Future Biochemistry and Bioscience
Yenilikçi Biyokimya ve Biyobilim

FBB 2023;5(1):22-27
http://doi.org/10.48086/ASD0023

Araştırma Makalesi/Research Article

Determination of DNA Adducts By ^{32}P-Postlabelling

Abdullah TULİ[1], Umut KÖKBAŞ[2] Fatma Telli KARAKOÇ[3], Alan HEWER[4]

[1]Department of Medical Biochemistry, Faculty of Medicine, Çukurova University, Adana, Turkey.
[2]Department of Medical Biochemistry, Dental Faculty, Nevşehir Hacı Bektaş Veli University, Nevşehir, Turkey
[3]Institute of Marine Sciences, METU, Mersin, Turkey.
[3]Institute of Cancer Research, Haddow Laboratories, Sutton, Surrey, UK.

Öz

Amaç: Poliaromatik hidrokarbonlar gibi farklı nedenlerle oluşan DNA eklentilerinin ölçülmesinde, biyoizlenmesinde ve karakterize edilmesinde önemli rol oynayan 32P-postlabeling yöntemi, Çukurova Üniversitesi Tıp Fakültesi'nde kurmayı amaçladığımız karsinogenez laboratuvarı için anahtar bir tekniktir.

Gereç ve Yöntem: Çukurova bölgesinin kırsal bölgelerinden alınan kan örneklerinden ve benzo(a)piren uygulanmış örnekten DNA saflaştırılmıştır. DNA örnekleri, 3′-nükleosit monofosfatları verecek şekilde parçalandı. DNA eklentileri, nükleaz P1 ile işlenerek zenginleştirildi. [*-32P] ATP ile etiketlendiler ve iki boyutlu ince tabaka kromatografisi ile ayrıldılar. Plakalar -75 °C'de X-ışını filmine maruz bırakıldı ve numunelerin radyoaktivitesi bir β- sintilasyon sayacı kullanılarak sayıldı.

Bulgular: Çukurova Üniversitesi Tıp Fakültesi'nde (Türkiye) ve Haddow laboratuvarları Kanser araştırma enstitüsünde (İngiltere) ölçülen DNA eklentileri örbekleri için sırasıyla 9.6±8.7 ve 9.9±8.9 eklenti/108 nükleotit ve benzo(a)piren ile muamele edilmiş numune için sırasıyla 95.0 ve 110.0 eklenti/108 nükleotit olarak bulundu.

Sonuç: İki grubun verileri karşılaştırıldığında, istatistiksel olarak anlamlı bir fark bulunmadı, bu da Çukurova Üniversitesi'nde 32P-postlabeling yönteminin başarıyla oturtulduğunu gösteriyor.

Anahtar Kelimeler: DNA eklentileri, ^{32}P-postlabellin, Kanser.

Abstract

Objective: ^{32}P-postlabelling method that plays an important role in measuring, biomonitoring, and characterizing DNA adducts formed by different causes such as polyaromatic hydrocarbons, is a key technique for understanding carcinogenesis which we aimed to establish at the Faculty of Medicine, Çukurova University.

Material and Methods: DNA was purified from blood samples taken in the rural region of Çukurova and from a benzo(a)pyrene treated sample. DNA samples were digested to yield 3′-nucleoside monophosphates. DNA adducts were enriched by treating with nuclease P_1. They were labelled by [γ-^{32}P]ATP and separated by two-dimensional thin-layer chromatography. The plates were exposed to X-ray film at -75°C and the radioactivity of the samples were counted by using a β-liquid scintillation counter.

Results: DNA adducts were measured both at Faculty of Medicine, Çukurova University, Turkey, and Institute of Cancer Research Haddow Laboratories, UK were found as 9.6±8.7 and 9.9±8.9 adducts/10^8 nucleotides for rural region samples and 95.0 and 110.0 adducts/10^8 nucleotides for the benzo(a)pyrene treated sample, respectively.

Conclusion: When data of the two groups were compared no statistically significant difference was found, demonstrating that ^{32}P-postlabelling has been successfully established at Çukurova University.

Keywords: DNA adduct, ^{32}P-postlabelling, Cancer

İletişim adresi/Address for Correspondence:

Abdullah Tuli https://orcid.org/0000-0002-6475-1087
Çukurova Üniversitesi Tıp Fakültesi Tıbbi Biyokimya Anabilim Dalı 01330 Adana
Tel: 0533 457 04 08
E-mail: tuliabd@cu.edu.tr

Umut Kökbaş https://orcid.org/0000-0003-4028-3458

Fatma Telli Karakoç https://orcid.org/0000-0001-7666-7504

Geliş Tarihi/Received: 26 Şubat 2021. Kabul Tarihi/Accepted: 23 Kasım 2022. Çevrimiçi Yayın: Published Online: 01 Nisan 2023.

INTRODUCTION

Cancer is the second leading cause of death throughout the world. There is substantial evidence that such chemicals or potential carcinogens as polyaromatic hydrocarbons (PAHs) are biotransformed through the free radical pathway by p4501A1 and, if not quenched by the antioxidant enzymes or antioxidants present in situ, resulting in the formation of DNA adducts[1,2]. These adducts impair the normal control activity of the DNA[1,2,3]. In humans, aromatic DNA adducts are usually present in concentrations of the order 1 per 10^8 nucleotides, but when environmental carcinogens are present in high concentrations, for example as a result of smoking or to exposure of carcinogens, concentrations may increase, and should they persist beyond the ability of the body to repair DNA, then carcinogenesis becomes inevitable [4]. This has been validated by isolation and characterization of the aromatic DNA adducts and determination of their concentrations by ^{32}P-postlabelling [3, 4]. Accordingly, postlabelling is a key technique for understanding carcinogenesis.

In this paper, we describe the successful establishment of the ^{32}P-postlabelling technique at Çukurova University, the success of the technique being confirmed by the analyses of DNA both from normal blood samples and from DNA in mouse skin treated with benzo (a) pyrene and in particular by the agreement between the results obtained at Çukurova University and the Institute for Cancer Research (Haddow Laboratories, ICR).

MATERIAL AND METHODS

Samples for analysis

a)10 ml samples of blood were withdrawn into EDTA (10 mg/mL) from the antecubital vein of each of the three farmers in the Çukurova region and brought in the cold to the laboratory.

b)A sample of DNA was isolated from the epidermis of male Parkes mice 24 hours after application of 1 μmol/mouse of benzo (a) pyrene. This sample, containing approximately 890 adducts per 10^8 nucleotides, was originally prepared by ICR for a fifteen laboratory intercalibration exercise.

The concentrations of aromatic adducts present in the DNA of both samples were determined at both Çukurova University and the ICR. The specific detail provided here applies to experimentation at Çukurova University; the procedure at the ICR was virtually identical.

Isolation of DNA

Since it is of vital importance for postlabelling that high quality, RNA/protein-free DNA is prepared, residual RNA in solutions of DNA isolated according to Poncz et al. [5], was removed by digestion with a mixture of $RNaseT_1$ (150 U/total volume) and RNase A (30 μg/total volume) and extractions with sevag (chloroform/isoamyl alcohol, 24:1).

Hydrolysis of DNA

The initial step in the postlabelling assay is the hydrolysis of DNA to 3′-nucleoside monophosphates by digestion with a mixture of micrococcal nuclease (MN) and spleen phosphodiesterase (SPD). We digested DNA with 0.6 mill units SPD and 0.14 unites MN in 8 mM $CaCl_2$ and 17 mM sodium succinate, pH 6.0 (total volume 4.8 μl) at 37°C in overnight, however, both amount of enzymes and enzymatic incubation time could vary in this step [6, 7].

Adduct enrichment by nuclease P_1

Nuclease P_1 enzyme has a base-specific 3′-phosphatase activity. Normal nucleoside monophosphates are easily hydrolyzed to their corresponding nucleosides by nuclease P_1, although certain adducted nucleotides are resistant to this hydrolysis. We hydrolyzed the digested DNA by adding 4.8 μL of a solution containing each of the following: 0.96 μL of nuclease P_1 (1.25 μg/μl) 2.4 μL of 0.25 M sodium acetate, pH 5.0 and 1.4 μL of 0.3 mM $ZnCl_2$. After incubation at 37°C for 30 min 1.92 μL of 0.5 M tris base was added to stop the reaction [7, 8].

^{32}P-postlabelling of digested DNA samples

Radiolabelling following enrichment involves polynucleotide kinase-catalyzed enzymatic transfer of ^{32}P from [γ-^{32}P]ATP to the 5'-position of adducted nucleotides to create 3',5'-bisphosphate nucleotides. Crucial to this step is the presence of adequate polynucleotide kinase and a molar excess of [γ-^{32}P]ATP. Although there are widespread variations in both the recommended amounts of polynucleotide kinase and [γ-^{32}P]ATP, we used 50 μCi of [γ-^{32}P]ATP 3 units of polynucleotide kinase for labeling the digested DNA [9].

Thin-layer chromatography (TLC) of ^{32}P radioactive adducts

Resolution of ^{32}P-labelled adducts was carried out on polyethyleneimine-cellulose TLC sheets

which were developed overnight with 1 M sodium phosphate, pH 6.0, on a filter paper wick and then, after removal of the wick, developed in the reverse direction with 3.5 M lithium formate, 8.5 M urea, pH 3.5. The sheets were turned 90°, then developed from left to right first with 8.0 M LiCl, 0.5 M Tris-HCl, 8.5 M urea, pH 8.0, and then with 1.7 M sodium phosphate, pH 6.0, in the latter case using a filter paper wick [9].

Visualization of the TLC plates by autoradiography

The TLC plates were exposed to X-ray film (Kodak) at -80°C for three days and developed in an automatic X-ray developer.

Determination of the DNA adduct concentration

Determination of the aromatic DNA adduct concentrations from measurements of the radioactivity of the appropriate areas of the TLC plates using a Packard 1500 β-liquid scintillation counter.

Measurement of the specific activity of the [γ-^{32}P]ATP used for postlabelling

The commercial sample of [γ-^{32}P]ATP used for postlabelling being of uncertain purity, its specific activity was determined by postlabelling a known amount of standard nucleotide and measuring the radioactivity of the product. 3 μL of standard 3′-dAp monophosphate (6 pM) were mixed with T_4 kinase (6 U) in kinase buffer (4.8 μL) and tris buffer at pH 9.0 (2.5 μL, 10 mM). Excess, a nominal 60 μCi, of [γ-^{32}P]ATP was added and the whole was incubated for 30 min at 37°C. The volume was increased to 1 mL with tris buffer (10 mM, pH 9.0). The labeled 3′,5′-dAp bisphosphates were separated from 5 μL samples of the resulting solution on polyethyleneimine-cellulose TLC sheets developed overnight with 1 M sodium phosphate (pH 6.0) on a filter-paper wick. The TLC was visualized by autoradiography. The radioactivity of the spot due to dAp on the TLC sheet was determined and, the amount of the dAp being known, the specific activity of the ATP was calculated [8].

Statistical analyses

SPSS program was used for statistical studies. SPSS program was used for statistical studies. The same samples were studied at Çukurova University and ICR, and the results were compared whether they were statistically different.

RESULTS

Table 1 shows the absorption of the DNA extracted from the blood samples.

Table 1. Concentration and the purity of isolated DNA.

Samples	OD_{230}	OD_{260}	OD_{280}	OD_{230}/OD_{260}	OD_{260}/OD_{280}	Concentration (μg/ml)
1	0.040	0.111	0.060	0.36	1.8	333
2	0.062	0.155	0.085	0.40	1.8	465
3	0.096	0.253	0.133	0.38	1.9	759
4	0.040	0.110	0.060	0.36	1.8	330
5	0.037	0.096	0.051	0.39	1.9	288
6	0.100	0.265	0.445	0.38	1.8	795
7	0.040	0.110	0.058	0.36	1.9	330
8	0.045	0.124	0.064	0.36	1.9	372
9	0.043	0.118	0.062	0.36	1.9	354
10	0.042	0.120	0.063	0.35	1.9	360
11	0.075	0.195	0.104	0.38	1.9	585
12	0.114	0.285	0.150	0.40	1.9	855
13	0.100	0.250	0.136	0.40	1.8	750
14	0.101	0.235	0.124	0.43	1.9	705

Table 2 shows the specific activity of the [γ-^{32}P]ATP used in Çukurova University.

Table 2. Measurements of specific activities of used [γ-^{32}P] ATP

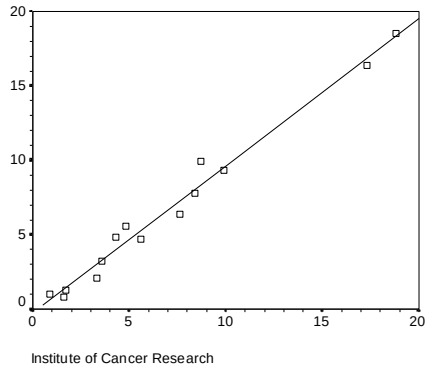
Number of used [γ-^{32}P]ATP	Mean cpm values	dpm/mL	dpm/mole	Ci/mM
1	77416	42629956	7104993	3200
2	125366	6268300	10447167	4706

Table 3 shows concentrations of aromatic DNA adducts found in the three blood samples and the sample treated with benzo(a)pyrene.

Table 3. Calculated DNA adduct concentrations both at Çukurova University and at ICR

Samples	Adducts/10^8 nucleotides	
	FMÇU	ICR
1	9.3	9.9
2	9.9	8.7
3	1.0	0.9
4	0.8	1.6
5	18.5	18.8
6	16.4	17.3
7	5.6	4.8
8	7.8	8.4
9	6.4	7.6
10	2.1	3.3
11	3.2	3.6
12	4.8	4.3
13	1.3	1.7
14	4.7	5.6
Benzo(a)pyrene	95.0	110.0

Figure 1 is typical autoradiography of TLC plates obtained from measurements of the specific activity of the [γ-^{32}P]ATP.



The presence of DNA adducts in DNA is determined sequentially by the ^{32}P-postlabelling technique. The DNA is isolated from the samples being investigated uncontaminated by both proteins and RNA. This DNA is completely hydrolyzed to give 3′-nucleoside (and nucleoside adduct) monophosphates; the monophosphates are completely converted to ^{32}P labeled 3′,5′-nucleoside (and nucleoside adduct) bisphosphates; the nucleoside adduct bisphosphates are separated by TLC, and these TLCs are visualized by autoradiography and their radioactivity. The last step necessitates the determination of the specific activity of [γ-

^{32}P]ATP used for labeling. None of these steps is simple; pure solvents and chemicals and stable enzymes are required and enzyme-catalyzed reactions have to be conducted so that they proceed quantitatively to completion. Whereas the optimization conditions for each step have been investigated [10], the procedures have yet to be standardized. Indeed, a recent intercalibration exercise in which 15 laboratories analyzed identical samples produced analytical results ranging over a factor of 5 [11]; it is necessary to establish the reliability and reproducibility of our results.

Isolation of DNA

DNA giving ratios of absorbance at 230/260 nanometers and at 260/280 nanometers of ~0.4 and ~1.8, respectively is uncontaminated by proteins and RNA [10]. Table 1 shows that in this sense DNAs isolated at Çukurova from the three blood samples were pure.

The specific activity of [γ-^{32}P]ATP

This activity is needed in subsequent calculations of aromatic DNA adduct concentrations. Table 2 shows the specific activity of the carrier-free [γ-^{32}P]ATP used in this work; activities measured by two independent workers were in agreement. The method of measuring specific activities implies that the agreement of independent results ensures that postlabelling and subsequent (one-dimensional) TLC separations and autoradiography steps were performed consistently. Figure 1 is of a typical autoradiograph showing good resolution of the dAp spot. First attempts to measure specific activity failed because the kinase used in the postlabelling was unsatisfactory; subsequent success then testified to the purity of all the reagents used in postlabelling.

Determination of aromatic DNA adducts

Previous paragraphs have shown that DNA could be isolated uncontaminated and that 3'-nucleoside phosphates could be postlabelled satisfactorily. Figure 2 and Table 3 show the results of the complete process, including visualization of DNA adducts and of determining aromatic DNA adduct concentrations in the three normal blood samples and the sample pretreated with benzo (a) pyrene. These measurements were made independently at Çukurova University and ICR. Figure 2d shows the autoradiograph of the TLC obtained from the benzo (a) pyrene-treated sample. The spot due to the benzo (a) pyrene dihydrodiolepoxide adduct is shown very clearly in exactly that area of the TLC plate -the diagonal zone- in which it has been found by previous workers. In principle, each benzo (a) pyrene dihydrodiolepoxide isomer should form an adduct with each nucleoside though published results suggest either that this does not happen or that the resulting spots overlap [12]. Figures 2a, 2b, and 2c shows autoradiographs of the TLCs obtained from blood from three farmers in the Çukurova region. Again, the presence of adducts in the diagonal zone is clear. One expects the farming environment to include several carcinogens, including perhaps pesticide residues, and it is not surprising that the diagonal zone shows several overlapping spots. It is hoped that future work will characterize these more precisely. Table 3 shows the aromatic DNA adducts concentration. The agreement between the results obtained at Çukurova University and ICR is better than (about the same as) the agreement between different laboratories in the recent intercalibration exercise [11] and demonstrates Çukurova competence to carry out the entire procedure. The benzo (a) pyrene-treated sample can be stored for about a year and will be used as a standard to ensure that procedures used to determine aromatic DNA adducts of Çukurova University remain optimized.

DISCUSSION

As a result, the concentrations of aromatic DNA adducts present in 4 samples (three normal blood samples and one sample pretreated with benzo (a) pyrene have been determined independently by Çukurova University and the ICR. The results, which cover a thirty-fold range of adduct DNA concentrations, are in satisfactory agreement demonstrating that the techniques of isolating DNA, hydrolyzing it, concentrating the adducted nucleotides, labelling them with ^{32}P, and separating the adducts by TLC can be carried out under optimized conditions at Çukurova University. As a result of our studies, we came to the conclusion that we have successfully implemented 32P-post labeling studies in our own laboratory.

Acknowledgements

It has taken more than two years to reach the stage when we can modestly say that the work of isolating and determining the adducts formed

between carcinogens and DNA at Çukurova University is beginning. We would not have got this far without the support of TÜBİTAK and the British Council and the sympathetic encouragement of David Phillips and Alec Gaines.

Etik Onay:-
Çıkar Çatışması: Yazarlar çıkar çatışması beyan etmemektedir
Finansal Destek: Yok
Ethical Approval: -
Conflict of Interest: Authors declared no conflict of interest.
Financial Support: None

REFERENCES

1. Reed, L., Jarvis, I. W., Phillips, D. H., & Arlt, V. M. Enhanced DNA adduct formation by benzo [a] pyrene in human liver cells lacking cytochrome P450 oxidoreductase. Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2020, 852, 503162. https://doi.org/10.1016%2Fj.mrgentox.2020.503162.
2. Rodwell, V. W. Harper's illustrated biochemistry. McGraw-Hill Education.2015
3. Jafari, F., Baghayi, H., Lavaee, P., Hadizadeh, F., Soltani, F., Moallemzadeh, H., et al. Design, synthesis and biological evaluation of novel benzo-and tetrahydrobenzo-[h] quinoline derivatives as potential DNA-intercalating antitumor agents. European journal of medicinal chemistry, 2019, 164, 292-303. doi: 10.1016/j.ejmech.2018.12.060.
4. Punetha, A., Saraswat, S., & Rai, J. P. N. An insight on microbial degradation of benzo [a] pyrene: current status and advances in research. World Journal of Microbiology and Biotechnology, 2022 Feb 24;38(4):61, 1-18. doi: 10.1007/s11274-022-03250-3.
5. Lattanzi, A., Camarena, J., Lahiri, P., Segal, H., Srifa, W., Vakulskas, C. A. et al. Development of β-globin gene correction in human hematopoietic stem cells as a potential durable treatment for sickle cell disease. Science translational medicine, 2021, Jun 16;13(598), eabf2444. https://doi.org/10.1126%2Fscitranslmed.abf2444.
6. Clergé, A., Le Goff, J., Lopez-Piffet, C., Meier, S., Lagadu, S., Vaudorne, I., et.al. Investigation by mass spectrometry and 32P post-labelling of DNA adducts formation from 1, 2-naphthoquinone, an oxydated metabolite of naphthalene. Chemosphere, 2021, Jan;263:128079. doi: 10.1016/j.chemosphere.2020.128079.
7. Yang, L., Liu, Q., Zhang, X., Liu, X., Zhou, B., Chen, J., & Song, E. DNA of neutrophil extracellular traps promotes cancer metastasis via CCDC25. Nature, Jul;583(7814):133-138. doi: 10.1038/s41586-020-2394-6.
8. Tang, S., Liu, X., He, L., Liu, B., Qin, B., & Feng, C. Application of postlabeling delay time in 3-dimensional pseudocontinuous arterial spin-labeled perfusion imaging in normal children. Journal of Computer Assisted Tomography, Sep/Oct;43(5):697-707. doi: 10.1097/RCT.0000000000000911.
9. Zimmermann, M., Li, T., Semrad, T. J., Wu, C. Y., Yu, A., Cimino, G.,et al.. Oxaliplatin–DNA Adducts as Predictive Biomarkers of FOLFOX Response in Colorectal Cancer: A Potential Treatment Optimization StrategyOxaliplatin–DNA Adducts as Biomarkers of FOLFOX Response. Molecular cancer therapeutics, 2020 Apr;19(4):1070-1079. doi: 10.1158/1535-7163.MCT-19-0133.
10. Yao, X., & Bierbach, U. DNA Adduct Detection after Post-Labeling Technique with PCR Amplification (DNA-ADAPT–qPCR) Identifies the Pre-ribosomal RNA Gene as a Direct Target of Platinum–Acridine Anticancer Agents. Chemistry–A European Journal, 2021, 27(59), 14681-14689. https://doi.org/10.1002/chem.202102263.
11. Totsuka, Y., Watanabe, M., & Lin, Y. New horizons of DNA adductome for exploring environmental causes of cancer. Cancer science, 2021, 112(1), 7-15. https://doi.org/10.1111/cas.14666.
12. Fielden, J., Wiseman, K., Torrecilla, I. et al. TEX264 coordinates p97- and SPRTN-mediated resolution of topoisomerase 1-DNA adducts. Nat Commun 2020,11, 1274 . https://doi.org/10.1038/s41467-020-15000-w.