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Optimization of a radiolabel DNA-binding assay in cultured mammalian cells

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ABSTRACT

An improved protocol for the radiolabel DNA-binding assay, which gives a high yield of highly pure DNA has been developed by use of mouse lymphoma cells. The critical difference from previously published methods is the use of enzymatic degradation of proteins in the later DNA purification steps rather than during the homogenisation procedure.

Different DNA-purification methodologies were first compared and the protocol of choice was optimized later on; both steps were performed with [^{35}S]-labelled amino acids for labelling of cellular protein, which enabled both the quantification of cellular protein contaminating the DNA sample and the distinction between cellular and enzyme-derived protein.

The assay was later evaluated and shown to give reproducible results based on the data obtained with benzo[*a*]pyrene (B[*a*]P) and doxorubicin in two different laboratories. In addition, two further reference compounds, dopamine and diazepam and one proprietary AstraZeneca compound were also tested in mouse lymphoma cells in one laboratory. The two compounds B[*a*]P and doxorubicin were identified as suitable positive controls for routine testing in the presence and absence of S9, respectively.

Exposing 90–100 $\times$ 10^6 cells to ^{14}C-labelled compound with a molar radioactivity of 2 MBq/μmol, yields approximately 500 μg DNA with <3% total protein contamination, of which approximately 7% is of cellular origin (<0.2%). The detection level is approximately 2 adducts/10^8 dNTP.

1. Introduction

A great number of mutagens and carcinogens are electrophiles, known to interact either directly or through generation of metabolites, with nucleophilic centres in DNA [1]. The resulting DNA adducts may be pro-mutagenic lesions, which play a major role in the mode of action of chemical mutagenesis and carcinogenesis [2–4]. Consequently, DNA-binding assays to evaluate the ability of a compound to form stable chemical adducts with DNA [1,5,6] have been used as part of the risk-assessment process when substances including pharmaceuticals have yielded positive results in various tests for genetic toxicity. Although the subject of considerable debate, according to the current regulatory view, any compound that reacts directly with DNA will have no safe exposure level and this is stated in the CHMP Guideline on the Limit of Genotoxic Impurities [7] "*According to current regulatory practice it is assumed that (in vivo) genotoxic compounds have the potential to damage DNA at any level of exposure and that such damage may lead/contribute to tumour development. Thus for genotoxic carcinogens it is prudent to assume that there is no discernible threshold and that any level of exposure carries a risk*". Hence a positive finding from a DNA-binding assay may have serious implications for drug development, so it is important to optimize experimental procedures in order to prevent artefacts and false-positive results.

Various pitfalls in the radiolabel DNA-binding assay have been noted [5,8–10]. First, contamination of DNA by protein-bound compounds, which gives uncertainty as to the amount of compound actually bound to DNA and second, the DNA yield which may be too low to allow detection of biologically significant amounts of DNA binding. Since the primary aim of the assay is to determine covalent binding to DNA, it is thus essential that thorough extraction and purification of the DNA from proteins and lipids is achieved.

The purpose of this work was to develop a robust, sensitive and specific DNA-binding assay protocol that would quantify the extent of irreversible binding of a compound to both DNA and cellular protein. Emphasis was given to optimize DNA extraction and purification methods, specifically purification from cellular proteins and lipids or unbound test compound as well as reliable quantification methods for DNA and protein.

The first part consisted of the evaluation of different DNA-purification methodologies, including one with a commercial kit, i.e. Genomic-tip (QIAGEN), and two different methods of purification with hydroxyapatite and digestion and removal of the proteins

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Table 1
Schematic description of protocol optimization and evaluation.

	Choice of protocol	Protocol optimization	Protocol evaluation
Labelling	Cellular proteins with ^{35}S-cysteine and ^{35}S-lysine	Cellular proteins with ^{35}S-cysteine and ^{35}S lysine	Not applicable
Cell treatment	Not applicable	Not applicable	^{14}C-labelled test compound, 90×10^6 cells, 3 h, 37 °C
Cell lysis	1. Repeated freezing and thawing + vortex + pipetting	1. Vortex 2. Vortex + pipetting 3. Vortex + pipetting + sonication	2. Vortex + pipetting 3. Vortex + pipetting + sonication
Extraction protein and DNA	Salt precipitation of proteins Centrifugation Ethanol precipitation of DNA	Salt precipitation of proteins Centrifugation Ethanol precipitation of DNA	Salt precipitation of proteins Centrifugation Ethanol precipitation of DNA
DNA purification	1. Genomic tip (QIAGEN) 2. Hydroxyapatite KPO_4 3. Hydroxyapatite $NaPO_4$ 4. Pronase 5. Proteinase K	Proteinase K treatment 0.12-120 μg/10^6 cells Gel filtration	Proteinase K treatment 1.5 μg/10^6 cells Gel filtration
Protein purification	Wash with organic solvents Gel filtration	Wash with organic solvents Gel filtration	Wash with organic solvents Gel filtration
DNA quantification	OD 260 nm and diphenylamine assay	OD 260 nm and diphenylamine assay	OD 260 nm and/or diphenylamine assay
Protein quantification	Peterson and quantification of radioactivity	Peterson and quantification of radioactivity	Peterson
Quantification of radioactivity	100 μg DNA and 100 μg protein	100 μg DNA and 100 μg protein	≥200 μg DNA and 1000 μg protein

by either pronase (a mixture of various types of endo- and exo-peptidases) or proteinase K (a stable serine protease with broad substrate specificity). Both the evaluation of methodologies as well as the following optimization of the protocol of choice were performed using [^{35}S]-labelled amino acids for labelling cellular proteins, which allowed both the quantification of cellular proteins contaminating the DNA sample, and the distinction between cellular and enzyme-derived protein. The resulting standardised protocol was subsequently evaluated in two laboratories with the reference compounds, benzo[*a*]pyrene and doxorubicin. Two further reference compounds, dopamine and diazepam, and one proprietary AstraZeneca compound (AZ compound A) were tested in one laboratory. Dopamine, diazepam and AZ compound A were tested for DNA binding in mouse lymhoma L5178Y cells, with compound A tested in the presence of S9 after giving positive results in a thymidine kinase (*Tk*) gene mutation assay. An overview of the choice of protocol, protocol optimization and protocol evaluation is presented in Table 1.

2. Materials and methods

2.1. Test chemicals

[7,10-^{14}C]Benzo[*a*]pyrene (2.22 GBq/mmol), [14-^{14}C]doxorubicin hydrochloride (2.07 GBq/mmol) and [^{14}C]-diazepam hydrochloride (2.07 GBq/mmol) were purchased from GE-Healthcare. [^{14}C]-Dopamine (1.85 GBq/mmol) and non-radiolabelled B[*a*]P, doxorubicin, dopamine and diazepam were purchased from Sigma–Aldrich (Poole, UK). [^{14}C]-labelled compound A (2.2 GBq/mmol) was synthesised in-house.

DMSO was used as the solvent for all compounds. [^{14}C]-Dopamine, [^{14}C]-diazepam and the AstraZeneca proprietary compound were supplied in ethanol and [^{14}C]-B[*a*]P was supplied in toluene. In each case, solvents were evaporated under a stream of nitrogen and heat and the solid was then dissolved in DMSO.

2.2. Cells and culture conditions

Mouse lymphoma L5178Y cells, clone 3.7.2c, were originally obtained from Dr. J. Cole, MRC Cell-Mutation Unit, University of Sussex, Brighton, UK. Stock cell cultures were screened to confirm the absence of mycoplasma, and karyotyped. Cells were cultured in RPMI 1640 medium (Invitrogen, Paisley, UK) with 2 mmol/L L-glutamine, 2 mmol/L sodium pyruvate, 200 IU/mL penicillin and 200 mg/mL streptomycin, and maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air in complete medium supplemented with 10% heat-inactivated donor horse serum (DHS).

2.3. ^{35}S-labelling of mouse lymphoma L5178Y cellular proteins

Approximately 160 μCi (20 μL, 8.06 mCi/mL) of PRO-MIX™ L-[^{35}S] (GE Healthcare), a mixture of ^{35}S-labelled methionine and cysteine in the ratio of 70:30, was added to four cultures containing 160 mL L5178Y cells at a cell density of 4×10^5 cells/mL and incubated, with gentle mixing, for 3 h at 37 °C. Cells were pooled, counted for viability by means of trypan-blue exclusion and then centrifuged at $700 \times g$ for 10 min in aliquots of 5×10^7 cells/tube. The cell pellets were washed three times with 10 mL HBSS and stored at −80 °C prior to DNA and protein extraction.

2.4. Treatment with reference chemicals and proprietary compounds

L5178Y cells (9×10^7 in 36 mL) were treated in duplicate with test chemicals in DMSO (1%, v/v) or water, in complete RPMI with 5% heat-inactivated donor horse serum (DHS) for 3 h. For B[*a*]P, treatment was done in the presence and absence of 2% (final concentration) liver post-mitochondrial fraction (S9) from Aroclor 1254-induced rats (Molecular Toxicology Inc., Boone, NC, USA) and, therefore, additional extraction controls and controls for non-specific binding were included. Extraction controls were prepared by addition of [^{14}C]-B[*a*]P to chilled L5178Y cells immediately before the end of treatment of the incubated cultures, followed by simultaneous processing of all samples. Non-specific binding controls consisted of cells exposed to [^{14}C]-B[*a*]P with S9-proteins but without the cofactors needed for metabolic activation, i.e. nicotine-adenine dinucleotide phosphate (NADP) and glucose-6-phosphate. These controls were incubated for 3 h at 37 °C.

Doxorubicin, dopamine and diazepam were only tested in the absence of a metabolic activation system and these tests included solvent and extraction controls.

AZ compound A was tested only in the presence of the S9 system used in the original genotoxicity tests [11] and included solvent and extraction controls and B[*a*]P as a positive control.

Following treatment, cells were centrifuged at $700 \times g$ for 10 min and 10 μL medium supernatant was taken from each treatment into vials for liquid scintillation counting (LSC) counting. The cell pellets were washed three times with 20 mL Hank's buffered salt solution (HBSS) with intermittent centrifugation.

2.5. Cell lysis

Cell pellets (50×10^6 cells for the choice of protocol and the protocol optimization, 90×10^6 cells for the protocol evaluation) were lysed in 10 mL 1% SDS, 1 mmol/L EDTA. For the choice of protocol the cells were disrupted by repeated freezing and thawing of the cell pellet, followed by vortexing and pipetting repeated times. During protocol optimization the homogenate was thoroughly dispersed comparing three methods, (1) vortexing, (2) vortexing and pipetting and (3) vortexing and pipetting followed by a mild sonication at 30% amplitude for 30 s. Methods (2) and (3) were thereafter used for the protocol evaluation with reference chemicals and AZ compound A. A 500-μL aliquot was retained at −80 °C for determination of total cellular protein and DNA quantification, as well as total internal radioactivity.