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FOREWORD

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Eleanor H. Rogan
PI - Signature _____ Date _____

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INTRODUCTION

The role of estrogens on the induction of breast cancer can be related to initiation, promotion, or both. We hypothesize that the initiating event for human breast cancer and certain other human cancers is associated with activation of endogenous catechol estrogens (CE), which are hydroxylated metabolites of estrone (E_1) and 17β -estradiol (E_2). In mammalian cells, CE are predominantly conjugated to their 2-, 3- or 4- monomethoxy derivatives by catechol-O-methyltransferases (COMT). In our hypothesis, CE-3,4-quinones (CE-3,4-Q) are the ultimate carcinogenic forms of estrogens because they bind to DNA to form depurinating adducts that could initiate cancer by mutating critical genes. Because only nonmethylated CE can be oxidized to CE-Q, the ratio of nonmethylated vs methylated CE should be higher in women with breast cancer compared to women without disease. In this project, we established methods to determine the levels of CE in breast tissue from pre- and post-menopausal women with and without breast cancer. The CE are identified and quantified by HPLC with multichannel electrochemical detection after extraction from tissue. We have obtained preliminary results consistent with the hypothesis that women with breast cancer form high levels of 4-hydroxyestrogens and have poor ability to protect them by methylation.

BODY

The role of estrogens on the induction of breast cancer can be related to initiation, promotion, or both. We hypothesize that the initiating event for human breast cancer and certain other human cancers is associated with activation of endogenous catechol estrogens (CE), which are hydroxylated metabolites of estrone (E_1) and 17β -estradiol (E_2), 2-OH E_1 (E_2) and 4-OH E_1 (E_2) [1].

In mammalian cells, CE are predominantly conjugated to their 2-, 3- or 4- monomethoxy derivatives by catechol-O-methyltransferases (COMT) [2]. These enzymes are considered to be protective enzymes because only nonmethylated CE can be oxidized to their quinones (CE-Q) by peroxidases and cytochrome P-450 (Fig. 1). If the formation of CE exceeds the capacity of a cell to catalyze O-methylation, an accumulation of CE might ensue [3,4]. In our hypothesis, CE-3,4-Q are the ultimate carcinogenic forms of estrogens because these electrophiles can covalently bind to the nucleophilic groups of DNA to form depurinating adducts that could initiate cancer by mutating critical genes [1]. Depurinating adducts are lost from DNA by hydrolysis of the glycosidic bond, leaving apurinic sites, which if not repaired, could be mis-replicated to produce oncogenic mutations.

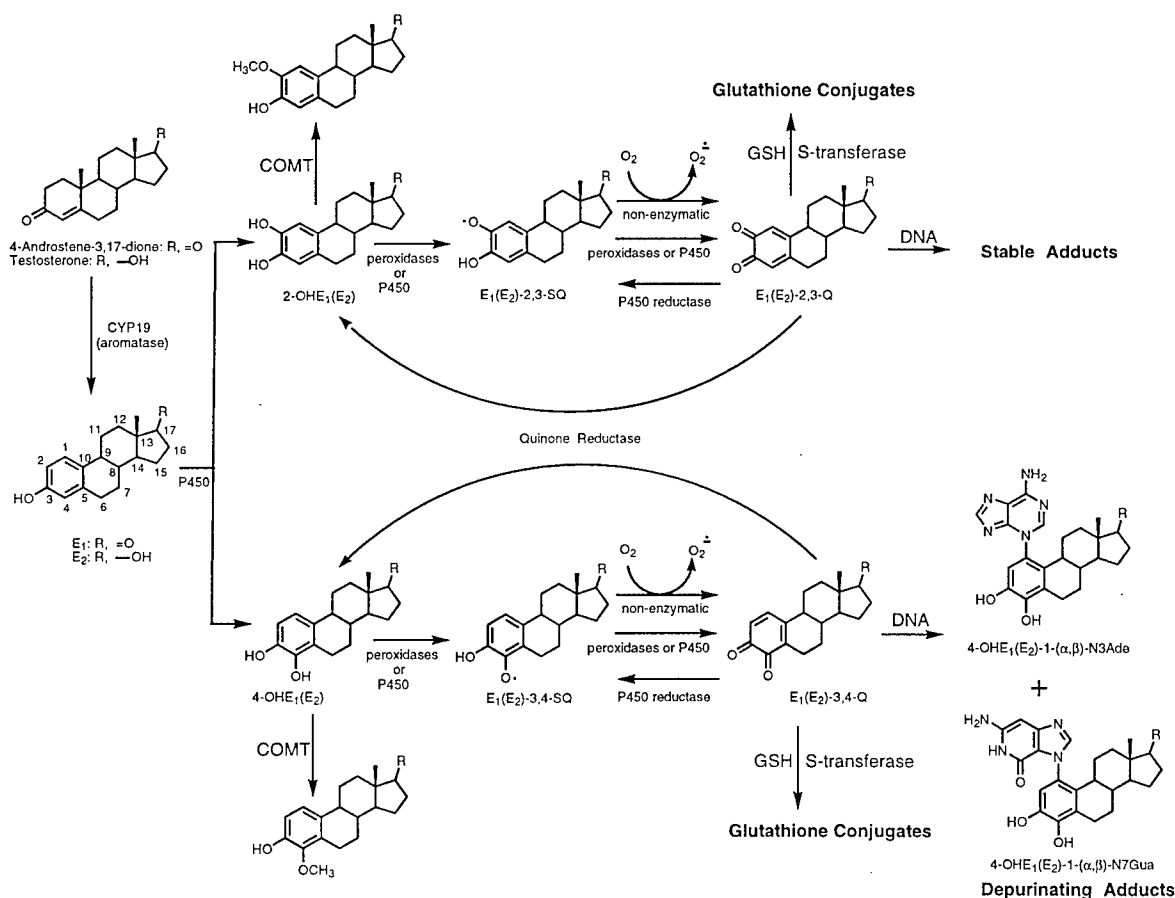


Figure 1. Formation, metabolism and DNA Adducts of estrogens.

Because only nonmethylated CE can be oxidized to CE-Q, the ratio of nonmethylated vs methylated CE should be higher in women with breast cancer compared to women without disease [3,4]. We expect to find that the ratio of nonmethylated vs methylated CE is higher in women with breast cancer and/or the level of COMT in breast tissue is lower. This approach presumes that the levels of nonmethylated vs methylated CE and COMT are characteristic of a woman and, thus, the results obtained with breast cancer patients are similar to those that would have been obtained before development of the tumor.

In this project, we planned to determine the levels of CE and COMT in breast tissue from pre- and post-menopausal women with and without breast cancer. CE were to be quantified by gas chromatography/mass spectrometry (GC/MS) analysis after extraction from tissue, and COMT activity was to be quantified by an assay using [methyl-³H]S-adenosyl methionine. At the end of this project we expected to have collected sufficient data on the levels of CE and COMT in breast tissue to make preliminary comparisons of women with and without breast cancer. As described below, every aspect of this proposal turned out to be much more time-consuming than expected.

The results of these studies, however, are expected to provide the basis for very early **detection** of women at risk for developing breast cancer and to lay the foundation for developing approaches to **preventing** manifestation of this disease. In addition, new information concerning the **etiology** of breast cancer will be obtained.

An integral part of our hypothesis is that a high level of nonmethylated vs methylated CE and/or low level of COMT is a persistent characteristic of a woman susceptible to breast cancer. Thus, by analyzing breast tissue from patients we expect to find results similar to those that would have been found prior to tumor induction. It is necessary to conduct these analyses with breast tissue because only the tissue susceptible to the tumor will presumably reflect anomalies. Technology for determination of CE in breast tissue has been reported [5], but we were not able to use these methods with success, and we developed new procedures to obtain the desired specificity and sensitivity. We began this project by using GC/MS to analyze the CE metabolites, but found that this method was not as good as using HPLC coupled with a multichannel electrochemical detector (ESA CoulArray), which enables us to analyze 20 compounds with picomole sensitivity.

The technical objectives proposed for this project were (1) to determine the levels of CE in breast tissue samples from pre- and post-menopausal women with and without breast cancer, and (2) to determine the levels of COMT in breast tissue samples from pre- and post-menopausal women with and without breast cancer. As described in the following section, our goal of analyzing a large number of breast tissue samples was not met because we had to spend a great deal of time developing the methods to prepare the samples and carry out the analyses. We did not begin the research on COMT for two reasons: first, we spend all our time working out the CE analyses, and, second, we have realized that there now are much better assays for COMT. These include analyzing the COMT mRNA by RT-PCR, the COMT protein by western blots, and the

methoxyCE products by HPLC with electrochemical detection.

We accomplished the following research objectives, described with respect to the related task(s).

Task 1 (Years 1 and 2) - Collection of breast tissue samples. Female patients available for study are those scheduled for breast biopsy because of suspected breast cancer or other breast disease. We have collected approximately 400 samples of "normal" breast tissue (not tumor tissue) from women undergoing breast surgery or biopsy at the Nebraska Health System. During the procedure, a sample was immediately cut by a pathologist from excess tissue, put into a labeled plastic cassette and frozen in liquid nitrogen. At the end of the day, Ms S. Higginbotham, Research Technologist II in our research group, picked up the container of liquid nitrogen with the samples and the signed consent forms and transferred the samples to our -80 °C freezer, entered the sample information into our database and filed the consent forms.

Task 2 (Year 1) - Analysis of CE and metabolites (sample preparation methods). We spent a considerable amount of time and effort to establish effective methods for preparing these samples for HPLC analysis. In our procedure, the tissue (approximately 1 g) is ground in liquid nitrogen, suspended in 50 µM ammonium acetate, pH 4.4, and divided into two portions. One portion is incubated with β-glucuronidase/sulfatase (to release any further conjugated estrogens) for 6 h at 37 °C, and the other is left untreated. Methanol is added to each portion (to 60% volume) and the mixture extracted with hexane. The aqueous phase is diluted to approximately 30% methanol and applied to a Certify I Sep-Pak cartridge. The cartridge is eluted with increasing amounts of methanol in buffer and fractions are collected. Ascorbic acid is added to the eluting buffer to minimize oxidation of the CE derivatives. The fractions are analyzed by HPLC (see below) and, in some cases, identification is subsequently confirmed by electrospray mass spectrometry.

Task 3 (Years 1 and 2) - Analysis of CE and metabolites (MS methods). Methodology was worked out. Samples are derivatized in N,O-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) and analyzed by GC/MS at mass resolution of 10,000, monitoring the molecular ion of each derivatized compound. A DB-5 capillary column (30 m x 0.32 mm, 0.32 µ) is used and data are acquired on a Kratos MS-50 double-focusing mass spectrometer/Kratos-Mach 3 data system. The high resolution mass spectrometry operated in mass profiles mode, combined with the capillary GC separation, provides good method specificity. Deuterated analogues, approximately 50 ng each of E₂(d₂), 2-OCD₃E₁ and 4-OCD₃E₂ were used as internal standards. After derivatization, each sample and a reference standard received a 50 ng of previously derivatized 4-OHE₁(d₂) to aid in determining recovery. The procedure detection limit should have been in the sub-nanogram range with excellent accuracy and precision. We proposed to quantify the levels of twelve available analytes: E₁ and E₂; 2-OH-, 4-OH-, and 16α-OH- E₁ and E₂; and 2-OCH₃- and 4-OCH₃- E₁ and E₂. In fact, problems were encountered in conducting analyses of breast samples, and recovery was at best 5%.

Because HPLC with multichannel electrochemical detection allows us to detect CE metabolites and conjugates in human breast tissue samples, we abandoned the GC/MS approach. Mass spectrometry will be incorporated into our analyses in the future, but we will use an on-line electrospray mass spectrometry approach.

Task 4 (Years 1 and 2)- Analysis of CE and metabolites (initial analyses of breast tissue samples).

By using HPLC with multichannel electrochemical detection, we have achieved our goal of identifying and quantifying CE metabolites in human breast tissues. HPLC is conducted by using a Phenomenex Luna(2) C18 reverse phase column (250 x 4.6 mm, 5 μ) on an HPLC system equipped with an 8-channel ESA CoulArray electrochemical detector to determine the levels of the major estrogen metabolites, methoxyCE conjugates, depurinating 4-OHE₁(E₂)-N7Gua adduct,

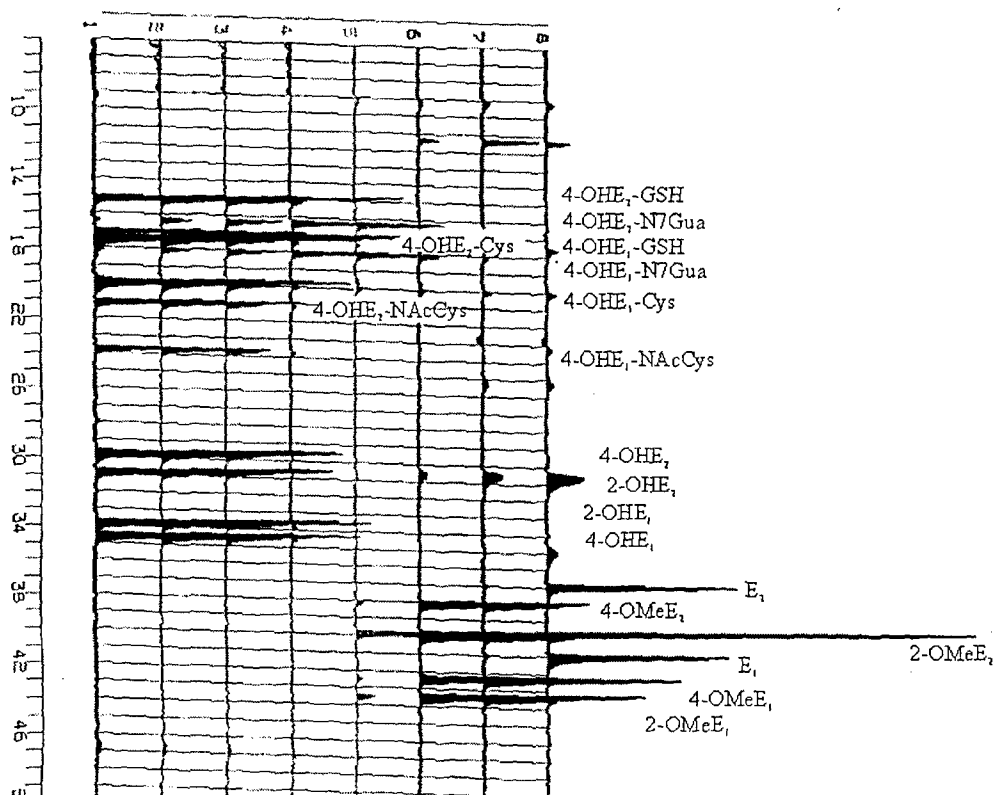


Figure 2. HPLC separation of standard estrogen metabolites, conjugated metabolites and depurinating DNA adducts with an ESA 8-channel CoulArray electrochemical detector. The 16 α -OHE₁ and 16 α -OHE₂ are missing from this chromatogram.

and CE-GSH and CE-Cys conjugates. The channels are each set at successively increasing potentials between 0 mV and 750 mV. A linear gradient starting from acetonitrile/methanol/water/1 M ammonium acetate, pH 4.4 (15:5:70:10) to acetonitrile/methanol/water/1 M ammonium acetate, pH 4.4 (50:20:20:10) over 50 min at a flow rate of 1 mL/min (Fig. 2). Metabolites, conjugates and adducts are identified by comparison with authentic standards, based on their retention time, as well as peak height ratios between the dominant peak and the two peaks in adjacent channels in the chromatogram. The CE-GSH conjugates are further metabolized to 4-OHE₁(E₂)-2-Cys and 4-OHE₁(E₂)-2-NAcCys by the mercapturic acid biosynthesis pathway; thus any of these conjugates may be present in the tissues.

Initial analyses have been conducted on a few (10) specimens of "normal" breast tissue from women with breast cancer. We prepared the samples as described above and subjected them to HPLC separation with electrochemical detection (Table 1). From these results, we can make several conclusions: (1) It is possible to detect estrogen metabolites and CE-GSH conjugates at the picomole level in human breast specimens of approximately 1 g by using HPLC with electrochemical detection. (2) In 7/10 samples, the level of 4-CE was several-fold greater than that of 2-CE, as anticipated (in normal subjects the level of 2-CE is four times greater than that of 4-CE [25]). (3) The methoxyCE, if present, were under the limit of detection, indicating at best low COMT activity. (4) Some samples contained 4-CE conjugated with Cys or NAcCys, which are products of mercapturic acid biosynthesis from GSH conjugates. These demonstrate that the CE-3,4-Q were formed in the breast tissue of these patients, suggesting that DNA damage from CE-3,4-Q may also have occurred. **These initial results underscore our hypothesis that the 4-CE oxidative pathway feeds into the etiology of human breast cancer. Furthermore, it offers great promise for the power of this approach to investigate the 4-CE oxidative pathway in the etiology of other human cancers.**

Table 1. Analysis of estrogen metabolites, GSH conjugates and depurinating DNA adducts in breast tissue from women with breast cancer*

Sample	Wgt (g)	picomoles								
		E ₂	4-OHE ₂	4-OHE ₁	2-OHE ₂	2-OHE ₁	4-OHE ₂ -2-NAcCys	4-OHE ₁ -2-NAcCys	4-OHE ₂ -2-Cys	4-OHE ₁ -2-Cys
1	0.62	-	3	-	-	-	-	-	-	-
2	1.2	20	-	-	-	-	-	-	-	-
3	0.75	-	17	3	1	3	-	-	-	-
4	0.30	-	-	1	-	-	-	-	-	-
5	0.83	-	1	3	1	-	-	2	-	-
6	0.76	-	4	-	3	-	-	-	2	-
7	0.82	-	6	1	2	3	-	6	2	1
8	0.47	-	4	1	-	-	-	4	2	-
9	0.54	-	3	20	1	2	-	4	-	-
10	0.17	-	2	2	-	1	7	2	-	1

*Samples were analyzed by HPLC with electrochemical detection for E₁(E₂), 2-OHE₁(E₂), 4-OHE₁(E₂), 2-OCH₃E₁(E₂), 4-OCH₃E₁(E₂), 4-OHE₁(E₂)-2-SG, 4-OHE₁(E₂)-2-Cys, 4-OHE₁(E₂)-2-NAcCys and 4-OHE₁(E₂)-1-N7Gua. The analytes not reported in the table, including the 2-OCH₃E₁(E₂) and 4-OCH₃E₁(E₂), were below the limit of detection.

Task 5 (Year 1) - Analysis of COMT (establishment of method). This task was not begun because we spent all of our time and effort establishing the assay for CE metabolites and conjugates and because we now know how to conduct better assays of COMT activity.

Task 6 (Years 1 and 2) - Analysis of COMT (initial analyses of breast tissue samples). This task was not begun because we spent all of our time and effort establishing the assay for CE metabolites and conjugates and because we now know how to conduct better assays of COMT activity.

Task 7 (Year 2) - Analysis of data. We do not yet have sufficient data to conduct statistical analyses. Nonetheless, we can make some preliminary conclusions from the analysis of the initial ten samples of human breast tissue:

Table 2. Summary of the first 10 breast cancer samples

Number	weight, g	4-OHE/2-OHE	Methylated estrogen	NAcCys or Cys (shows formation of E-3,4-quinone)
1	0.6			
2	1.2			
3	0.75	20/4	0	0
4	0.3			
5	0.83	4/1	0	2
6	0.76	4/3	0	2
7	0.82	7/5	0	9
8	0.47	5/0	0	6
9	0.54	23/3	0	4
10	0.17	4/1	0	10

Conclusions:

1. In 7 of the 10 samples, 4-hydroxyestrogens were greater than (range, 1:1-8:1) 2-hydroxyestrogens (in normal women, the 2-hydroxyestrogens are 4 times higher than the 4-hydroxyestrogens).
2. Methylated ("protected") catechol estrogens were not found, indicating that protection by the enzyme COMT was not working.
3. In 6 of the 10 samples, conjugates formed by the catechol estrogen-3,4-quinones were detected, demonstrating that the quinones were formed (and, presumably, also attacked DNA to form the depurinating DNA adducts that generate mutations leading to cancer).

These results demonstrate that we successfully completed the goal of this research project, namely, to establish methods of analysis for catechol estrogen metabolites in human breast tissue samples. In addition, we also analyze catechol estrogen conjugates that demonstrate formation of the catechol estrogen quinones that also react with DNA to form mutagenic depurinating DNA adducts

KEY RESEARCH ACCOMPLISHMENTS

1. The methodology has been developed to analyze estrogens, catechol estrogen metabolites, methylated catechol estrogens, depurinating catechol estrogen-DNA adducts and catechol estrogen conjugates in tissue and urine samples.
2. Initial analyses of ten samples of "normal" breast tissue from women with breast cancer detected more 4-hydroxyestrogens than 2-hydroxyestrogens in seven samples, no methylated catechol estrogens, and catechol estrogen conjugates formed by catechol-estrogen-3,4-quinones. These results indicate that the profile of catechol estrogen metabolites and conjugates in breast tissue from women with breast cancer support the hypothesis guiding this research.

REPORTABLE OUTCOMES

Publications, Abstracts and Presentations

Rogan, E., Stack, D., Cerny, R., Edney, J., Johansson, S., Higginbotham S. and Cavalieri, E. GC/MS analysis of catechol estrogen metabolites in breast tissue samples. Proc. The Dept. of Defense Breast Cancer Res. Prog. Era of Hope Meeting, 743-744 (1997) Washington, DC, Oct. 31-Nov. 4, 1997.

Funding Applied for Based on This Work

The research conducted under this grant has provided key methods and results to support the major emphasis of research in the competitive renewal application for the program project grant entitled "Molecular Origin of Cancer: Catechol Estrogen-3,4-Quinones", E. Cavalieri, Principal Investigator, submitted to the National Cancer Institute on June 1, 1999. Dr. Rogan is a Project Leader in this ongoing program project grant.

CONCLUSIONS

The research conducted under the auspices of this grant has been key for our developing methodology to analyze human tissue and urine samples for estrogen compounds. We have, indeed, successfully developed methods to detect picomole amounts of estrogen metabolites, DNA adducts and conjugates in tissue samples weighing approximately 1 g. Although we began this research by using a gas chromatography/mass spectrometry analytical method, we found that HPLC separation with detection by a multichannel electrochemical (ESA CoulArray) detector is the successful method.

Because we had to spend many months developing the methods of sample preparation and compound identification and quantification, we have analyzed only ten samples of human

breast tissue. Nonetheless, these initial analyses have provided us with very valuable results. Several possible parameters may reflect risk of developing breast cancer. These include the ratio of 2-hydroxyestrogens to 4-hydroxyestrogens. In normal women, the level of 2-hydroxyestrogens is approximately four times that of the 4-hydroxyestrogens. It was previously reported that breast fluid from women with breast cancer contained approximately four times more 4-catechol estrogens than 2-catechol estrogens. Our initial results are consistent with this finding, indicating that the level of 4-catechol estrogens (which are carcinogenic) is higher in women with breast cancer than in the control population. In addition, methylation of 4-catechol estrogens by COMT has been reported to be low in women with breast cancer. Our initial results also support this very important finding, in that the levels of methoxyestrogens were below the limit of detection in our analyses. This finding indicates that protection of 4-catechol estrogens from oxidation to catechol estrogen-3,4-quinones is poor in these women with breast cancer. Finally, in our initial analyses, we found catechol estrogen conjugates, which had to be formed by catechol estrogen-3,4-quinones. This finding demonstrates that the quinones are formed in the breast tissue of women with breast cancer and suggests that the quinones also formed the depurinating DNA adducts that are thought to generate apurinic sites leading to oncogenic mutations.

Therefore, (1) the results of this research are of fundamental importance for understanding the events leading to initiation of breast cancer in women, and (2) this research has led to the development of methodology to analyze relevant estrogen compounds in human samples with high sensitivity. This forms the basis for determining which estrogen compounds can serve as biomarkers for risk of developing breast cancer and for developing methods for routine screening of women.

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APPENDICES

None.

Personnel Receiving Pay from this Research Effort

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E. Cavalieri (effort, no salary)

R. Cerny (effort, no salary)

J. Edney (effort, no salary)

S. Johansson (effort, no salary)

P. Devanesan (effort and salary)

R. Todorovic (effort and salary)

D. Stack (effort and salary)



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