Characterization of the T-cell response in a patient with phenindione hypersensitivity

Authors: Dean J. Naisbitt, John Farrell, Peter J. Chamberlain, Josephine E. Hopkins, Neil G. Berry, Munir Pirmohamed, B. Kevin Park.

Departments: Departments of Pharmacology (DJN, JF, PJC, JEH, MP, BKP) & Chemistry (NGB), The University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, England.

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: The role of T-cells in drug hypersensitivity

Corresponding Author: Dr. Dean J. Naisbitt, Department of Pharmacology and Therapeutics, Sherrington Building, Ashton Street, The University of Liverpool, PO Box 147, Liverpool, L69 3GE, England.

Tele: ++44 151 7945791; FAX: ++44 151 7945540; e-mail: dnes@liv.ac.uk

Text Pages: 28

Tables: 1

Figures: 5

References: 31

Words in Abstract: 178

Words in Introduction: 464

Words in Discussion: 858

Section Assignment: Toxicology

Non-standard abbreviations: none used.

Abstract

The oral anticoagulant phenindione is associated with hypersensitivity reactions in 1.5-3% of patients, the pathogenesis of which is unclear. We describe a patient who developed a severe hypersensitivity reaction that involved both the skin and lungs. A lymphocyte transformation test showed proliferation of T cells from the hypersensitive patient, but not from 4 controls, on exposure to phenindione in vitro. Drug-specific T-cell clones were generated and characterised in terms of their phenotype, functionality, and mechanism of antigen presentation. Forty three-HLA class II restricted CD4+ $\alpha\beta$ T-cell clones were identified. T-cell activation resulted in the secretion of IFN-γ and IL-5. Five out of seven clones proliferated with phenindione alone, while two clones also proliferated with 2-phenylindene. Certain T-cell clones were also stimulated by R- and S-warfarin; computer modelling revealed that warfarin can adopt a phenindione-like structure. Phenindione was presented to T-cells via two pathways: first, bound directly to MHC; and secondly, bound to a processed peptide. Our data show that CD4+ T-cells are involved in the pathophysiology of phenindione hypersensitivity. There may be cross-sensitivity with warfarin in some phenindione hypersensitive patients.

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Phenindione (2-Phenyl-1*H*-indene-1,3(2*H*)-dione), an indanedione derivative, is an oral anticoagulant with similar clinical indications as warfarin. Phenindione was introduced in the early 1950s but its usage has remained low because of its potential to cause hypersensitivity reactions in 1.5-3% of individuals. The skin is the commonest organ to be affected, although occasionally extra-cutaneous manifestations may be predominant. The reactions vary in severity and occasionally can be fatal (Mohamed, 1965; McMenamin et al., 1976). Clinical symptomatology, the delayed onset of the reaction and the demonstration of phenindione-specific lymphocyte proliferation (Potier et al., 1975) are all suggestive of an immune pathogenesis; however, the nature of the immune response and how phenindione is presented in an antigenic form has not been delineated.

Hypersensitivity reactions in general are an important clinical problem. In a recent 6-month prospective study of cutaneous drug reactions, the prevalence was estimated to be 3.6/1000 hospitalised patients (Fiszenson-Albala et al., 2003). Recent studies that have characterised drug-specific T-cell clones from the skin and blood of hypersensitive individuals have led to a better understanding of the cellular pathophysiology (Pichler et al., 2002; Naisbitt, 2004). Interestingly, studies using sulfamethoxazole as a paradigm have shown that drugs can be presented to T-cells in several ways. First, T-cells from experimental animals can proliferate in the presence of a MHC-restricted processed peptide derived from cells haptenated with high levels of a protein-reactive nitroso metabolite (Naisbitt et al., 2001, Naisbitt et al., 2002). These data are consistent with the hapten hypothesis of immune recognition of drugs and chemicals (Landsteiner and Jacobs, 1935). Secondly,

lymphocytes from hypersensitive patients can proliferate in the presence of the nitroso metabolite bound covalently, but directly to the MHC (Schnyder et al., 2000; Burkhart et al., 2001). Direct binding to MHC avoids the requirement for antigen processing. Finally, lymphocytes from the same patients can also proliferate with sulfamethoxazole bound reversibly in a labile fashion to both MHC and the T-cell receptor (Schnyder et al., 1997; Schnyder et al., 2000). Detailed cross reactivity studies have shown that antigen specificity is determined by the avidity of the drug for the MHC T-cell receptor complex and the T-cell receptor density (von Greyerz et al., 2001; Depta et al., 2004). Further studies using structurally unrelated compounds such as lidocaine, mepivacaine, lamotrigine, carbamazepine, phenobarbital and p-phenylenediamine have also shown that specific T-cells can be stimulated through a non-covalent interaction of a drug with the MHC and the T-cell receptor (Zanni et al., 1998; Zanni et al., 1999; Hashizume et al., 2002; Naisbitt et al., 2003a, 2003b).

In this study, we describe a patient who developed a severe hypersensitivity reaction to phenindione. We have investigated the pathophysiology of the hypersensitivity reaction in this patient by defining the phenotype and functionality of phenindione-specific T-cells and by using T-cell clones we have explored drug recognition by MHC-restricted T-cell receptors.

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Patient case summary

The patient, a 46-year-old woman, developed a deep venous thrombosis, and was prescribed warfarin. However, she did not respond to warfarin, and was therefore changed to phenindione, with which she managed to become therapeutically anticoagulated. Thirty days after starting phenindione, the patient developed a maculopapular rash on her legs. This was not initially suspected to be due to phenindione, which was continued until the patient was admitted to hospital 13 days later, at which time her symptoms had progressed. At the time of hospital admission, the patient was found to have a generalised maculopapular erythematous rash associated with a temperature of 39^0C, generalised lymphadenopathy, mild jaundice (peak bilirubin 35μmol/l, normal range <17), and bilateral patchy consolidation on the chest X-ray. Her eosinophil count was elevated at 2.5 x 10^9/l (normal range 0-0.4), while alanine aminotransferase level peaked at 1115 U/l (normal range <35). On admission, phenindione was stopped, although the diagnosis was unclear at that time. Non-drug induced causes of persistent eosinophilia and pulmonary symptoms were excluded by investigations (including stools for ova and parasites, viral and parasite serology, autoantibodies, skin biopsy, liver ultrasound, CT scan of lungs and pulmonary function tests). A sepsis screen, included repeated blood cultures, was found to be negative. The patient did not have a history of atopy. On the basis of the above, a clinical diagnosis of phenindione hypersensitivity was made. The patient was also treated with prednisolone after which she improved slowly over the next 6 weeks, during which time the steroids were gradually withdrawn.

Blood was obtained from the patient 3 weeks after complete resolution of her symptoms and 6 weeks after stopping prednisolone. Blood was also obtained from

four phenindione exposed non-allergic controls. Lymphocytes were obtained by centrifugation of blood layered onto Lymphoprep®. Approval for the study was obtained from the Liverpool local research ethics committee and informed consent was obtained from each participant.

Culture medium and chemicals

Culture medium consisted of RPMI-1640 supplemented with 10% pooled heat-inactivated human AB serum, HEPES buffer (25 mM), L-glutamine (2 mM), transferrin (25 μg ml^{-1}), streptomycin (100 μg ml^{-1}) and penicillin (100 U ml^{-1}). The above reagents were obtained from Sigma Chemical Co. (Poole, Dorset, UK). For culture of the T-cell clones the media was enriched with human recombinant IL-2 (60 U ml^{-1}; PeproTech EC Ltd, London, UK).

An Epstein-Barr virus-transformed B-lymphoblastoid cell line, for use as an autologous population of antigen presenting cells, was generated from the peripheral blood of the drug allergic donor by transformation of B-cells with supernatant from the Epstein-Barr virus producing cell line B9-58 (obtained from Dr. D. Neumann-Haefelin, University of Freiburg, Freiburg, Germany). Transformed cells were cultured in RPMI-1640 supplemented with 10% foetal calf serum, HEPES buffer (25 mM) and L-glutamine (2 mM).

Phenindione, indan, indanone, 1,3 indandione, 2-phenylindene, 2-phenylindole, 3-iminoisoindolinone and S- and R-enantiomers of warfarin were obtained from Sigma Chemical Company (Poole, Dorset, UK). For T cell culture, drugs were used at concentrations that did not inhibit the T-cell proliferative response to the mitogen phytohemagglutinin (1 μg ml^{-1}). Stock solutions (10 mg ml^{-1}) were prepared in a

mixture of culture media and DMSO (4:1 v/v) and diluted before use. All general reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK) and were of the best available grade.

Lymphocyte proliferation with phenindione

Proliferation of lymphocytes from the allergic patient and controls was measured using the lymphocyte transformation test, as described previously (Nyfeler and Pichler, 1997). Briefly, freshly isolated lymphocytes (1.5 x 10^5; total volume 0.2 ml) were incubated with phenindione (1–500 μg ml^{-1}) or tetanus toxoid (0.1μg ml^{-1}; positive control) in 96 well U-bottomed tissue culture plates for 6 days (37^{o}C; 5% CO_2). Proliferation was determined by the addition of [^{3}H] thymidine (0.5 μCi) for the final 16 h of the incubation period. Proliferative responses were calculated as stimulation indices (SI; cpm in drug-treated cultures / cpm in cultures with DMSO alone).

Generation and characterization of phenindione-specific T-cell clones

Lymphocytes (2 x 10^6; total volume 1 ml) from the phenindione allergic patient were incubated with phenindione (10 - 50μg ml^{-1}). On day 6 and 9, IL-2 was added to maintain antigen-specific proliferation. After 14 days, T-cells were cloned by serial dilution as described previously (Schnyder et al., 1997). To test the specificity of the clones (28 days after serial dilution), T-cells (0.5 x 10^5; total volume 0.2 ml) were incubated with autologous irradiated (60 Gy) antigen presenting cells (0.1 x 10^5) and phenindione (10 and 50 μg ml^{-1}). After 48 h, [^{3}H] thymidine was added and proliferation was measured by scintillation counting as described above. T-cell clones with an SI of greater than or equal to 2.5 were restimulated and expanded in IL-2 containing medium.

Phenindione-specific T-cell clones were characterized in terms of CD phenotype and T-cell receptor Vβ expression by flow cytometry. MHC-restriction was determined by the addition of specific anti-HLA blocking antibodies (anti-class 1, anti-DR, anti-DP and anti-DQ [all obtained from Dr. E. Padovan, University of Basel, Switzerland]) to the proliferation assay at concentrations known to inhibit MHC-restricted stimulations of T-cell clones. To exclude self-presentation by HLA-positive T cells or presentation in the absence of additional antigen presenting cells, certain incubations contained T-cells and phenindione in the absence of antigen presenting cells. The functionality of the T-cell clones was investigated by measurement of phenindione-specific (1–500 μg ml^{-1}) proliferation by thymidine incorporation and cytokine secretion by ELISA. Cell cultures containing T-cells and antigen presenting cells in the absence of phenindione was taken as a control. The following ELISA kits were used: IL-4, IL-10, IFN-γ (Diaclone, Besancon, France) and IL-5 (PharMingen, San Diego, California, USA). The detection limits were 1 pg ml^{-1} for IL-4, 8 pg ml^{-1} for IL-5 and 13 pg ml^{-1} for IL-10 and IFN-γ.

Determination of the specificity of the interaction of phenindione with MHC and the T-cell receptor

To investigate the fine specificity of the interaction between phenindione and the T-cell receptor, T-cell clones were incubated with antigen presenting cells and phenindione (50 – 250 μg ml^{-1}) or 6 structurally related compounds (all 50 – 250 μg ml^{-1}). Chemicals were selected based on a structure disconnection approach, removing the major structural motifs of phenindione, namely the benzene ring (1,3-indandione), the ketone groups (2-phenylindene) or both the benzene ring and ketone groups (indan). In addition, R- and S-enantomers of warfarin (50-250μg ml^{-1})

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

were added to the proliferation assay. Proliferation was determined after 48 h by assessment of [^{3}H] thymidine incorporation, as described above.

Generation of 3-dimensional energy minimized molecular models of phenindione and warfarin

3-Dimensional structure building and molecular modelling studies were performed using SYBYL 6.91 (St. Louis, USA) installed on a PC workstation with Red Hat Linux 7.3 operating system (http://www.redhat.com). Atomic charges were calculated by the method of Gasteiger and Marsili (Gasteiger and Marsili, 1981). Energy minimizations were performed using Tripos force field with the default force field settings and charges with distance dependent dielectric and conjugate gradient method with a convergence criterion of 0.01kcal mol^{-1} (Clark et al., 1989). The molecules were overlaid by performing a least squares fit between pairs of atoms of two molecules. Phenindione was used as the reference and the two enantiomers of warfarin were translated so that the centroid of the atoms to be fitted was superimposed on the centroid of the corresponding atoms in phenindione. The warfarin enantiomers were then rotated about this point to obtain the best possible fit between the specified pairs of atoms (Nyburg, 1974). The atoms used to match the structures are shown by asterisks in Fig 4.

Determination of the mechanism of phenindione presentation to T-cells

The role of processing in phenindione presentation was determined using previously described methodology (Schnyder et al., 1997; Zanni et al., 1998): first, by chemical fixation of antigen presenting cells; and secondly, by measurement of the kinetics of T-cell receptor internalisation, which is an early parameter of antigen T-cell receptor engagement. Antigen presenting cells (2 x 10^6; 0.5 ml) were fixed by the addition of

glutaraldehyde (2 %; 12.5 μl) for 30 sec at room temperature. To determine the kinetics of T-cell receptor internalisation, T-cell clones (5×10^4; total volume 0.2 ml) and antigen presenting cells (2.5×10^4) were incubated with phenindione (10 and 50 μg ml^{-1}) in 96 well U-bottomed tissue culture plates. After 1, 4 and 16 h, cells were harvested, washed and stained for 30 min at 4^0C with a FITC-labelled anti-CD3 mAb (Serotec Ltd., Oxford, UK). CD3 fluorescence was measured by flow cytometry (Coulter Epics, XL software; Beckman Coulter, Luton, UK). 100% values represent the CD3 fluorescence of T-cells incubated with antigen presenting cells in the absence of phenindione.

Statistical Analysis

Values to be compared were initially analyzed for non-normality by the Shapiro-Wilks test. Since the data were found to be non-normally distributed, we used the Mann-Whitney test for comparison of the two groups, accepting $P < 0.05$ as significant.

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Lymphocyte proliferation with phenindione

Lymphocytes from the phenindione allergic patient proliferated vigorously in vitro in the presence of phenindione and the positive control tetanus toxoid (Fig. 1). Concentration-dependent proliferation was observed at 10 – 500 μg ml^{-1} phenindione (maximum SI, 141.6; 25 μg ml^{-1} phenindione). Lymphocytes from phenindione exposed non-allergic control patients proliferated with tetanus toxoid (SI 15.1 – 38.4; 0.1 μg ml^{-1} tetanus toxoid), but not with phenindione (maximum SI did not exceed 1.5).

Phenotype and functionality of phenindione-specific T-cell clones

In preliminary experiments, phenindione (10 and 50 μg ml^{-1}) specific proliferation of over 600 serially diluted T-cell cultures generated from the phenindione allergic patient was measured by the addition of [^{3}H] thymidine. Forty-three cultures proliferated in the presence of phenindione (mean SI 5.0 ± 5.4 [10 μg ml^{-1}], 10.5 ± 6.1 [50 μg ml^{-1}]; cpm 407.5 ± 253 [control]; $P<0.0001$ at both concentrations); 12 were randomly selected for further analysis. Monoclonality was assessed by flow cytometric determination of T-cell receptor Vβ expression. All cultures expressed a single, but differing, T-cell receptor Vβ chain and are therefore, from this point onwards, referred to as clones (Table 1). All of the T-cell clones were CD4+.

Proliferation of phenindione-specific T-cell clones was concentration-dependent; maximal proliferation was observed with 25 – 100 μg ml^{-1} phenindione. Concentrations of 250 μg ml^{-1} and above inhibited proliferation. No significant proliferation was seen when T-cell clones were incubated with phenindione in the

absence of antigen presenting cells. Blocking experiments with antibodies against HLA-DR, HLA-DQ, HLA-DP and HLA-class I showed that phenindione was presented on HLA-DR (5/7 clones) or HLA-DQ (2/7 clones). MHC restriction was noted when the proliferative response in the presence of blocking antibody was less than 80% of that seen with drug alone. Analysis of cytokines secreted (IL-4, IL-5, IL-10 and IFN-γ) from eight phenindione-stimulated T-cell clones revealed a mixed cytokine secretion profile. Moderate to high levels of IFN-γ (9189 ± 11001 pg ml^{-1}; range 1024 - 23980), was secreted by five clones, four clones secreted IL-5 (3459 ± 4009 pg ml^{-1}; range 642 - 9364) and one clone secreted IL-4 (4914 pg ml^{-1}). The phenotype and functionality of eight phenindione-specific T-cell clones is summarized in table 1.

Proliferation of T-cell clones with phenindione-related structures and generation of energy minimized 3-dimensional molecular models

Phenindione-specific T-cell clones were tested for their ability to proliferate in the presence of several structurally related chemicals (Fig. 2). Five out of the seven clones were highly specific and proliferated in the presence of phenindione alone. Two clones proliferated with phenindione and weakly in the presence of 2-phenylindene; further structural modification completely inhibited the proliferative response (Fig. 2).

To study potential cross reactivity of phenindione-specific T-cells with warfarin, eight T-cell clones were incubated with phenindione or R- and S-enantiomers of warfarin and antigen-specific proliferation measured. Four T-cell clones proliferated with phenindione alone, three T-cell clones proliferated with phenindione and S-warfarin

and one T-cell clone proliferated with all three compounds (Fig. 3). A stimulation index of 3 or greater was considered significant. Energy minimized models of phenindione (in the preferred enol confirmation) (Pipkin and Stella, 1982) and R- and S-warfarin show significant differences in their preferred spatial arrangement (Fig. 4A). By forcing R- and S-warfarin to take up a similar 3D disposition of groups as phenindione, then energy minimising, it was possible to obtain structures with a similar spatial arrangement (Fig. 4B). The energy required to generate the conformers of R-warfarin and S-warfarin is 0.056kcal mol^{-1} and 1.437kcal mol^{-1} higher than that of the global minimum, respectively.

Processing-dependent and independent presentation of phenindione to T-cells

The role of antigen processing in phenindione presentation to specific T-cell clones was investigated initially by fixing antigen presenting cells with glutaraldehyde. Fixed antigen presenting cells express MHC but are incapable of processing. Phenindione was presented to six T-cell clones by fixed antigen presenting cells (Fig. 5A). However, fixation of antigen presenting cells inhibited phenindione-specific proliferation of six T-cell clones. These data indicate that phenindione is presented to T-cells via two pathways, one dependent, and the other independent of processing. To confirm these data, the kinetics of T-cell receptor internalisation of two T-cell clones (one potentially processing dependent clone [clone id 21] and one potentially processing independent clone [clone id 59]) was monitored by flow cytometry following phenindione stimulation. Fast (less than 1h) and slow (4-16h) internalisation of T-cell receptors following antigen stimulation is indicative of processing independent and processing dependent drug presentation, respectively (Zanni et al., 1998). A significant down regulation in T-cell receptor expression was observed when both T-cell clones were incubated in the presence of phenindione and antigen

presenting cells (Fig. 5B). For T-cell clone 59, down regulation in T-cell receptor expression occurred within 1h. The extent of T-cell receptor internalisation reached a plateau after 4h. In contrast, addition of phenindione to T-cell clone 21 did not alter T-cell receptor expression between 0-4h. However, significant T-cell receptor down regulation was observed after 16h, the time taken for antigen processing.

Discussion

Phenindione hypersensitivity is characterised by delayed onset of cutaneous eruptions following primary drug exposure and rapid onset following drug re-exposure, (Mohamed, 1965; McMenamin et al., 1976). These features together with the detection of phenindione-specific lymphocyte proliferation in vitro (Potier et al., 1975), suggest an immune-mediated pathogenesis. Our patient developed a severe maculopapular erythematous rash and eosinophilic pneumonia after 4 weeks treatment, which necessitated drug withdrawal. Taken together with the fact that the patient was not on any other drugs, and that non-drug induced disease was excluded, this was consistent with phenindione being the cause of her symptoms and signs.

The lymphocyte proliferation assay, which has previously been utilised to study hypersensitivity reactions to carbamazepine, lamotrigine and sulfamethoxazole (Schnyder et al., 2000; Naisbitt et al., 2003a, 2003b; Farrell et al., 2003), was again used in this patient to help in making a diagnosis and investigating the pathogenesis. In accordance with the clinical features, lymphocytes from the phenindione hypersensitive patient, but not from phenindione exposed non-hypersensitive controls, proliferated extremely vigorously at concentrations within the therapeutic range (6-29$\mu g\ ml^{-1}$) (Schulert and Weiner, 1954). The phenindione-specific T-cells were CD4+, and T cell activation required drug presentation in the context of MHC class II (both HLA DR and DQ, but not HLA DP), but not class I, molecules. T-cell receptor activation also resulted in the secretion of high levels of the pro-inflammatory cytokine IFN-γ, and moderate-to-low levels of IL-5 (from four T-cell clones), and IL-4 (from one T-cell clone), but not the regulatory cytokine IL-10. The cytokine profile seen in this patient provides some insights into the

immunopathogenesis of this hypersensitivity reaction. First, the secretion of IFN-γ is a common feature of T-cells from drug-induced maculopapular skin eruptions in the presence or absence of systemic symptoms (Yalwalkar and Pichler, 2001; Pichler et al., 2002). IFN-γ is known to up-regulate MHC expression on keratinocytes, rendering them more susceptible to FAS or perforin-mediated T-cell killing (Schnyder et al., 1998; Kuechler et al., 2004). Second, the secretion of IL-5 by some of the T cell clones is consistent with the clinical manifestation of eosinophilic pneumonia in this patient. Indeed, it has been suggested that IL-5 may be involved in the pathogenesis of eosinophilic pneumonia (Allen et al 1996). However, IL-5 is also important for cutaneous and peripheral blood eosinophilia in the absence of any lung involvement (Hashizume et al., 2002; Yawalkar et al., 2000; Yawalkar et al., 2000b). Indeed, IL-5 production by cultured T-cells has been suggested as a possible method to diagnose drug hypersensitivity (Sachs *et al*. 2002). Based on the above, it can be surmised that the phenindione hypersensitivity described in this patient is an example of a type IV hypersensitivity reaction, according to the original classification proposed by Gell and Coombs. This can probably be further sub-divided into types IVa and IVb, according to the recently modified classification proposed by Pichler (2003).

In order to gain insight into the chemistry of drug recognition by T-cells, we undertook detailed cross reactivity studies. Modification of the phenindione structure revealed that both aryl groups and the ketone contribute to the recognition of phenindione. To explore the 3-dimensional aspects of drug recognition, we investigated the enantiomers of warfarin because phenindione can tautomerize to yield an enol form (Pipkin and Stella, 1982), which more closely resembles warfarin. Although some clones only recognised phenindione, three clones proliferated with phenindione and S-warfarin and one clone proliferated in the presence of phenindione and both

enantiomers of warfarin (Fig. 3). *In silico* energy minimised molecular models revealed that both the R- and S-enantiomers of warfarin can adopt conformers with a spatial arrangement similar to phenindione thus providing an explanation for the cross reactivity of certain clones with the two drugs. These data indicate that warfarin administration to phenindione allergic patients could lead to hypersensitivity. However, we are not aware of any case reports of this in the literature. Nevertheless, it is interesting to note that warfarin by itself can cause cutaneous eruptions, with rapid recurrence on re-exposure, which is consistent with an immune-mediated pathogenesis (Spyropoulos et al., 2003). Whether the converse occurs, i.e. the development of cross-sensitivity with phenindione following an initial reaction to warfarin, is unclear, but may be more important in the clinical setting given the widespread use of warfarin.

Finally, we analysed the role of antigen processing in phenindione presentation to T-cells. Glutaraldehyde-fixed antigen presenting cells present pre-processed antigens, but not antigens that require antigen processing (Zanni et al., 1998). In keeping with previous studies of drugs such as sulfamethoxazole, lidocaine, carbamazepine, and lamotrigine (Schnyder et al., 2000; Zanni et al., 1998; Naisbitt et al., 2003a, 2003b), certain phenindione-specific T-cell clones proliferated in the presence of phenindione bound directly to MHC in the absence of antigen processing (Fig. 5A), while others required antigen processing for T-cell receptor activation. These data were confirmed by evaluation of the kinetics of T-cell receptor internalisation, an early measure of T-cell receptor activation (Fig. 5B). Thus, internalisation of T-cell receptors was either rapid signifying processing-independent drug presentation (Zanni et al., 1998) or did not occur until 16h, the time required for antigen processing of haptenated proteins (Brander et al., 1995). There are two possible scenarios that may result in processing

dependent phenindione-specific T-cell activation: first, phenindione may be metabolised in vitro to a protein-reactive intermediate that binds covalently to cellular proteins. This possibility is difficult to explore since the metabolism of phenindione in liver cells in vitro and in patients has not been defined and mass spectrometric analysis of cell culture supernatant was not sufficiently sensitive to identify a protein reactive species derived from phenindione (results not shown). Secondly, phenindione is unstable in light and air; major oxidation products are 3-aryl-4-hydroxyisocoumarin and the dimer 2,2'diaryl-[2,2'-biindan]-1,1',3,3'-tetraone (De Vries et al., 1977). In addition, phenindione is converted to a light rearrangement product 3-benzylidenephthalide, which has the potential to covalently modify lysine rich protein (Bundgaard, 1975). Thus, T-cells may be exposed to both parent drug and a covalently modified protein within the same in vitro system. The advent of proteomic technology may assist identification of the nature and origin of the modified processed peptide presented to T-cells.

In conclusion, we have characterised T-cells in a patient with phenindione hypersensitivity. Phenindione can be presented *in vitro* in an MHC class II restricted fashion to CD4+ T-cells, via two mechanisms, one dependent, and the other independent of antigen processing. T-cell receptor activation resulted in proliferation and the secretion of IFN-γ and IL-5, consistent with the involvement of both the skin and lungs in this patient. Stimulation of certain phenindione-specific T-cell clones with warfarin suggests that warfarin administration to phenindione hypersensitive patients could lead to the development of cross-sensitivity and the occurrence of a hypersensitivity reaction to warfarin.

Acknowledgements

The authors would like to thank the Departments of Immunology and Tropical Medicine (The University of Liverpool) for use of equipment. Thanks are also extended to all blood donors.

References

Allen JN, Liao Z, Wewers MD, Altenberger EA, Moore SA and Allen ED (1996) Detection of IL-5 and IL-1 receptor antagonist in bronchoalveolar lavage fluid in acute eosinophilic pneumonia. *J Allergy Clin Immunol* 97:1366-1374.

Brander C, Mauri-Hellweg D, Bettens F, Rolli H, Goldman M and Pichler WJ (1995) Heterogeneous T-cell responses to beta-lactam-modified self structures are observed in penicillin-allergic individuals. *J Immunol* 155:2670-2678.

Bundgaard H (1975) Identification and quantitation of a 3-benzylidenephthalide contaminant of phenindione tablets and its characterization as a potentially immunogenic substance. *Acta Pharm Suec* 12:333-348.

Burkhart C, von Greyerz S, Depta JP, Naisbitt DJ, Britschgi M, Park KB and Pichler WJ (2001) Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells. *Br J Pharmacol* 132:623-630.

Clark M, Cramer RD and Van Opdenbosh N (1989) Validation of the general-purpose TRIPOS 5.2 force-field. *J Comput Chem* 10:982-1012.

Depta JP, Altznauer F, Gamerdinger K, Burkhart C, Weltzien HU and Pichler WJ (2004) Drug interaction with T-cell receptors: T-cell receptor density determines degree of cross-reactivity. *J Allergy Clin Immunol* 113:519-527.

De Vries J, Verboom CN, De Neef JH, Groot EJ and Nauta WT (1977) High pressure liquid chromatographic separation of 2-phenyl-1,3-indandione and its oxidation and rearrangement products. *J Chromatogr* 152:557-559.

Farrell J, Naisbitt DJ, Drummond NS, Depta JP, Vilar FJ, Pirmohamed M and Park BK (2003) Characterization of sulfamethoxazole and sulfamethoxazole metabolite-specific T-cell responses in animals and humans. *J Pharmacol Exp Ther* 306:229-237.

Fiszenson-Albala F, Auzerie V, Mahe E, Farinotti R, Durand-Stocco Crickx B and Descamps (2003) A 6-month prospective survey of cutaneous drug reactions in a hospital setting. *Br J Dermatol* 149:1018-1022.

Gasteiger J and Marsili M (1981) Prediction of proton magnetic-resonance shifts – the dependence on hydrogen charges obtained by iterative partial equalization of orbital electronegativity. *Organ Magn Reson* 15:353-360.

Hashizume H, Takigawa M and Tokura Y (2002) Characterization of drug-specific T cells in phenobarbital-induced eruption. *J Immunol* 168:5359-5368.

Kuechler PC, Britschgi M, Schmid S, Hari Y, Grabscheid B and Pichler WJ (2004) Cytotoxic mechanisms in different forms of T-cell-mediated drug allergies. *Allergy* 59:613-622.

Landsteiner K and Jacobs J (1935) Studies on the sensitization of animals with simple chemical compounds. *J Exp Med* 61:643-656.

McMenamin RA, Davies LM and Craswell PW (1976) Drug induced interstitial nephritis, hepatitis and exfoliative dermatitis. *Aust N Z J Med* 6:583-587.

Mohamed SD (1965) Sensitivity reaction to phenindione with urticaria, hepatitis, and pancytopenia. *Br Med J* 5476:1475-1476.

Naisbitt DJ, Gordon SF, Pirmohamed M, Burkhart C, Cribb AE, Pichler WJ and Park BK (2001) Antigenicity and immunogenicity of sulphamethoxazole: demonstration of metabolism-dependent haptenation and T-cell proliferation in vivo. *Br J Pharmacol* 133:295-305.

Naisbitt DJ, Farrell J, Gordon SF, Maggs JL, Burkhart C, Pichler WJ, Pirmohamed M, and Park BK (2002) Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol* 62:628-637.

Naisbitt DJ, Britschgi M, Wong G, Farrell J, Depta JP, Chadwick DW, Pichler WJ, Pirmohamed M and Park BK (2003a) Hypersensitivity reactions to carbamazepine: characterization of the specificity, phenotype, and cytokine profile of drug-specific T cell clones. *Mol Pharmacol* 63:732-741.

Naisbitt DJ, Farrell J, Wong G, Depta JP, Dodd CC, Hopkins JE, Gibney CA, Chadwick DW, Pichler WJ, Pirmohamed M and Park BK (2003b) Characterization of drug-specific T cells in lamotrigine hypersensitivity. *J Allergy Clin Immunol* 111:1393-1403.

Naisbitt DJ (2004) Drug hypersensitivity reactions in skin: understanding mechanisms and the development of diagnostic and predictive tests. *Toxicology* 194:179-196.

Nyburg SC (1974) Some uses of a best molecular fit routine. *Acta Cryst* B30:251-253.

Nyfeler B and Pichler WJ (1997) The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Allergy* 27:175-181.

Pichler WJ, Yawalkar N, Britschgi M, Depta J, Strasser I, Schmid S, Kuechler P and Naisbitt D (2002) Cellular and molecular pathophysiology of cutaneous drug reactions. *Am J Clin Dermatol* 3:229-238.

Pichler WJ (2003) Delayed drug hypersensitivity reactions. *Ann Intern Med* 139:683-993.

Pipkin JD and Stella VJ (1982) Tautomerism of phenindione, 2-phenyl-1,3-indandione, in dipolar aprotic hydrocarbon solvent mixtures. *J Am Chem Soc* 104: 672-680.

Potier JC, Bories H, Foucault JP and Olivier J (1975) Letter: A new case of severe intolerance to phenindione. Value of the lymphoblastic transformation test. *Nouv Presse Med* 4:2041.

Sachs B, Erdmann S, Malte Baron J, Neis M, al Masaoudi T and Merk HF (2002) Determination of interleukin-5 secretion from drug-specific activated ex vivo peripheral blood mononuclear cells as a test system for the in vitro detection of drug sensitization. *Clin Exp Allergy* 32:736-744.

Schnyder B, Mauri-Hellweg D, Zanni M, Bettens F and Pichler WJ (1997) Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest* 100:136-141.

Schnyder B, Frutig K, Mauri-Hellweg D, Limat A, Yawalkar N and Pichler WJ (1998) T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazole-induced skin reaction. *Clin Exp Allergy* 28:1412-1417.

Schnyder B, Burkhart C, Schnyder-Frutig K, von Greyerz S, Naisbitt DJ, Pirmohamed M, Park BK and Pichler WJ (2000) Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals. *J Immunol* 164:6647-6654.

Schulert AR and Weiner M (1954) The physiologic disposition of phenylindanedione in man. *J Pharmacol Exp Ther* 110:451-457.

Spyropoulos AC, Hayth KA and Jenkins P (2003) Anticoagulation with anisindione in a patient with a warfarin-induced skin eruption. *Pharmacotherapy* 23:533-536.

von Greyerz S, Bultemann G, Schnyder K, Burkhart C, Lotti B, Hari Y and Pichler WJ (2001) Degeneracy and additional alloreactivity of drug-specific human alpha beta(+) T cell clones. *Int Immunol* 13:877-885.

Yawalkar N and Pichler WJ (2001) Pathogenesis of drug-induced exanthema. *Int Arch Allergy Immunol* 124:336-338.

Yawalkar N, Egli F, Hari Y, Nievergelt H, Braathen LR and Pichler WJ (2000a) Infiltration of cytotoxic T cells in drug-induced cutaneous eruptions. *Clin Exp Allergy* 30:847-855.

Yawalkar N, Shrikhande M, Hari Y, Nievergelt H, Braathen LR and Pichler WJ (2000b) Evidence for a role for IL-5 and eotaxin in activating and recruiting eosinophils in drug-induced cutaneous eruptions. *J Allergy Clin Immunol* 106:1171-1176.

Zanni MP, von Greyerz S, Schnyder B, Brander KA, Frutig K, Hari Y, Valitutti S and Pichler WJ (1998) HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *J Clin Invest* 102:1591-1598.

Zanni MP, von Greyerz S, Hari Y, Schnyder B and Pichler WJ (1999) Recognition of local anesthetics by alphabeta+ T cells. *J Invest Dermatol* 112:197-204.

Footnotes

These studies were funded by the Wellcome Trust. DJN was a Wellcome Trust Research Career Development Fellow (Jan 2000 – Dec 2003).

The data has previously been presented in part as a poster communication [Naisbitt DJ, Farrell J, Chamberlain PJ, Park BK, Pirmohamed M. Characterization of T-cell responses in phenindione hypersensitivity. Br. J. Clin. Pharmacol. 57;675:2004] at the British Pharmacological Society meeting, London, UK, December 2003.

Send reprint requests to: Dr Dean J. Naisbitt, Department of Pharmacology, Sherrington Building, Ashton Street, The University of Liverpool, P.O. Box 147, Liverpool, UK, L69 3BX

Legends for Figures

Fig. 1. Phenindione-specific lymphocyte proliferation in vitro. Results are expressed as the mean of triplicate incubations. Statistical analysis was performed by comparing incubations in the presence of drug with those in the absence of drug ($^{*}P < 0.05$). The co-efficient of variation was consistently less than 20%.

Fig. 2. Stimulation of T-cell clones with phenindione and phenindione related structures. Results are given as mean [^{3}H] thymidine incorporation of triplicate cultures. The co-efficient of variation was consistently less than 20%. A stimulation index of 3 or greater was considered significant.

Fig. 3. Stimulation of T-cell clones with phenindione and R- and S-enantomers of warfarin. Results are given as mean [^{3}H] thymidine incorporation of triplicate cultures. The co-efficient of variation was consistently less than 20%. A stimulation index of 3 or greater was considered significant.

Fig. 4. In silico modelling of phenindione and warfarin. (a) Generation of energy minimized molecular model of phenindione in its enol form (blue) overlaid with r-(red) and s-warfarin (green) enantomers. (b) Phenindione (blue) and r-(red) or s-(green) warfarin enantiomers overlaid by forcing warfarin to take up a similar 3D disposition of groups as phenindione. Atoms used to match the structures of phenindione and warfarin are shown by asterisks.

Fig. 5. Phenindione is presented to T-cell clones, via two mechanisms, one dependent, and the other independent of antigen processing. (a) T-cell clones were incubated with irradiated or glutaraldehyde fixed antigen presenting cells and

phenindione. Each shape represents proliferation data from an individual clone. The black bar indicates mean proliferative response. (b) Kinetics of T-cell receptor internalisation following stimulation with phenindione. Following phenindione stimulation, T-cells were stained at the indicated time-points for T-cell receptor surface expression using an anti-CD3 antibody. Results show the percentage CD3+ mean fluorescence calculated from values without antigen. Data presented shows results from 2 individual T-cell clones. Co-efficient of variation was consistently less than 20%.

Tables - Table 1. Phenotype, proliferation and cytokine secretion from phenindione-specific T-cell clones

Clone ID	HLA restriction	Phenotype CD & Vβ	Proliferation (cpm)[c]						Cytokine Secretion[c]			
			0	5[a]	10	25	50	100	250	IL-4[b]	IL-5[b]	IL-10[b]	IFN-γ[b]
21	DR	CD4+ (16)	127	96	194	557	1476	3092	108	nd	nd	nd	nd
29	DQ	CD4+ (13.6)	494	494	243	1158	2142	2670	107	nd	nd	nd	1716
53	DR	CD4+ (13.1)	144	441	781	877	1381	1511	141	nd	642	nd	nd
56	DR	CD4+ (12.1)	132	2078	3463	7395	8093	6881	234	nd	721	nd	1173
59	np	CD4+ (np)	280	526	1263	3318	6395	4099	130	nd	nd	nd	1024
60	DR	CD4+ (17)	583	3596	2493	3381	3275	2545	155	nd	nd	nd	18050
69	DQ	CD4+ (5.2)	482	1836	2459	5259	7045	4890	149	4914	9364	nd	nd
79	np	CD4+ (21.3)	294	7936	11263	13615	12000	10550	283	nd	3107	nd	23980

[a] Phenindione concentrations expressed as μg ml^{-1}

[b] Cytokine secretion measured by ELISA. Cytokine secretion expressed as pg ml^{-1}

[c] Co-efficient of variation consistently less than 20%

np, not performed due to lack of cells; nd, not detectable

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758

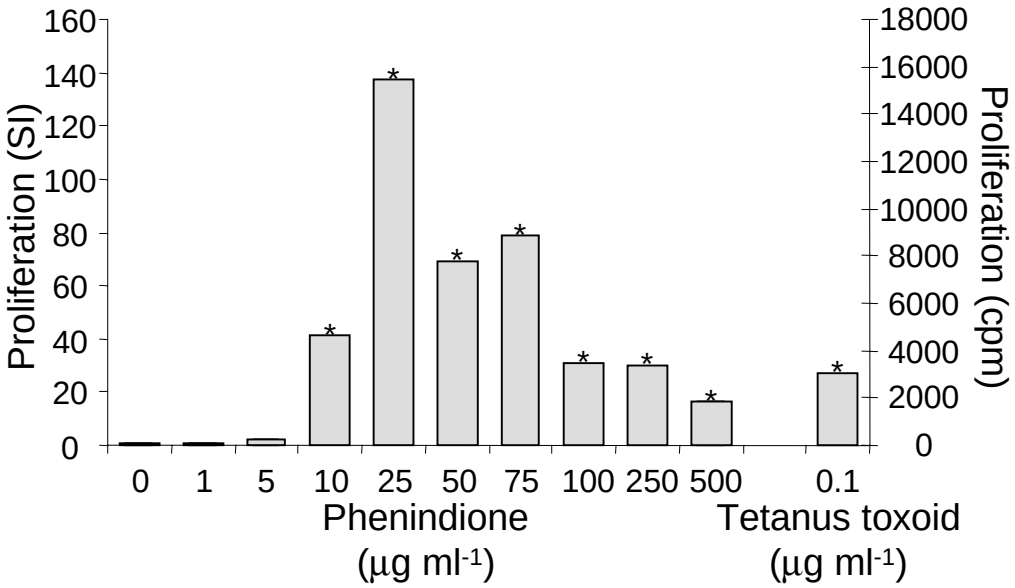
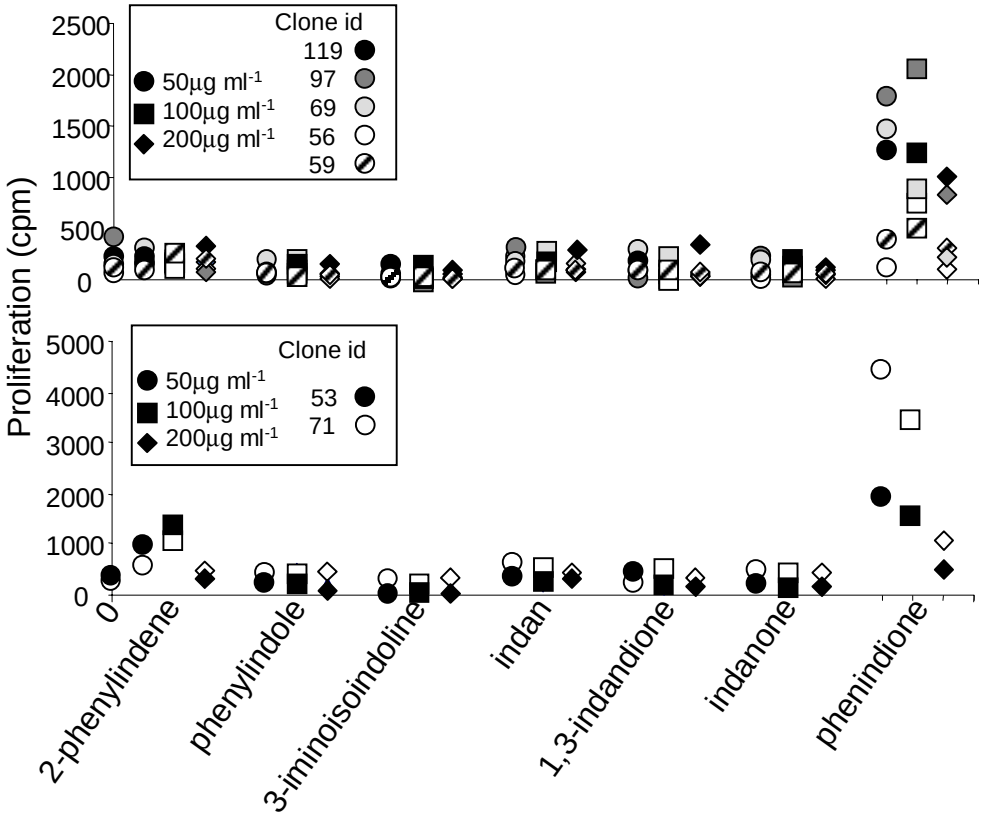


Figure 1

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.



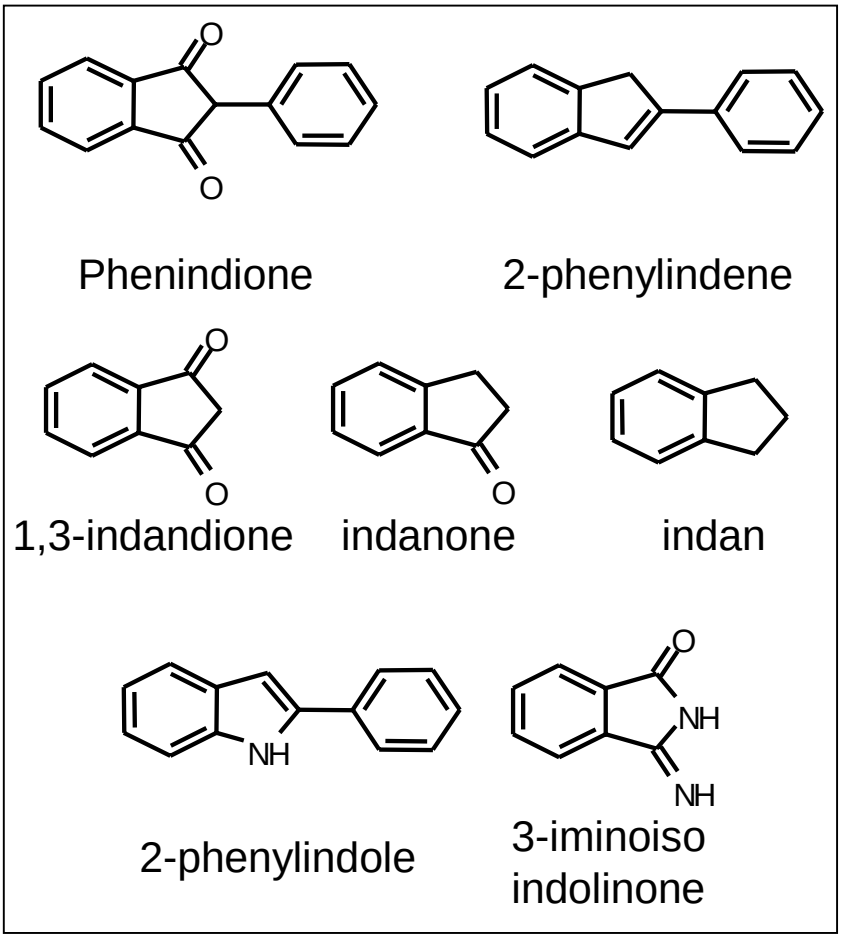


Figure 2

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

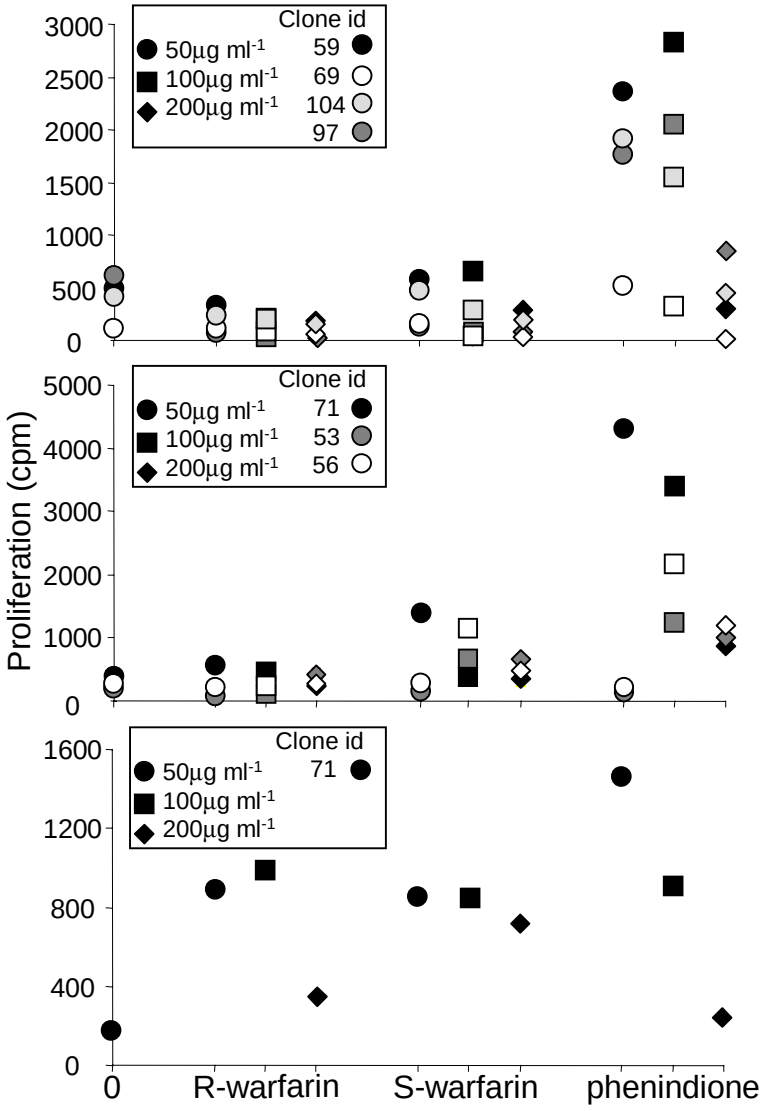
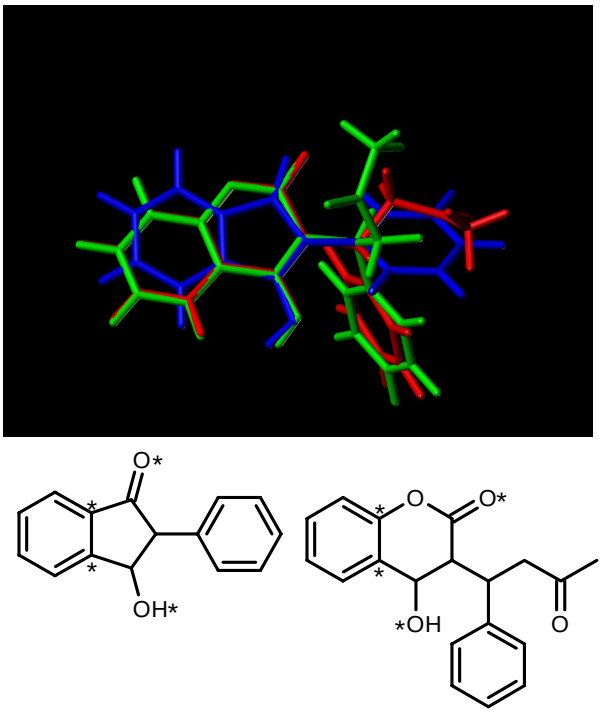


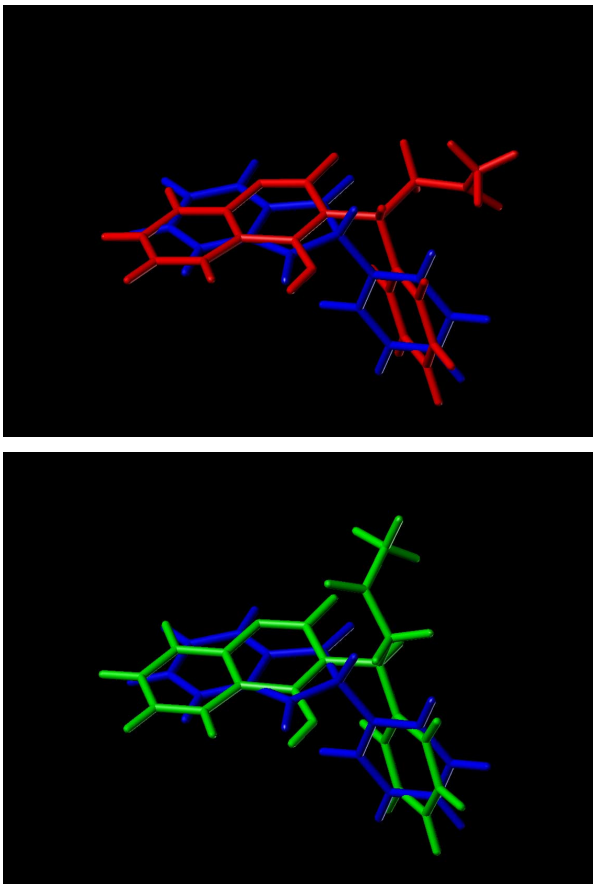
Figure 3

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

a



b



phenindione warfarin

Figure 4

JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.105.083758
This article has not been copyedited and formatted. The final version may differ from this version.

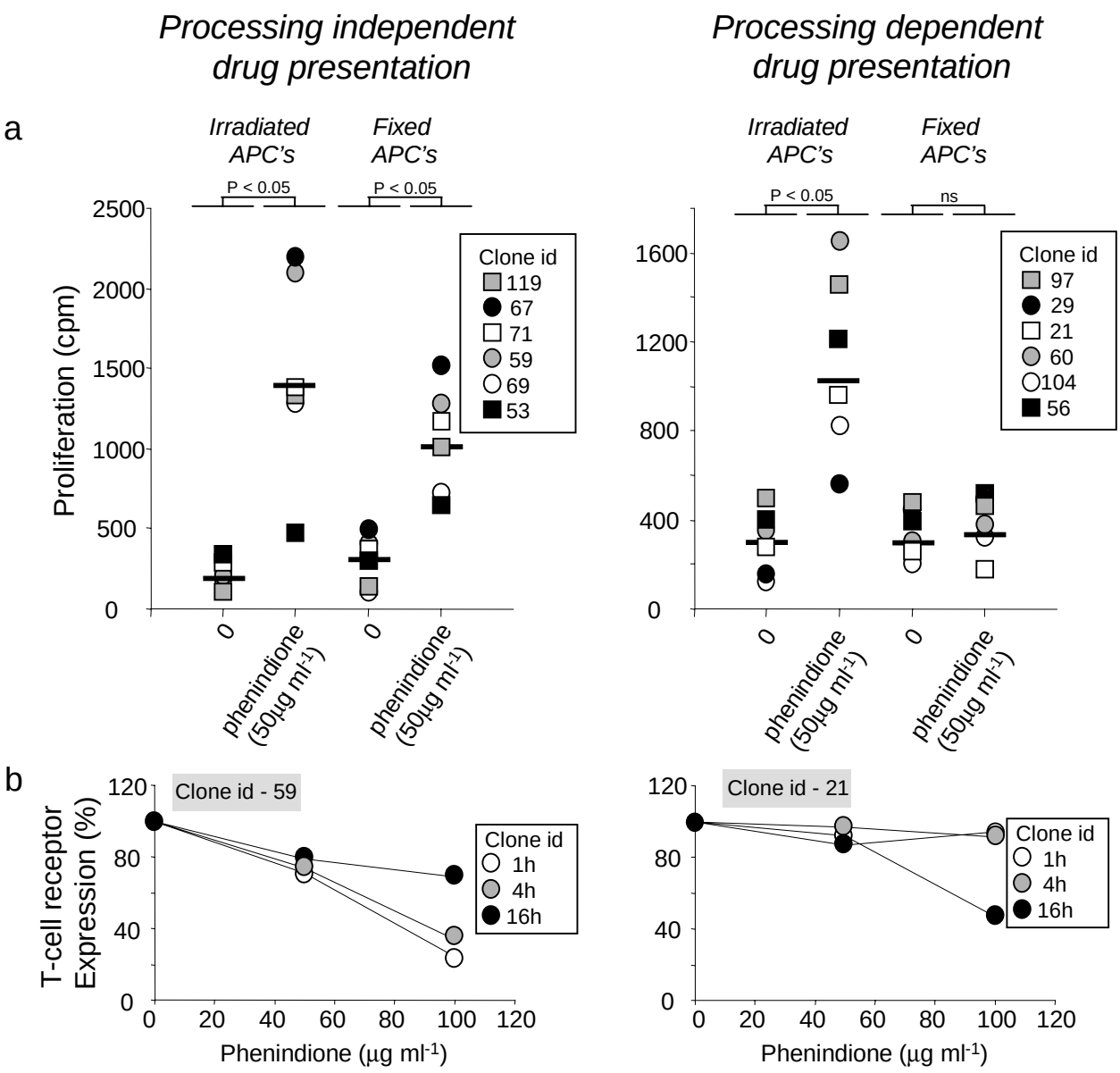


Figure 5