Detection of Adriamycin–DNA adducts by accelerator mass spectrometry at clinically relevant Adriamycin concentrations

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ABSTRACT

Limited sensitivity of existing assays has prevented investigation of whether Adriamycin–DNA adducts are involved in the anti-tumour potential of Adriamycin. Previous detection has achieved a sensitivity of a few Adriamycin–DNA adducts/10⁴ bp DNA, but has required the use of supra-clinical drug concentrations. This work sought to measure Adriamycin–DNA adducts at sub-micromolar doses using accelerator mass spectrometry (AMS), a technique with origins in geochemistry for radiocarbon dating. We have used conditions previously validated (by less sensitive decay counting) to extract [¹⁴C]Adriamycin–DNA adducts from cells and adapted the methodology to AMS detection. Here we show the first direct evidence of Adriamycin–DNA adducts at clinically-relevant Adriamycin concentrations. [¹⁴C]Adriamycin treatment (25 nM) resulted in 4.4 ± 1.0 adducts/10⁷ bp (~1300 adducts/cell) in MCF-7 breast cancer cells, representing the best sensitivity and precision reported to date for the covalent binding of Adriamycin to DNA. The exceedingly sensitive nature of AMS has enabled over three orders of magnitude increased sensitivity of Adriamycin–DNA adduct detection and revealed adduct formation within an hour of drug treatment. This method has been shown to be highly reproducible for the measurement of Adriamycin–DNA adducts in tumour cells in culture and can now be applied to the detection of these adducts in human tissues.

INTRODUCTION

The anthracyclines are a group of chemotherapeutics that include Adriamycin (doxorubicin), daunorubicin, idarubicin and epirubicin, and which have been widely used in the clinic since the 1960s (1). Adriamycin (Figure 1) is active against a broad range of tumours and is now commonly used in the treatment of breast cancer, Hodgkin’s lymphoma, lung cancer, multiple myeloma and re-occurring ovarian cancer (2). Adriamycin remains the anti-cancer agent with the widest spectrum of anti-tumour activity; however, resistance and side effects are major limitations to the success of Adriamycin therapy (3).

Although the exact mechanism by which Adriamycin exerts its anti-tumour activity is uncertain, the dominant mechanism of action appears to involve impairment of topoisomerase IIα activity (4,5), consistent with observed DNA intercalation and nuclear localization of Adriamycin (6–8). Many potential alternative mechanisms of action have been cited and comprehensively reviewed (3,4). It is likely that Adriamycin acts by many different mechanisms to kill tumour cells, and this would be consistent with its broad spectrum activity.

One of these possible mechanisms, Adriamycin–DNA adduct formation, was first reported in 1979 by Sinha and Chignell (9), but the adducts were difficult to isolate and characterize. A complicating factor in the chemistry of the DNA adduct formation is the participation of formaldehyde in vivo, which activates the compound to a more reactive electrophile capable of forming covalent adducts with DNA (10). The requirements for formation of formaldehyde-mediated Adriamycin–DNA adducts have now been well characterized and reviewed recently by Cutts and co-workers (10,11) and their structure characterised (12–14). It is uncertain whether the adducts first...
reported by Sinha are of the same chemical composition as the formaldehyde-mediated adducts. Early findings by Konopa (15) and many other researchers since (16–21) supported the hypothesis that Adriamycin could form adducts that functioned essentially to cross-link DNA in cells, although methods to detect the adducts were limited in their sensitivity and required the use of supra-clinical drug concentrations (typically ≥10μM Adriamycin) (Table 1). In comparison, plasma concentrations of Adriamycin usually achieved clinically for extended periods of time are <250 nM (4). Initially, isolation of Adriamycin–DNA adducts was also hindered by their intrinsic instability to both heat and alkali. Now that the stability of the adduct has been extensively characterized, conditions have been established to facilitate their isolation (22–24).

Detection of [14C]Adriamycin–DNA adducts by liquid scintillation counting (LSC) is now a straightforward procedure with few manipulations, hence minimal adduct losses. However LSC is relatively insensitive and is unable to accurately measure small quantities of 14C, necessitating high specific activity radiolabelled compounds and artificially high levels of the radiolabelled drug. Previous LSC studies have used 2–10μM Adriamycin for the detection of Adriamycin–DNA adducts in cells (17). The lowest Adriamycin–DNA adduct frequency typically measured by LSC in cells (with good reproducibility) is a few adducts per 10^4 bp DNA following treatment with 2μM Adriamycin (16), which is highly toxic to cells at this concentration. Detection of such low concentrations of radiocarbon by decay counting may be of limited relevance because Adriamycin concentrations that achieve growth inhibition in vitro are typically in the range of 20–400 nM (25).

Table 1. Quantitative techniques used to detect Adriamycin–DNA adducts

| Methodology | Minimum Adriamycin dose | Adducts per 10^7 bp DNA | Sample source | Reference |
|-------------|-------------------------|-------------------------|---------------|-----------|
| Chromatography specific for covalently bound Adriamycin using an intercalator affinity column | 50μM | ~1220 | Cultured cells | 66 |
| Gene-specific and cross-linking assays | 7.5μM | 100 | Cultured cells | 17,67,68 |
| ^32P DNA post-labelling | 0.27 μmol/2.5 g tumour | 10 | Intra-tumoural injection into rat mammary carcinoma (in vivo) | 19 |
| [14C]Adriamycin bound to cellular DNA detected by decay counting | 1 μmol/200 g rat | 70 | Rat liver (in vivo) | 69 |
| | 1 μM | 1 | Cultured cells | 16,25,44,70 |

^With HPLC detection.
^Detected 3 h post-injection.
compounds as low as 1µg/kg body weight and target molecules have been measured in samples at levels as low as one radiolabelled molecule per cell (29–31). AMS has been utilized in the detection of DNA adducts (e.g. DNA alkylating agents such as benzene, atrazine and heterocyclic amines) (30) and has been cited as the gold standard for DNA adduct detection because of its extreme sensitivity (32). More recently, AMS has been used to study the pharmacokinetics and cellular metabolism of several drugs and nucleosides that result in DNA adducts (33–37).

Previous reports have used high drug concentrations (2–50µM Adriamycin), or formaldehyde activation of Adriamycin, to enhance Adriamycin–DNA adduct formation [e.g. a Tris/iron-containing system (38) and formaldehyde-releasing drugs (11,16)]. In the present study we tested the sensitivity of detection of Adriamycin–DNA adducts by AMS using sub-micromolar concentrations of Adriamycin in cultured breast cancer cells. An aim of this work was to evaluate the use of AMS as a highly sensitive methodology for quantifying total cellular Adriamycin–DNA adducts and to assess the sensitivity and specificity of AMS detection of Adriamycin–DNA adducts at clinically relevant Adriamycin concentrations.

MATERIALS AND METHODS

Materials and cell lines

[^14C]Adriamycin hydrochloride (55 mCi/mmol, 95.9% chemical purity) was obtained from GE Healthcare (Piscataway, NJ, USA) and dissolved in sterile Milli-Q H2O to a concentration of 1 mM and stored at −20°C.

Tris-saturated phenol and trypsin-EDTA were obtained from Invitrogen, Carlsbad, CA, USA; chloroform from BDH, Dorset, UK; QIAamp Blood Kit from QIAGEN, Valencia, CA, USA; glycogen from Roche Molecular Biochemicals, Nutley, NJ, USA; Ready-Safe Scintillation fluid from Beckman Coulter, Fullerton, CA, USA and Whatman GF/A filters from Whatman, Kent, UK.

Calf thymus DNA (CT-DNA) was obtained from Worthington Biochemical Corporation, Lakewood, NJ, USA and prepared by dissolving 60 mg overnight at 4°C in 20ml Tris-EDTA pH 8 buffer with agitation and sonicated for six 30s bursts, resulting in 100 bp to 5 kb DNA fragments. Fragmented DNA was then ethanol precipitated and fully dissolved in 0.1 x Tris-EDTA buffer to a concentration of 2.5 mM base pair DNA and particulate matter removed by 5 µm filtration.

Distilled water passed through a four-stage Milli-Q purification system was used to prepare all solutions. Unless otherwise specified, analytical grade reagents were used.

Cell lines

MCF-7 breast adenocarcinoma cells (39) and the MCF-7/Dx Adriamycin-resistant sub-line were obtained from Dr Rosanna Supino (Laboratorio Oncologia, Milan, Italy) and maintained in RPMI Scientific, Melbourne, Australia). MCF-7/Dx cells were generated by growth in escalating Adriamycin concentrations that resulted in increased expression of the multidrug resistance pump P-glycoprotein, and were maintained under selective pressure of 300 nM Adriamycin as previously reported (25,40). Antibiotics were not used in cell line maintenance and all cell lines were routinely tested for mycoplasma by fluorescence microscopy. All cell culture was carried out at 37°C in a humidified atmosphere with 5% CO2.

Drug treatment of cells

On day 1 of Adriamycin–DNA adduct detection experiments, 7.5 x 10^4 cells in 2 ml of growth medium were seeded in 35mm wells and allowed to attach overnight. On day 2 the media was refreshed with new growth media that had been equilibrated to 37°C in 5% CO2 overnight, followed by drug treatment as required. Samples were harvested using trypsin-EDTA at specified times and washed twice with PBS. Cell pellets were stored at −80°C until required for further processing.

Clonogenic survival

Cells were seeded as described above and treated with Adriamycin for 4 h. Cells were then harvested and replated at densities to give between 150 and 250 colonies/dish (5 ml/60 mm dish in four replicate plates). Dishes were incubated for 10 days, then colonies stained with 0.25% (w/v) crystal violet in 2% (v/v) formaldehyde and 80% (v/v) methanol. Colonies consisting of ≥50 cells were counted. For each treatment the plating efficiency was calculated and the surviving fraction assessed relative to the untreated control.

Purification of Adriamycin–DNA adducts

Following drug treatments, cell pellets were resuspended in 150 µl PBS and cellular DNA extracted using a QIAamp Blood Kit according to the manufacturer’s instructions, with the following four modifications to the standard procedure to minimize loss of the heat-labile adducts:

1. 2 mg/ml RNase A digestion was included during the lysis;
2. cell lysis was conducted at 50°C for 30 min (to minimize the loss of heat-labile adducts);
3. column-wash buffer AW2 was used in place of buffer AW1 and
4. DNA was eluted into 200 µl Milli-Q H2O.

These modifications were previously established to maximize DNA yield with minimal adduct losses (16). DNA was then subjected to a cleanup procedure involving extraction twice with phenol and once with chloroform, and then ethanol precipitated (2 volumes ethanol, 0.1 volume of 3 M sodium acetate, 20 µg glycogen) DNA pellets were thoroughly washed twice with 70% ethanol and allowed to dry.

Detection of Adriamycin–DNA adduct by LSC

DNA pellets were resuspended in 100 µl of Tris-EDTA buffer pH 8, and the DNA concentration and quality were determined using a Nanodrop ND-1000
spectrophotometer (NanoDrop, Wilmington, DE, USA). Incorporation of $[^{14}C]$Adriamycin was determined by LSC of a 90 μl aliquot of each sample with 1 ml of Ready-Safe Scintillation fluid using a Wallac 1410 Liquid Scintillation Counter (LSC). The amount of Adriamycin present in each scintillated sample was determined by a LSC standard curve of $[^{14}C]$Adriamycin standards (40–200 pmol Adriamycin). DNA concentrations were determined spectrophotometrically using an extinction coefficient of 13 200/M bp/1 cm at 260 nm. The frequency of Adriamycin–DNA adducts was then calculated per $10^7$ bp DNA.

**AMS procedure**

The procedure for measurement of Adriamycin–DNA adducts by AMS was adapted from a method for preparation of DNA from tissues for AMS measurement (41) with further modifications to minimize sample contamination. AMS samples are highly susceptible to $^{14}$C contamination and hence rigorous procedures must be observed to minimize the possibility of contamination and to ensure reproducible results. Many experimental aspects for the prevention of contamination during preparation of AMS samples have been documented previously (41,42).

Once DNA samples were purified as described above, samples were prepared for submission to the AMS facility at Lawrence Livermore National Laboratories (LLNL). Details for the preparation and submission of samples are described below. This procedure is a newly developed methodology for the detection of Adriamycin–DNA adducts and the first reported detection of Adriamycin–DNA adducts with such high sensitivity.

DNA pellets were thoroughly dissolved in 200 μl of 1 mM NaCl and 100 μl of each sample was sub-sampled to a separate tube to be used for DNA quantification and scintillation (designated LAB). The remainder (designated AMS) was stored at −20°C for sample submission to the AMS facility at LLNL for $^{14}$C measurement.

DNA concentrations of LAB samples (2 μl per sample) were determined using a Nanodrop ND-1000 spectrophotometer and the majority (90 μl) of these samples was subjected to LSC as described above. This preliminary LSC measurement of each sample was taken to establish that $^{14}$C was within background levels as a precaution against contaminating the AMS instrument.

DNA concentrations of AMS samples were adjusted by dilution to the required concentration with 1 mM NaCl. Samples were prepared to give between an estimated 0–100 Fraction Modern (F. Mod.) per sample. Samples were prepared for submission to LLNL for AMS measurement as described above. For each sample the results were reported as F. Mod. (a ratio of C total:$^{14}$C in a sample as a fraction of the standard Modern value, which is 9.79 × $10^{-17}$ mole $^{14}$C/mg of total carbon). The F. Mod. was then calculated per μg of DNA and then corrected for the vehicle control.

The frequencies of Adriamycin–DNA adducts were determined using the following calculations. In order to determine the quantity of Adriamycin bound to DNA, F. Mod. was converted to an absolute quantity of $^{14}$C per sample compared to the 0.62 mg of carrier carbon (C carrier). The quantity of DNA in each case added negligible carbon to the sample. For one F. Mod. in a 0.62 mg sample of carbon, the number of moles of $^{14}$C [n(Adr)] is defined by:

$$n(^{14}C) = 1 \text{ modern }^{14}C \times \frac{9.79 \times 10^{-17} \text{ }^{14}C}{\text{mg C}} \times 0.62 \text{ mg } C_{\text{carrier}} = 6.07 \times 10^{-17} \text{ mol }^{14}C$$

For one F. Mod., the number of moles of Adriamycin, n(Adr), was then calculated from the specific activity of $[^{14}C]$Adriamycin (55 mCi/mmol; 0.8814 atoms $^{14}$C/molecule of Adriamycin).

$$n(Adr) = \frac{\text{Eqn}}{\text{specific activity}} = \frac{6.07 \times 10^{-17} \text{ mol }^{14}C}{0.8814 \text{ atoms }^{14}C/\text{molecule of Adr}} = 6.89 \times 10^{-17} \text{ mol Adr}$$
The quantity of DNA (μg) was converted to a molar quantity using MWbp of 653.72 g/mol and Adriamycin–DNA adducts per bp DNA were then calculated. One F. Mod. of 14C per μg DNA can then be converted to Adriamycin adducts per 107 bp DNA as defined by Equation (3) below, where \( f = n(14C) \) in F. Mod. and \( D = n(DNA) \) in μg.

Adriamycin adducts per 107 bp DNA \( = \frac{f}{D} \times 0.450 \)

**RESULTS**

The purification of Adriamycin–DNA adducts was dependent on the efficient extraction of non-covalently bound Adriamycin into phenol. This efficiency was confirmed by mixing [14C]Adriamycin with CT-DNA which was then subjected to stepwise phenol extractions followed by one chloroform extraction and the remaining 14C measured by LSC. The extraction efficiency of one phenol extraction (followed by one chloroform extraction) was found to be 90%; however, only 4% further removal of Adriamycin resulted from a second phenol extraction. No further removal of Adriamycin was observed in the two subsequent phenol extractions. A single chloroform extraction alone resulted in 13% removal of Adriamycin (without any phenol extractions). For subsequent experiments adequate removal of non-covalently bound Adriamycin was achieved with two phenol extractions, followed by one chloroform extraction.

Adriamycin–DNA adducts have previously been detected in cells using radiolabelled Adriamycin and decay counting (16, 25, 44), hence the more sensitive technique of AMS was expected to permit the detection of lower levels of adducts using a similar procedure for sample preparation. To test whether the LSC sample preparation procedure used previously and described here was suitable for AMS measurement of 14C, a dose response of Adriamycin–DNA adduct formation was tested in Adriamycin sensitive and resistant MCF-7 breast cancer cells with 1–500 nM Adriamycin treatments for 4 h. Importantly, no exogenous source of formaldehyde was used. Adriamycin–DNA adducts were clearly detectable at doses as low as 10 nM Adriamycin, with a linear dose response from 10–500 nM Adriamycin (Figure 2A). However, when the results were extrapolated to 2 μM, the absolute Adriamycin–DNA adduct levels were lower than expected, based on results obtained by LSC (Table 2). By AMS measurement, 500 nM Adriamycin resulted in approximately 130 adducts/107 bp DNA (Figure 2A) whereas by LSC, 2 μM Adriamycin resulted in 1400 ± 540 adducts/107 bp DNA as detected by LSC (Table 2). The lack of consistency is likely to be due to the limited accuracy and reliability of LSC detection at low levels of 14C. To address this variation, cells were treated with either 100 nM or 1 μM Adriamycin and samples prepared for AMS measurement. The 10-fold difference in Adriamycin concentration resulted in a proportional increase in Adriamycin–DNA adduct formation suggesting that the detection by AMS is linear from 0 to 1 μM (Table 2); however, adduct levels as detected by AMS and LSC remained inconsistent. The AMS and LSC sample preparation procedures differ in that AMS samples are dried onto filters, whereas solubilized DNA is subjected to LSC. Extra measures are also taken to prevent contamination in the ultra-sensitive AMS procedure, although these factors are unlikely to result in higher adduct levels as observed by LSC (Table 2). Using AMS detection, the sensitivity of Adriamycin–DNA adduct detection was increased by three orders of magnitude over LSC (Table 2 and Figure 2A).

MCF-7/Dx cells were also tested to investigate the effect of P-glycoprotein mediated resistance on
Adriamycin–DNA adduct formation compared to the Adriamycin-sensitive MCF-7 cells. The MCF-7/Dx cells exhibited a similar linear trend in adduct levels between 0 and 500 nM Adriamycin but overall lower adducts were detected, consistent with reduced accumulation of Adriamycin in the cells (Figure 2A). Overall, up to 3-fold lower Adriamycin–DNA adduct levels were detected in MCF-7/Dx cells compared to the Adriamycin-sensitive MCF-7 cells.

Adriamycin–DNA adducts were measured throughout the range of sensitivity achievable by AMS using the procedure described here. To assess the reproducibility of AMS measurement of Adriamycin–DNA adducts at various levels of detection, two different indicators of experimental error were examined:

1. intra-experimental error: the experimental error within a single experiment (three experimental replicates were utilized);
2. inter-experimental error: the experimental error between independent experiments (up to five independent experiments).

The reproducibility of AMS measurements of Adriamycin–DNA adducts is shown in Table 3. Intralexperimental error (SD) for Adriamycin-treated samples ranged from 7% to 13%, whereas inter-experimental error ranged from 15% to 23% indicating that neither independent experiments nor experimental replicates were subject to high error. The error in drug-treated samples was not elevated at low Adriamycin concentrations, indicating that low levels of Adriamycin–DNA adducts did not result in less reliable detection.

In order to assess the rate of formation of adducts (in the absence of exogenous formaldehyde) MCF-7 cells were treated with 100 nM Adriamycin and adduct formation observed for up to 8 h (Figure 2B). Adducts formed rapidly, with the earliest observed adduct levels at 30 min after treatment and increased in a linear fashion over 0–8 h, indicating that adduct formation was not limited by exhaustion of Adriamycin or formaldehyde available in the cellular environment, in this timeframe. Of the dose of Adriamycin administered, only <0.1% was retained on DNA as Adriamycin adducts after the cleanup procedure (8 h post-treatment with 100 nM Adriamycin), indicating the enormous potential for enhancement of Adriamycin–DNA adduct formation. It is expected that under these conditions Adriamycin–DNA adduct levels would be extensively enhanced by the supply of formaldehyde-releasing pro-drugs, as demonstrated previously (11,16,25).

The half-life of Adriamycin–DNA covalent lesions has previously been measured by various techniques as 5–40 h (24,45). These studies used high Adriamycin concentrations (up to 10 μM) due to the limited sensitivity of the techniques available, hence may not reflect the true half-life of Adriamycin–DNA adducts formed at clinically-relevant Adriamycin concentrations. A complex formaldehyde-generating system (DTT/iron) was also employed for formation of Adriamycin–DNA adducts in these earlier studies (38,46). The broad half-life observed was thought to reflect a range of Adriamycin–DNA monoaducts at isolated guanine residues, together with the preferred GpC dinucleotide binding site (most stable).

The decay of Adriamycin–DNA adducts at 37°C was measured by AMS for two purposes: to determine the temporal stability of Adriamycin–DNA adducts formed at clinically relevant Adriamycin concentrations and also to compare the stability of Adriamycin–DNA adducts, as previously described. Adriamycin–DNA adducts were subjected to extended times at 37°C and the results are presented in Figure 3. The half-life for the decay of Adriamycin–DNA adducts (in the first three days) was found to be ~13 h and this is expected to be of greater relevance to the clinical situation than previous estimates because of the lower doses employed and the fact that the adducts were formed in tumour cells as opposed to a cell-free system. The possibility that the observed loss of adducts in the first three days could be due to residual intercalated drug (that had not been extracted by phenol/chloroform treatments) is unlikely since intercalated Adriamycin has a half-life of less than 2 s (47). Of great potential significance is the fact that ~50% of the population of adducts persisted for up to 12 days, with no sign of decay after the initial decrease at early time points.

### Table 2. Comparison of Adriamycin–DNA adducts detected by LSC and AMS

| Method of detection | Adriamycin concentration | Adducts/10^7 bp DNA |
|---------------------|--------------------------|--------------------|
| AMS                 | 100 nM                   | 37.6               |
| AMS                 | 1 μM                     | 442                |
| LSC                 | 2 μM                     | 1400 ± 540         |

Cells were treated for 4 h and Adriamycin–DNA adducts were measured by AMS and LSC as described.

*Error is SEM, n = 5.*

![Figure 3. Stability of Adriamycin–DNA adducts. DNA from MCF-7 cells treated with 100 nM Adriamycin for 4 h was prepared as for AMS adduct measurement. DNA was then further subjected to incubation at 37°C for the indicated times. Samples were then subjected to a second phenol–chloroform extraction and prepared for AMS analysis as described.](image-url)

The relative 14C remaining (%) was measured by AMS for two purposes: to determine the temporal stability of Adriamycin–DNA adducts formed at clinically relevant Adriamycin concentrations and also to compare the stability of Adriamycin–DNA adducts, as previously described. Adriamycin–DNA adducts were subjected to extended times at 37°C and the results are presented in Figure 3. The half-life for the decay of Adriamycin–DNA adducts (in the first three days) was found to be ~13 h and this is expected to be of greater relevance to the clinical situation than previous estimates because of the lower doses employed and the fact that the adducts were formed in tumour cells as opposed to a cell-free system. The possibility that the observed loss of adducts in the first three days could be due to residual intercalated drug (that had not been extracted by phenol/chloroform treatments) is unlikely since intercalated Adriamycin has a half-life of less than 2 s (47). Of great potential significance is the fact that ~50% of the population of adducts persisted for up to 12 days, with no sign of decay after the initial decrease at early time points.
Adduct levels and colony survival. MCF-7 cells were treated with Adriamycin for 4 h and adduct levels and colony survival measured relative to an untreated control as described in Materials and methods section.

Table 3. Reproducibility of AMS measurement of Adriamycin-DNA adducts

| Adriamycin concentration (nM) | Level of Adriamycin-DNA adducts | Adducts/10^7 bp |
|-----------------------------|---------------------------------|-----------------|
|                             |                                 | Mean ± SD^a     | Mean ± SE^b |
|                             |                                 | (intra-expt)     | (inter-expt) |
| 0                           | None                            | 0.04 ± 0.012     | 0.04 ± 0.002 |
| 25                          | Low                             | 5.32 ± 0.48      | 4.24 ± 0.96  |
| 100                         | Intermediate                    | 22.8 ± 3.0       | 19.0 ± 2.8  |
| 500                         | High                            | 109.2 ± 7.6      | 131 ± 22    |

MCF-7 cells were treated with 0, 25, 100 and 500 nM Adriamycin for 4 h. The zero Adriamycin concentration samples were vehicle-treated samples. Cellular DNA was isolated and prepared for AMS analysis as described. Values are expressed as Adriamycin-DNA adduct frequency per 10^7 bp of DNA.

^aResults were obtained from three replicate samples prepared within one experiment, values shown are average ± SD; intra-expt, intra-experimental.

^bResults obtained from independent experiments, values presented are average ± SE (reproduced from Figure 2B: time course of Adriamycin–DNA adduct formation). MCF-7 cells were treated with 100 nM Adriamycin for 0, 0.5, 1, 2, 4 and 8 h. Cellular DNA was isolated and prepared for AMS analysis as described. Values are expressed as mean Adriamycin-DNA adduct frequency per 10^7 bp DNA and were obtained from two replicate experiments. Error bars represent range of two replicate experiments) inter-expt, inter-experimental.

DISCUSSION

AMS as a methodology for the detection of Adriamycin–DNA adducts

AMS measurement of radioisotopes exhibits higher sensitivity than decay counting, is applicable to many tracer radioisotopes (31) and has been cited as the most sensitive method available for the detection of various DNA adducts (32). AMS has exhibited over five orders of magnitude higher sensitivity for detection of radio-carbon-labelled Adriamycin compared to other routinely-used assays such as HPLC (48). DNA adduct studies with many DNA binders have also demonstrated linear dose responses using AMS detection (31).

To develop an AMS procedure for Adriamycin–DNA adduct measurement, adaptation of the LSC procedure (16) involved modifications to maintain sample quality and to the setting in which the procedure was carried out. Minor changes were also made to the final format of the DNA samples to adapt to the requirements for AMS detection (DNA samples are now routinely dried onto filters for shipping to LLNL for AMS measurement). Importantly, once the sample was dispensed onto the filter paper, it was no longer necessary to maintain intact Adriamycin–DNA adducts, and the filter could be stored and shipped at room temperature. The use of separate isolated reagents and equipment ensured sample contamination was kept to a minimum with extensive controls for routine monitoring of the process. The procedure presented here routinely produced samples of sufficient quality for AMS measurement.

The Adriamycin dose response (Figure 2A) demonstrates specificity for Adriamycin–DNA adducts evidenced by the linear dose response (down to 10 nM Adriamycin) and the virtual absence of detectable ‘adducts’ in untreated controls (Table 3). The half-life of ~13 h for the decay of adducts in the first three days is consistent with previous reports of the limited stability of these adducts in vitro of 5–40 h (10,11,23,24) and reflects the known lability of aminal linkages. The persistent sub-population of 14C lesions observed after 3 days (Figure 3) could possibly be due to metabolic-activation of the side chain of the C-12 of Adriamycin (leading to the formation of an inter-strand cross-link at some adduct sites), or to particularly stable lesions at specific DNA sequences.

Using the procedure described here, AMS detection of these adducts are of high reproducibility, both within experimental replicates and between separate experiments (Table 3). The day-to-day variation observed between separate experiments is probably due to experimental...
limitations in the dilution of the $[^{14}\text{C}]$Adriamycin stock to a working concentration (typically a 10- to 100-fold dilution). The variation within experimental replicates is low and is probably due to the precision limits of the equipment used, not sample contamination. The reproducibility of AMS measurement of radioisotopes itself is very high (49).

AMS has successfully been used here to measure Adriamycin–DNA adducts from cells in culture. There is potential to develop this procedure for other applications such as studies of Adriamycin treatment in mice and humans to establish the extent of formation of Adriamycin–DNA adducts in tumour and normal tissues. The same principle could also be applied to other DNA-binding chemotherapeutic drugs as a biomarker of adducts as a mechanism of action. For such applications the AMS technique would need to be optimized to develop an appropriate experimental process such as the need for radiolabelled compounds and suitable sample preparation.

**Adriamycin–DNA adduct formation as a mechanism of action of Adriamycin at clinically relevant drug concentrations**

There has been doubt whether adduct formation contributes significantly to the mechanism of action of Adriamycin at clinically relevant concentrations of the drug since adduct formation could not be proven at these levels using the existing conventional assays $[^{32}\text{P}]$ post-labelling, decay counting of radiolabelled Adriamycin and the gene-specific cross-linking assay (summarized in Table 1). The measurement of Adriamycin–DNA adducts at clinical Adriamycin concentrations was critical to the hypothesis that adducts contribute to the mechanism of action of Adriamycin and to substantiate this hypothesis the presence of Adriamycin–DNA adducts at low doses needed to be demonstrated. The results presented here indicate that adduct formation can readily be measured at 10–500 nM Adriamycin (Figure 2A), suggesting that significant levels of adducts form at clinically relevant Adriamycin concentrations. These results demonstrate adduct formation is a realistic mechanism of action of Adriamycin that may operate during clinical use of Adriamycin.

The commonly cited mechanism of action, topoisomerase inhibition, is likely to be a major player in the overall cytotoxicity and therapeutic activity exerted by Adriamycin; however, it should be noted that Adriamycin is also likely to be a multimodal chemotherapeutic that acts by many mechanisms in the clinical situation. The contribution of topoisomerase effects in this study are unknown; however, the lowest concentrations of Adriamycin that have been reported to give rise to detectable amounts of topoisomerase inhibition are in the vicinity of 1 μM (50–53). By comparison here, Adriamycin–DNA adducts have been demonstrated at concentrations as low as 10 nM Adriamycin. Many lines of evidence point to the possibility that topoisomerase II$\alpha$ inhibition is not the sole mechanism of action of Adriamycin. Topoisomerase II$\alpha$ activity in various cell lines does not correlate with sensitivity to Adriamycin, implying that cytotoxicity may be due to other factors (54). Similarly, gene expression analysis has indicated that topoisomerase II$\alpha$ involvement in the mechanism of action of supposed topoisomerase inhibitors is variable and that Adriamycin and Adriamycin-like drugs do not group with other topoisomerase inhibitors (55–58). Adriamycin–DNA adducts are now also known to induce apoptosis more rapidly and are more cytotoxic to cells than topoisomerase-mediated effects (44).

While uncertainty still exists regarding the mechanism of action of Adriamycin, given this new evidence using very low doses, Adriamycin–DNA adduct formation is a mechanism of action of Adriamycin that must be considered. It is likely that many observations of the cellular effects induced by Adriamycin have been due (at least in part) to Adriamycin–DNA adduct formation. This largely unfamiliar mechanism of action of Adriamycin should be more widely tested as a significant contributor to this important and widely used chemotherapeutic.

These findings are of particular significance in light of the widespread use of Adriamycin (and subsequent Adriamycin-induced resistance) and the knowledge that both Adriamycin treatment (59,60) and tumour burden (61) correlate with increased formaldehyde levels in the body. The finding that formaldehyde was involved in the formation of Adriamycin–DNA adducts (12) was of particular interest because Adriamycin has long been known to cause redox damage, a consequence of which is the production of aldehydes (62). Formaldehyde generated as a consequence of Adriamycin treatment has been observed as a urinary metabolite in rats (59), mice (60) and in MCF-7 cell lysates (46,63). The possibility exists that formaldehyde is available in Adriamycin-treated cells (whether it be from endogenous sources or as metabolic products of Adriamycin itself) and if so, this would facilitate the formation of Adriamycin–DNA adducts. Intracellular formaldehyde was measured in various cancer cell lines in culture and found to be 0–4 μM inside untreated cells (63). Tumour-bearing mice and patients exhale more formaldehyde (61) and have higher formaldehyde levels in tissue homogenates (64) than non-tumour bearing controls. Bladder and prostate cancer patients also have higher formaldehyde in their urine than control samples from healthy subjects (65) and a diagnostic test based on non-invasive measurements of formaldehyde has been suggested as marker of prostate and bladder cancer (65).

Although adduct formation accounts for only a small percentage of total Adriamycin, it is a potent mechanism of action that may be responsible for the reduced survival capacity of the cells shown in Figure 4. Since these effects were observed in the absence of any exogenous formaldehyde source there is vast potential for enhancing the anticancer activity of Adriamycin with formaldehyde sources such as formaldehyde-releasing pro-drugs. This drug activation process has been extensively investigated and is the subject of several recent reviews (10,11).

Highly sensitive detection of Adriamycin–DNA adducts has long been thought the critical evidence required to support the hypothesis that adducts are a likely mechanism of action of Adriamycin (4). Here, a novel method is presented for highly sensitive and specific detection of adducts at clinically relevant Adriamycin concentrations.
using AMS methodology. Adriamycin–DNA adducts form at doses and in a timeframe that correlate with plasma concentrations of Adriamycin in patients. Further support for this hypothesis could now be sought using an in vivo model and AMS detection to demonstrate whether Adriamycin–DNA adducts form in whole organisms at clinically relevant drug doses.

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