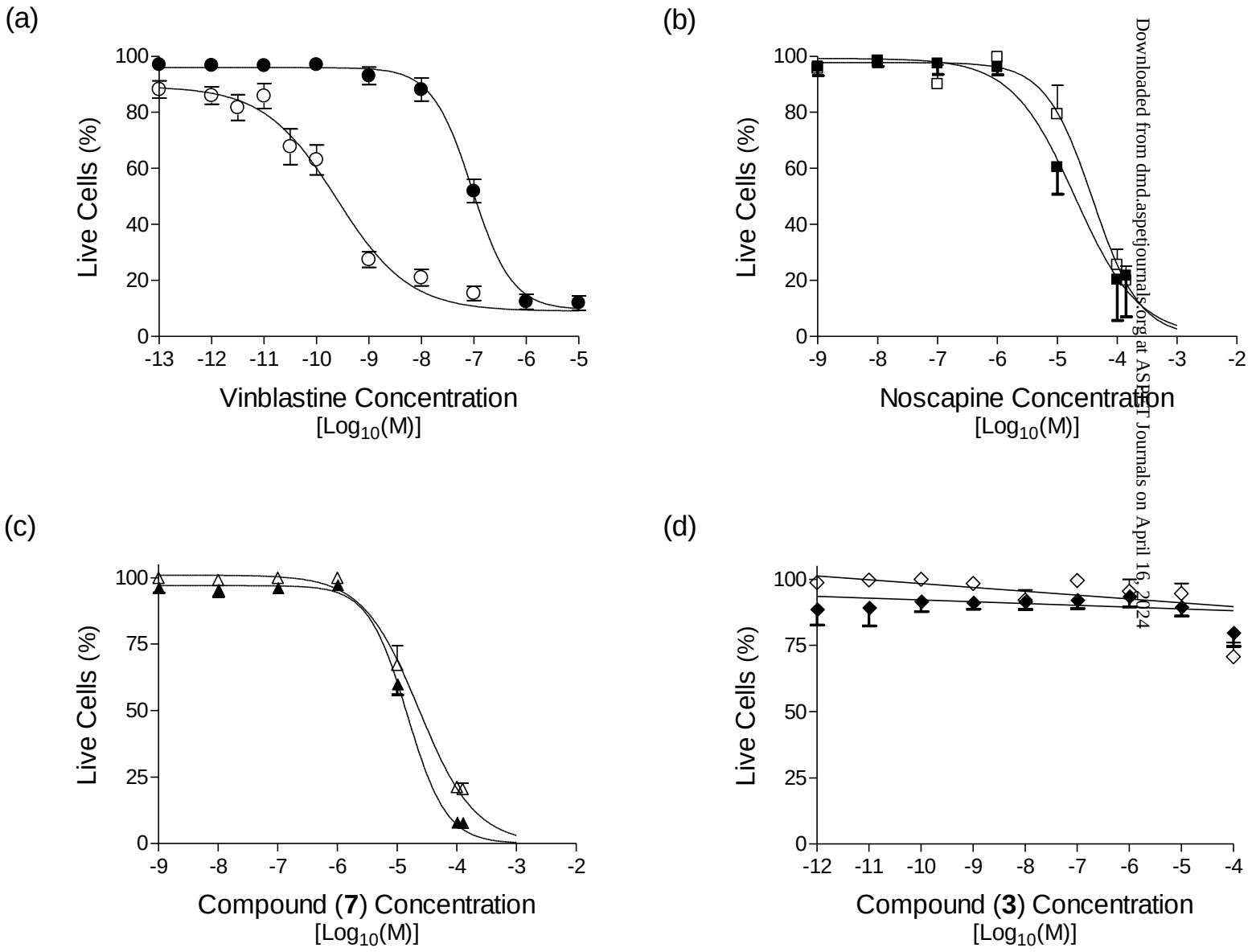


Figure 2

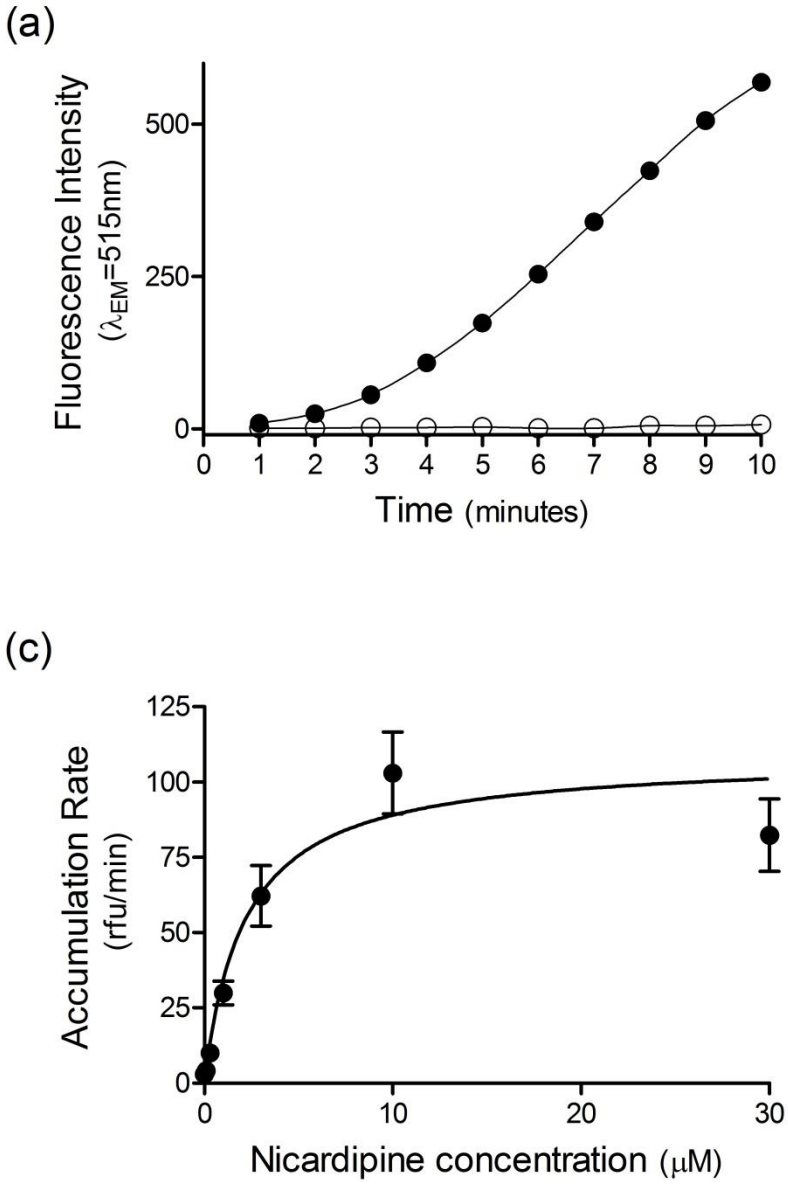


Figure 3

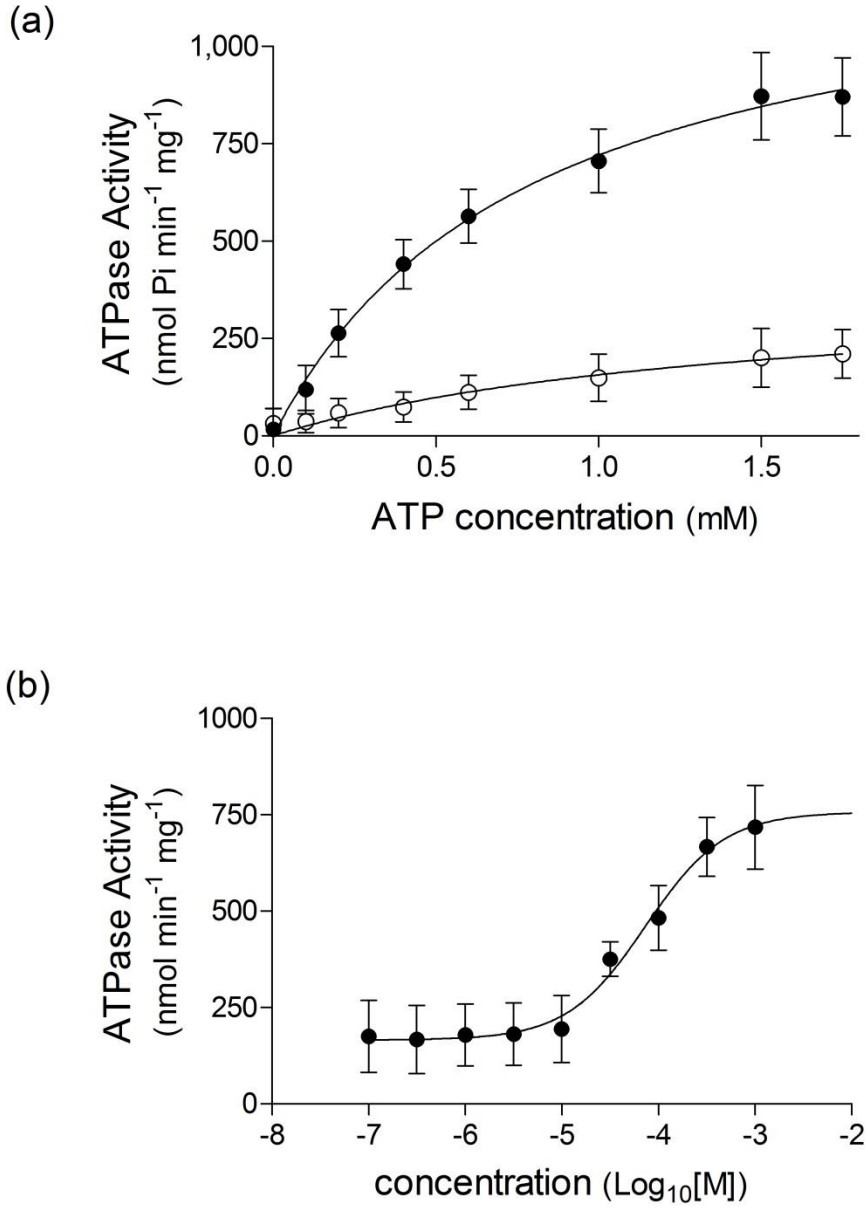


Figure 4

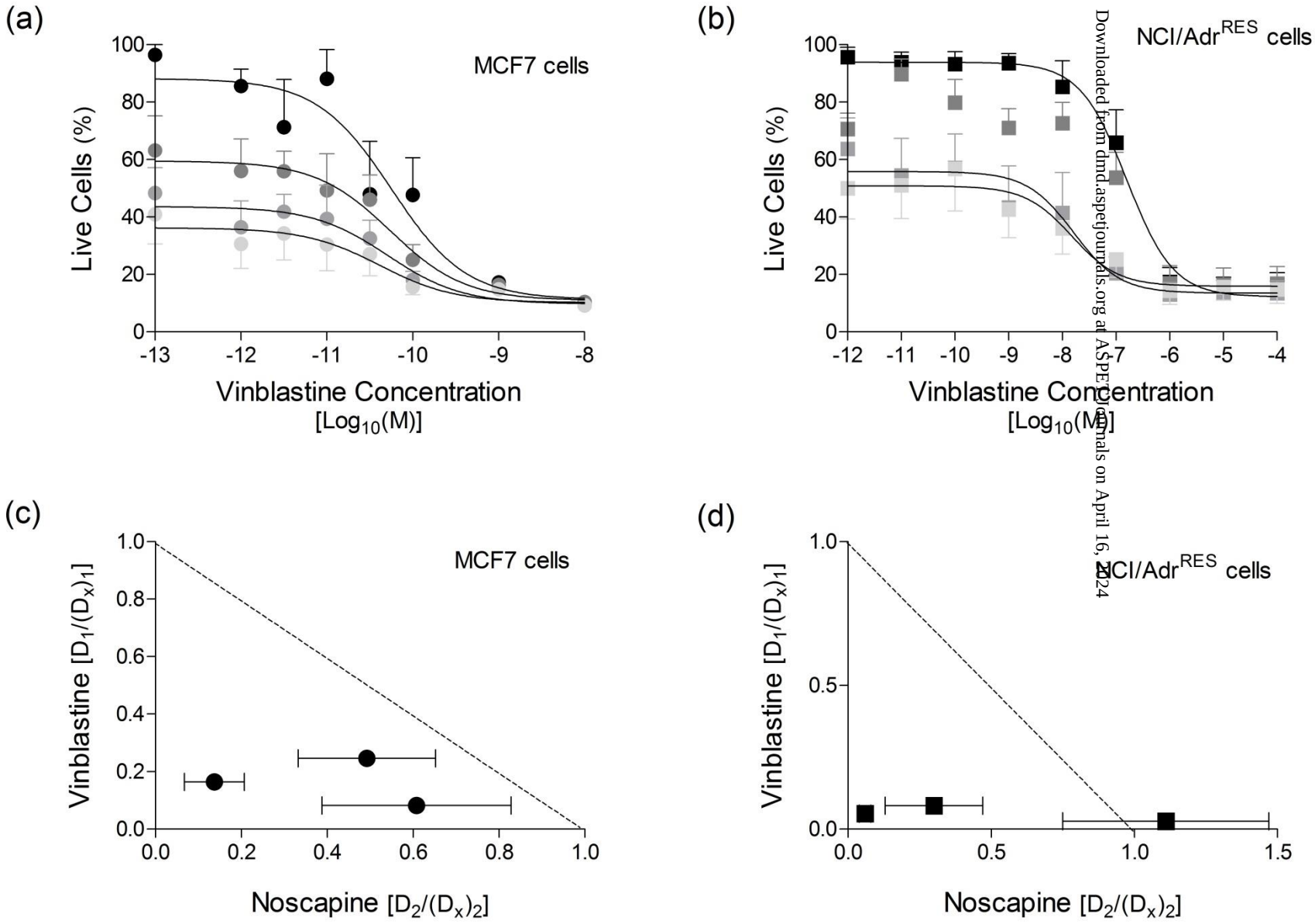


Figure 5