

**Formation of Tamoxifen-DNA Adducts via *O*-Sulfonation,
not *O*-Acetylation, of α -Hydroxytamoxifen in Rat and Human Livers**

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Abbreviations:

TAM, tamoxifen; α -OHTAM, α -hydroxytamoxifen; dG- N^2 -TAM, α -(N^2 -deoxyguanosinyl)tamoxifen; dG- N^2 -*N*-desTAM, α -(N^2 -deoxyguanosinyl)-*N*-desmethyltamoxifen; dG- N^2 -TAM *N*-oxide, α -(N^2 -deoxyguanosinyl)tamoxifen *N*-oxide; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; Acetyl-CoA, acetyl coenzyme A; HST, hydroxysteroid sulfotransferases; PAGE, polyacrylamide gel electrophoresis.

ABSTRACT

Tamoxifen (TAM) is used as the standard endocrine therapy for breast cancer patients and as a chemopreventive agent for women at high risk for this disease. Unfortunately, treatment of TAM increases the incidence of endometrial cancer; this may be due to the genotoxic damage induced by TAM metabolites. Formation of TAM-DNA adducts in rat liver correlates with the development of hepatocarcinoma. TAM-DNA adducts are proposed to be formed through *O*-sulfonation and/or *O*-acetylation of α -hydroxylated TAM and its metabolites. However, the role of *O*-sulfonation and *O*-acetylation in the formation of TAM-DNA adducts has not extensively investigated. Rat or human hydroxysteroid sulfotransferases (HST), acetyltransferases and liver cytosol was incubated with calf thymus DNA, α -OHTAM, and either 3'-phosphoadenosine 5'-phosphosulfate (PAPS) or acetyl coenzyme A (acetyl-CoA) as a cofactor and analyzed for TAM-DNA adduct formation, using ^{32}P -postlabeling/polyacrylamide gel electrophoresis analysis. TAM-DNA adduct was formed when PAPS, not acetyl-CoA, was used. No TAM-DNA adducts were produced using human *N*-acetyltransferase I and II. HST antibody inhibited approximately 90 % of TAM-DNA adduct formation generated by the cytosol or HST, suggesting that HST is primarily involved in the formation of TAM-DNA adducts. The formation of TAM-DNA adducts with rat liver cytosol and HST was much higher than that of human liver cytosol and HST. Our results indicate that TAM-DNA adducts are formed via *O*-sulfonation, not *O*-acetylation, of α -hydroxylated TAM and its metabolites.

INTRODUCTION

Tamoxifen (TAM) has been widely used for breast cancer therapy and also used as a chemopreventive agent for healthy women at high risk for this disease (Osborne, 1998; Fischer et al., 1998). Beside the significant benefit, long-term TAM treatment to women increases the risk of developing endometrial cancer (van Leeuwen et al., 1994; Fischer et al., 1998). TAM was listed in 1996 as a human carcinogen by the International Agency of Research on Cancer (IARC, 1996).

The cellular mechanism underlying TAM-induced carcinogenesis may be due to its partial estrogenic effect through the estrogen receptors and/or genotoxic damage (reviewed by Kim et al., 2004). Actually, a high level of TAM-DNA adducts produced in the liver of rats (Han and Liehr, 1992; Osborn et al., 1996) initiates the development of hepatocellular carcinomas (Hard et al., 1993). TAM-DNA adducts detected in the endometrium of women treated with TAM (Shibutani et al., 2000; Martin et al., 2003) may also be involved in the development of endometrial cancer.

Several phase I and phase II enzymes are involved in the metabolism of TAM. Cytochrome P450 (CYP) converts TAM to α -hydroxytamoxifen (α -OHTAM), *N*-desmethyltamoxifen (*N*-desTAM), 4-hydroxytamoxifen (4-OHTAM) (Figure 1) (reviewed by Kim et al., 2004). Tamoxifen *N*-oxide (TAM *N*-oxide) is produced from TAM by flavin containing monooxygenase. α -Hydroxylated forms of TAM and its metabolites are further metabolized by phase II enzymes and react with cellular DNA, resulting in the formation of TAM-DNA adducts (Phillips et al., 1994; Dasaradhi and Shibutani, 1997).

In general, sulfation effectively decreases the toxicity of xenobiotics; however, in some cases, sulfation increases the toxicity. Because certain sulfate conjugates are unstable, they can form potent electrophilic species. Like the sulfate esters, acetoxy esters generated by acetylation can also be highly reactive electrophilic species. In fact,

synthetic TAM α -sulfate and α -acetoxyTAM react rapidly with DNA, resulting in the formation of four diastereoisomers [two *trans*-forms (fr-1 and 2) and two *cis*-forms (fr-3 and 4)] of α -(N^2 -deoxyguanosinyl)tamoxifen (dG- N^2 -TAM) adducts (Figure 1) (Osborne et al., 1996; Dasaradhi and Shibutani, 1997). Similar adduct formation has been observed using α -sulfate and/or α -acetyl forms of α -OH-*N*-desTAM (Gamboa da Costa et al., 2000; Kitagawa et al., 2000), α -OHTAM *N*-oxide (Umemoto et al., 1999). Therefore, TAM-DNA adducts are considered to be formed through *O*-sulfonation and/or *O*-acetylation of α -hydroxylated TAM and its metabolites.

The formation of TAM-DNA adducts was increased in rat hepatocytes incubated with inorganic sulfate and was decreased in the hepatocytes treated with dehydroisoandrosterone-3-sulfate, an inhibitor of hydroxysteroid sulfotransferase (HST) (Davis et al., 1998). The increased formation of TAM-DNA adducts was observed when cells expressing rat STa were exposed to α -OHTAM, but not with cells expressing human HST; the authors concluded that α -OHTAM is a substrate for STa, but not for human HST; the authors concluded that α -OHTAM is a substrate for STa, but not for human SULT 2A1 (Glatt et al., 1998). In contrast, α -OHTAM can be *O*-sulfonated by both rat STa and human SULT 2A1 and react with DNA, forming dG- N^2 -TAM adducts (Shibutani et al., 1998a and 1998b). However, the contribution of *O*-acetylation in the formation of TAM-DNA adducts in rat or human tissues has not yet been determined.

We found, in the present study, that *O*-sulfonation, not *O*-acetylation, of α -hydroxylated TAM and its metabolites contributes primarily to the formation of TAM-DNA adducts in rat and human livers.

MATERIALS AND METHODS

Materials. TAM, calf thymus DNA, micrococcal nuclease, potato apyrase, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), and acetyl coenzyme A (Acetyl-CoA) were purchased from Sigma-Aldrich (St. Louis, MO). Spleen phosphodiesterase was obtained from Worthington Biochemical Corp. (Lakewood, NJ). 3'-Phosphatase-free T4 PNK and nuclease P1 were obtained from Roche Applied Science (Indianapolis, IN). Human arylamine *N*-acetyltransferase (NAT) I (2400 units/mg; enzyme units are expressed as nanomoles of acetic acid ester product formed from *para*-aminosalicylic acid per minute.), NAT II (220 units/mg; enzyme units are expressed as nanomoles of acetic acid ester product formed from sulfamethazine per minute.), and human liver cytosol were purchased from Discovery Labware, BD Biosciences (Bedford, MA). α -OHTAM (Shibutani et al., 2001), α -OH-*N*-desTAM (Kitagawa et al., 2000), and α -OHTAM-*N*-oxide (Umemoto et al., 1999) were prepared previously. TAM α -sulfate and diastereoisomers (fr-1 and fr-2) of *trans*-forms and diastereoisomers (fr-3 and fr-4) of *cis*-forms of dG_{3'p}-*N*²-tamoxifen (dG_{3'p}-*N*²-TAM) were prepared as described previously (Dasaradhi and Shibutani, 1997; Shibutani et al., 1998a). [γ -³²P]-ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Biosciences Corp. (Piscataway, NJ).

Preparation of cytosol fraction. Female Fisher 344 rats (8 weeks) were purchased from Taconic (Germantown, NY). The use of animals was in compliance with the guidelines established by the NIH Office of Laboratory Animal Welfare. Animals were housed in temperature (22 \pm 2 °C) and humidity (55 \pm 5 %) controlled rooms with a 12-hr light/dark cycle (light: 07:00–19:00 hr, dark: 19:00–07:00 hr) for one-week prior to use. Regular laboratory chow and tap water were allowed *ad lib*. The rats were

ethanized by CO₂ asphyxiation and open thoracotomy. Liver was removed quickly, frozen, and stored at -70 °C until fractionation of cytosol. The liver was rinsed several times to remove excess blood and fat and minced with scissors in ice cold homogenization buffer, 0.25 M sucrose containing 10 mM triethanolamine HCl, pH 7.4 and 5 mM mercaptoethanol. All subsequent steps were performed at 0 to 4 °C. The tissue was homogenized in 2 volumes of buffer per g wet tissue using a Potter-Elvehjem Tissue Grinder (Millville, NJ). The homogenates were immediately subjected to centrifugation at 105,000 xg for 1 hr at 4 °C and the resulting supernatant fraction was used as the cytosol fraction. Protein concentration in the cytosol was determined by Bradford method (Bradford MM, 1976).

O-Sulfonation and O-acetylation assay of α -OHTAM. 1) **cytosol.** To purify commercially available calf thymus DNA, the DNA was incubated with RNase A, RNase T1, and proteinase K, extracted with phenol/chloroform, and then precipitated by ethanol (Umemoto et al., 1994). These procedures were repeated to remove completely RNA and protein. The concentration of DNA was determined by UV spectroscopy as 50 μ g = O.D._{260 nm} 1.0. For determining O-sulfonation potential, calf thymus DNA (25 μ g) were incubated at 37 °C for 1 hr with cytosol (1 mg protein), 100 μ M α -OHTAM, 200 μ M PAPS and 100 μ M EDTA in 0.5 ml of 50 mM potassium phosphate, pH 6.5. For measuring O-acetylation potential, calf thymus DNA (25 μ g) were incubated at 37 °C for 1 hr with cytosol (1 mg of protein), 100 μ M α -OHTAM, 1 mM acetyl-CoA, 1 mM DTT and 100 μ M EDTA in 0.5 ml of 50 mM potassium phosphate, pH 7.4. The DNA was recovered using phenol/chloroform extraction following to the ethanol precipitation and used for analysis of TAM-DNA adducts.

2) rat and human hydroxysteroid sulfotransferase and acetyltransferase.

Recombinant rat liver STa (42.0 units/mg protein) (Sheng and Duffel, 2001) and human liver SULT 2A1 (63.0 units/mg protein) (Apak and Duffel, 2004) were prepared as described previously. The enzyme activity was determined using a methylene blue paired ion extraction method with dehydroepiandrosterone (DHEA) as the substrate (Sheng and Duffel, 2001; Apak and Duffel, 2004). Enzyme units are expressed as nanomoles of sulfuric acid ester product formed from DHEA per minute. DNA (25 μ g) was incubated at 37 °C for 1 hr with STa or SULT 2A1 (5 μ g each), human NAT I or NAT II (5 or 50 μ g each) in the buffer described above.

Effect of STa antibody on the formation of TAM-DNA adducts. To determine the inhibitory effect of TAM-DNA adduct formation, anti-STa-serum (0, 10, or 30 μ l) prepared previously (Ogura et al., 1990) was pre-incubated at 37 °C with cytosol (1 mg protein) for 30 min and then added to 0.2 ml of 50 mM potassium phosphate, pH 6.5, containing calf thymus DNA (10 μ g), 100 μ M α -OHTAM, 200 μ M PAPS and 100 μ M EDTA. After the reaction, the DNA was recovered as described above and used for analysis of DNA adducts.

Digestion of DNA Samples. DNA sample (2.5-5.0 μ g) was enzymatically digested at 37 °C for overnight in 100 μ l of 17 mM sodium succinate buffer (pH 6.0) containing 8 mM CaCl₂, using micrococcal nuclease (30 units) and spleen phosphodiesterase (0.15 unit) (Terashima et al., 2002). The reaction mixture was incubated for another 1 h with nuclease P1 (1 unit). After the incubation, 200 μ l of water was added. The reaction samples were then extracted twice with 200 μ l of butanol. The butanol fractions were combined, back-extracted with 50 μ l of distilled water, and evaporated to dryness.

³²P-postlabeling/PAGE analysis. The DNA digests were incubated at 37 °C for 40 min with 10 μCi of [γ -³²P]-ATP and 3'-phosphatase-free T4 PNK (10 units), and then incubated with apyrase (50 milliunits) for another 30 min, as described previously (Terashima et al., 2002). Known amounts (0.152 pmol mol, 0.0152 pmol, 0.00152 pmol or 0.000152 pmol) of dG-N²-TAM-modified oligodeoxynucleotide prepared by a phosphoramidite chemical procedure (Terashima et al., 2002) were mixed with 5 μg of calf thymus DNA (15200 pmol) and served as a standard (1 adduct/10⁵ nucleotides, 1 adduct/10⁶ nucleotides, 1 adduct/10⁷ nucleotides, or 1 adduct/10⁸ nucleotides). As described previously (Terashima et al., 2002), the amount of TAM adducts detected increased linearly depending on the amounts of oligodeoxynucleotide used. A part of the ³²P-labeled sample was electrophoresed for 4-5 h on a non-denaturing 30% polyacrylamide gel (35 x 42 x 0.04 cm) with 1400-1600 V/20-40 mA, as described previously (Terashima et al., 2002). The position of ³²P-labeled adducts was established by β-phosphorimager analysis (Molecular Dynamics Inc.). To quantify the level of ³²P-labeled products, integrated values were measured using a β-phosphorimager and compared with the standards. The detection limit for 5 μg DNA was approximately 7 adducts/10⁹ nucleotides.

RESULTS

To determine the capability of forming TAM-DNA adducts through O-sulfonation and/or O-acetylation of α-OHTAM, calf thymus DNA was incubated in the mixture of α-OHTAM, rat hepatic cytosol and either PAPS or acetyl-CoA as a cofactor. Formation of TAM-DNA adducts was analyzed using a ³²P-postlabeling/PAGE. When PAPS was used, large amounts of TAM-DNA adducts were detected at the level of 94.5 ± 32.9

adducts/ 10^7 nucleotides (means \pm S.D., $n=3$) (Figure 2). The migration of major and minor TAM-DNA adducts was consistent with standards of $dG_{3p}-N^2$ -TAM. A *trans*-form (fr-2) was a major adduct at the level of 84.1 ± 28.6 adducts/ 10^7 nucleotides; another *trans*-form (fr-1) and *cis*-forms (a mixture of fr-3 and fr-4) were minor adducts at the level of 8.0 ± 2.6 adducts/ 10^7 nucleotides and 2.5 ± 1.9 adducts/ 10^7 nucleotides, respectively. An unknown adduct (5.9 ± 2.3 adducts/ 10^7 nucleotides) was observed at the lower position of $dG_{3p}-N^2$ -TAM standards. Only a trace of adduct (0.6 ± 0.2 adducts/ 10^7 nucleotides) was detected without PAPS. In contrast, when acetyl-CoA was used as a cofactor, no significant formation of TAM-DNA adducts were observed. The levels of adduct in the reaction condition with and without acetyl-CoA were 0.4 ± 0.1 adducts/ 10^7 nucleotides and 0.5 ± 0.1 adducts/ 10^7 nucleotides, respectively.

When rat STa (210 mU/5 μ g) or human SULT 2A1 (315 mU/5 μ g) was used for the reaction condition applied for rat hepatic cytosol and PAPS, TAM-DNA adducts were detected at the level of 70.3 adducts/ 10^7 nucleotides and 5.8 adducts/ 10^7 nucleotides, respectively (Figure 3). Comparing with equivalent units of enzyme activity, the amount of TAM-DNA adducts generated by SULT 2A1 was approximately 18 times less than that of STa. Since NATs have *O*-acetylation activity, in addition to *N*-acetylation (Land et al., 1989), NAT I and NAT II were used to determine the capability of *O*-acetylating α -OHTAM in the presence of acetyl-CoA. However, human NAT I (1,100 mU/5 μ g) and NAT II (12,000 mU/5 μ g) did not produce any TAM-DNA adducts (Figure 3). Even though 10-fold amounts of NATs were used, no reactivity to DNA was observed (data not shown).

The formation of TAM-DNA adducts with rat hepatic cytosol and PAPS increased depending on the incubation time (Figure 4A). The presence of STa in the cytosol was confirmed by Western blot analysis using STa antibody, as described previously (Ogura

et al., 1990). The addition of STa antibody prior to the reaction with the cytosol decreased the formation of TAM-DNA adducts to 9.8 %, as observed with STa (8.2 %) (Figure 4B). The DHEA sulfation activity was reduced by addition of STa antibody in parallel to the formation of TAM-DNA adducts (data not shown).

To explore the substrate specificity for *O*-sulfonation, the reactivities of α -OH-*N*-desTAM and α -OH-TAM *N*-oxide to calf thymus DNA were determined in the reaction mixture containing rat liver cytosol and PAPS and compared with that of α -OHTAM (Table 1). The formation of DNA adducts with α -OH-*N*-desTAM and α -OH-TAM *N*-oxide were 50 % and 2.8 %, respectively, than that observed with α -OHTAM.

When liver cytosol (1 mg) from Caucasian women (23-71 years-old) was incubated with DNA, α -OHTAM and PAPS, the formation of TAM-DNA adducts was observed at the level of 0.91 ± 0.52 adducts/ 10^7 nucleotides (Figure 5). A liver cytosol pooled from 22 humans (9 Caucasian women, 13 Caucasian men and one Hispanic man) also promoted similar level of TAM-DNA adducts (0.74 adducts/ 10^7 nucleotides). In the reaction condition without PAPS, no TAM-DNA adducts was observed. In addition, no adducts were formed when acetyl-CoA was used as a cofactor (data not shown).

Discussion

The capability of forming TAM-DNA adducts from α -OHTAM was investigated with rat and human liver cytosol in the presence of PAPS or acetyl-CoA as a co-factor. TAM-DNA adducts were generated only when PAPS was used. In addition, rat and human hydroxysteroid sulfotransferases (STa and SULT 2A1) generated TAM-DNA adducts from α -OHTAM; however, human NATs did not form any TAM-DNA adducts. Since synthetic α -acetoxyTAM is highly reactive to DNA, forming TAM-DNA adducts (Osborne

et al., 1996; Dasaradhi and Shibutani, 1997), our results indicate that α -OHTAM may not be a substrate for acetyltransferases; therefore, no TAM-DNA adducts are formed. Thus, TAM-DNA adducts are formed through O-sulfonation, not O-acetylation, of α -OHTAM.

When α -OHTAM was incubated with DNA and rat liver cytosol in the absence of cofactors, a small amount of TAM-DNA adduct was observed at the level of 0.4-0.6 adducts/ 10^7 nucleotides. The TAM-DNA adduct formation may be reflected by an intrinsic reactivity of α -OHTAM to DNA (Phillips et al., 1994). However, such adduct formation was not detected under the similar reaction conditions with human liver cytosol. The trace of TAM-DNA adduct formation may be due to endogenous PAPS remaining in the rat liver cytosol, rather than in human liver cytosol, thus serving as a cosubstrate for a very small amount of O-sulfonation.

When a polyclonal antibody to STa was pre-incubated with rat liver cytosol before reacting with DNA, α -OHTAM and PAPS, STa antibody inhibited 90 % of TAM-DNA adduct formation, as similarly observed with STa. Since this antibody has no cross-reactivity with other rat isoforms including phenol sulfotransferase and estrogen sulfotransferase (Ogura et al., 1990), our result supported that STa is a principal enzyme involved in O-sulfonation of α -OHTAM. The formation of TAM-DNA adduct was not completely inhibited. The antibody may not completely deny the enzyme active site; therefore, a partial sulfating activity may remain.

Mass-spectroscopy and ^{32}P -postlabeling/HPLC analyses demonstrated that dG- N^2 -TAM and α -(N^2 -deoxyguanosinyl)- N -desmethyltamoxifen (dG- N^2 - N -desTAM) are the major hepatic DNA adducts of rodents treated with TAM (Rajaniemi et al., 1999; Umemoto et al., 2001). The dG- N^2 -TAM- and dG- N^2 - N -desTAM-DNA adducts

accounted for over 95% of DNA adducts induced by TAM in rat liver (Umemoto et al., 2001); however, α -(N^2 -deoxyguanosinyl)tamoxifen *N*-oxide (dG- N^2 -TAM *N*-oxide) was not detected in the livers of rats. Supporting the animal studies, α -OHTAM *N*-oxide was a poor substrate for rat liver cytosol, as compared with α -OHTAM and α -OH-*N*-desTAM, for the formation of DNA adducts. The liver cytosol catalyzed α -OHTAM more rapidly than α -OH-*N*-desTAM, as observed previously with STa (Shibutani et al., 2002). However, the ratio of dG- N^2 -TAM and dG- N^2 -*N*-desTAM adducts observed in rat liver may vary depending on the amounts of α -OHTAM and α -OH-*N*-desTAM generated by CYP enzymes from TAM and *N*-desTAM, respectively.

The formation of TAM-DNA adduct with human liver cytosol (0.91 adducts/ 10^7 nucleotides) was approximately 105 times lower than that when same amount of rat liver cytosol (94.5 adducts/ 10^7 nucleotides) was used. As observed with rat STa and human SULT 2A1 having same enzyme activity for DHEA, the amount of TAM-DNA adducts generated by human SULT 2A1 was approximately 18 times less than that of STa. The lower formation of TAM-DNA adducts using the human cytosol may be due to either a low substrate specificity of SULT 2A1 for α -OHTAM and/or the lower presence of SULT 2A1 in human liver. In an earlier report, no TAM-DNA adducts were detected in livers of women treated with TAM (Martin et al., 1995). However, in the livers of monkeys given six times the human-equivalent dose of TAM, TAM-DNA adducts including *trans*-form (fr-2) of dG- N^2 -TAM were detected at levels of 4.4 adducts/ 10^8 nucleotides, using ^{32}P -postlabeling/HPLC analysis developed in our laboratory (Shibutani et al., 2003). Similar results were observed for the same monkey DNA samples, using chemiluminescence immunoassay and HPLC electrospray tandem mass spectrometry (Schild et al., 2003). The failure of detecting TAM-DNA adducts in

human livers in some studies may be due to differences in the sensitivity of ^{32}P -postlabeling analysis used (Shibutani et al., 2002). Since human liver SULT 2A1 is capable of *O*-sulfonating α -OHTAM, our results indicate the possibility that women receiving TAM may form genotoxic damage in the liver, as observed in monkey (Shibutani et al., 2003; Schild et al., 2003).

The level of each TAM and its metabolites in plasma of patients treated with TAM was 0.1-5.0 μM (Etienne et al., 1989). The concentration of TAM and its metabolites in human tissues are approximately 10-60 fold higher than in serum (Lien et al., 1991). α -OHTAM is a minor metabolite, accounting for ~0.1% of the administered TAM dose (Jacolot et al., 1991). In our in vitro experiments, the formation of TAM-DNA adducts (0.63 adducts/ 10^7 nucleotides to 128 adducts/ 10^7 nucleotides) increased linearly using STa and α -OHTAM between 0.1 μM and 100 μM (data not shown). Although our in vitro study with relatively high level of α -OHTAM (100 μM) may not reproduce the physiological conditions found in the tissues of patients with long-term exposure to low levels of α -OHTAM, the ability of forming TAM-DNA adducts through *O*-sulfonation of α -OHTAM was proven with rat and human liver cytosol.

TAM-DNA adducts were detected in the endometrium of breast cancer patients treated with TAM (Shibutani et al., 2000) and in the cultured human endometrial explants exposed to α -OHTAM (Kim et al., 2005), suggesting that genotoxic mechanism is involved in the development of endometrial cancer. Although human HST was not detected in the endometrium of women with normal menstrual cycles (Rubin et al., 1999), no determination has been performed with postmenopausal women and women receiving TAM. Human HST may possibly be induced by TAM treatment, as observed in rats (Maiti and Chen, 2003). Other subfamily of HST such as SULT 2B1 expressed in

human uterus (Geese and Blanchard, 2001) may be involved in O-sulfonation of α -OHTAM. To explore the mechanism of forming TAM-DNA adducts in the endometrium, detailed analysis of hHST expression in the tissue of women exposed to TAM is required.

We conclude that α -hydroxylated tamoxifen metabolites are primarily activated via O-sulfonation, not O-acetylation, and then react with DNA, thereby exerting genotoxic effects in target tissues.

References

- Apak TI and Duffel MW (2004) Interactions of the stereoisomers of alpha-hydroxytamoxifen with human hydroxysteroid sulfotransferase SULT2A1 and rat hydroxysteroid sulfotransferase STa. *Drug Metab Dispos* **32**:1501-1508.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.
- Dasaradhi L and Shibutani S (1997) Identification of tamoxifen-DNA adducts formed by α -sulfate tamoxifen and α -acetytamoxifen. *Chem Res Toxicol* **10**:189-196.
- Davis W, Venitt S and Phillips DH (1998) The metabolic activation of tamoxifen and alpha-hydroxytamoxifen to DNA-binding species in rat hepatocytes proceeds via sulphation. *Carcinogenesis* **19**:861-866.
- Etienne MC, Milano G, Fischel JL, Frenay M, Francois E, Formento JL, Gioanni J and Namer M (1989) Tamoxifen metabolism: pharmacokinetic and in vitro study. *Br J Cancer* **60**: 30-35.
- Fischer B, Costantino JP, Wickerham L, Redmond CK, Kavanah M, Cronin WM, Botel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wormark N et al (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* **90**: 1371-1388.
- Gamboa da Costa G, Hamilton LP, Beland FA and Marques MM (2000) Characterization of the major DNA adduct formed by α -hydroxy-N-desmethyltamoxifen in vitro and in vivo. *Chem Res Toxicol* **13**:200-207.

- Geese WJ and Blanchard, R. (2001) Biochemical characterization and tissue distribution of human SULT2B1. *Biochem Biophys Res Commun* **288**: 280-289.
- Glatt H, Davis W, Meinl W, Hermersdorfer H, Venitt S and Phillips D (1998) Rat, but not human, sulfotransferase activates a tamoxifen metabolite to produce DNA adducts and gene mutations in bacteria and mammalian cells in culture. *Carcinogenesis* **19**:1709-1713.
- Han X and Liehr JG (1992) Induction of covalent DNA adducts in rodents by tamoxifen. *Cancer Res* **52**:1360-1363.
- Hard GC, Iatropoulos MJ, Jordan K, Radi L, Kaltenberg OP, Imondi AR and Williams GM (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. *Cancer Res* **53**:4534-4541.
- IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol 66. Some pharmaceutical drugs. Lyon: IARC, 1996.
- Jacolot F, Simon I, Dreano Y, Beaune P, Riche C and Berthou F. (1991) Identification of the cytochrome P-450 IIIA family as the enzymes involved in the *N*-demethylation of tamoxifen in human liver microsomes. *Biochem Pharmacol* **41**: 1911-1919.
- Kim SY, Suzuki N, Laxmi YRS and Shibutani S (2004) Genotoxic mechanism of tamoxifen in developing endometrial cancer. *Drug Metab Rev* **36**:199-218.
- Kim SY, Suzuki N, Santosh Laxmi YR, Barbara P, McGarrigle BP, Olson JR, Sharma M, Sharma M and Shibutani S. (2005) Formation of tamoxifen-DNA adducts in human endometrial explants exposed to α -hydroxytamoxifen. *Chem Res Toxicol* **18**: 889-895.

- Kitagawa M, Ravindernath A, Suzuki N, Rieger R, Terashima I, Umemoto A and Shibutani S (2000) Identification of tamoxifen-DNA adducts induced by α -acetoxy-N-desmethyltamoxifen. *Chem Res Toxicol* **13**:761-769.
- Land SJ, Zukowski K, Lee MS, Debiec-Rychter M, King CM and Wang CY (1989) Metabolism of aromatic amines: relationships of N-acetylation, O-acetylation, N,O-acetyltransfer and deacetylation in human liver and urinary bladder. *Carcinogenesis* **10**:727-731.
- van Leeuwen FE, Benraadt J, Coebergh JWW, Kiemeny LALM, Diepenhorst FW, van den Belt-Dusebout AW and van Tinteren H (1994) Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet* **343**:448-452.
- Lien EA, Solheim E and Ueland PM. (1991) Distribution of tamoxifen and its metabolites in rat and human tissues during steady-state treatment. *Cancer Res* **51**: 4837-4844.
- Maiti S and Chen G (2003) Tamoxifen induction of aryl sulfotransferase and hydroxysteroid sulfotransferase in male and female rat liver and intestine. *Drug Metab Dispos* **31**: 637-644.
- Martin EA, Rich KJ, White IN, Woods KL, Powles TJ and Smith LL (1995) ³²P-postlabelled DNA adducts in liver obtained from women treated with tamoxifen. *Carcinogenesis* **16**:1651-1654.
- Martin EA, Brown K, Gaskell M, Al-Azzawi F, Garner RC, Boocock DJ, Mattock E, Pring DW, Dingley K, Turteltaub KW, Smith LL and White IN (2003) Tamoxifen DNA damage detected in human endometrium using accelerator mass spectrometry. *Cancer Res* **63**:8461-8465.
- Ogura K, Sohtome T, Sugiyama A, Okuda H, Hiratsuka A and Watabe T (1990) Rat liver cytosolic hydroxysteroid sulfotransferase (sulfotransferase a) catalyzing the

formation of reactive sulfate esters from carcinogenic polycyclic hydroxymethylarenes. *Mol Pharmacol* **37**:848-854.

Osborne CK (1998) Tamoxifen in the treatment of breast cancer. *New Eng J Med* **339**:1609-1617.

Osborne MR, Hewer A, Hardcastle IR, Carmichael PL and Phillips DH (1996) Identification of the major tamoxifen-deoxyguanosine adduct formed in the liver DNA of rats treated with tamoxifen. *Cancer Res* **56**:66-71.

Phillips DH, Carmichael PL, Hewer A, Cole KJ and Poon GK (1994) α -Hydroxytamoxifen, a metabolite of tamoxifen with exceptionally high DNA-binding activity in rat hepatocytes. *Cancer Res* **54**:5518-5522.

Rajaniemi H, Rasanen I, Koivisto P, Peltonen K and Hemminki K (1999) Identification of the major tamoxifen-DNA adducts in rats liver by mass spectroscopy. *Carcinogenesis* **20**:305-309.

Rubin GL, Harrold AJ, Mills JA, Falany CN and Coughtrie MW (1999) Regulation of sulphotransferase expression in the endometrium during the menstrual cycle, by oral contraceptives and during early pregnancy. *Mol Hum Reprod* **5**: 995-1002.

Schild LJ, Divi RL, Beland FA, Churchwell MI, Doerge DR, Gamboa da Costa G, Marques MM and Poirier MC (2003) Formation of tamoxifen-DNA adducts in multiple organs of adult female cynomolgus monkeys dosed with tamoxifen for 30 days. *Cancer Res* **63**:5999-6003.

Sheng JJ and Duffel MW (2001) Bacterial expression, purification, and characterization of rat hydroxysteroid sulfotransferase STa. *Protein Expr Purif* **21**:235-242.

Shibutani S, Dasaradhi L, Terashima I, Banoglu E and Duffel MW (1998a) α -Hydroxytamoxifen is a substrate of hydroxysteroid (alcohol) sulfotransferase, resulting in tamoxifen DNA adducts. *Cancer Res* **58**:647-653.

- Shibutani S, Shaw P, Suzuki N, Dasaradhi L, Duffel MW and Terashima I (1998b) Sulfation of α -hydroxytamoxifen catalyzed by human hydroxysteroid sulfotransferase results in tamoxifen DNA adducts. *Carcinogenesis* **19**:2007-2011.
- Shibutani S, Ravindernath A, Suzuki N, Terashima I, Sugarman SM, Grollman AP and Pearl ML (2000) Identification of tamoxifen-DNA adducts in the endometrium of women treated with tamoxifen. *Carcinogenesis* **21**:1461-1467.
- Shibutani S, Ravindernath A, Terashima I, Suzuki N, Santosh LYR, Kanno Y, Suzuki M, Apak TI, Sheng JJ and Duffel MW (2001) Mechanism of lower genotoxicity of toremifene compared with tamoxifen. *Cancer Res* **61**:3925-3931.
- Shibutani S, Laxmi YRS and Suzuki N (2002) Inter-laboratory variation of detecting tamoxifen-DNA adducts using ^{32}P -postlabeling analysis. *Proc Am Assoc Cancer Res* **43**:1042.
- Shibutani S, Suzuki N, Laxmi YR, Schild LJ, Divi LJ, Divi RL, Grollman AP and Poirier MC (2003) Identification of tamoxifen-DNA adducts in monkeys treated with tamoxifen. *Cancer Res* **63**:4402-4406.
- Terashima I, Suzuki N and Shibutani S (2002) ^{32}P -Postlabeling/polyacrylamide gel electrophoresis analysis: application to the detection of DNA adducts. *Chem Res Toxicol* **15**:305-311.
- Umemoto A, Kajikawa A, Tanaka M, Hamada K, Seraj MJ, Kubota A, Nakayama M, Kinouchi T, Ohnishi Y, Yamashita K and Monden Y (1994) Presence of mucosa-specific DNA adduct in human colon: possible implication for colorectal cancer. *Carcinogenesis* **15**:901-905.
- Umemoto A, Monden Y, Komaki K, Suwa M, Kanno Y, Suzuki M, Lin CX, Ueyama Y, Momen MA, Ravindernath A and Shibutani S (1999) Tamoxifen-DNA adducts formed by α -acetytamoxifen *N*-oxide. *Chem Res Toxicol* **12**:1083-1089.

Umemoto A, Komaki K, Monden Y, Suwa M, Kanno Y, Kitagawa M, Suzuki M, Lin CX, Ueyama Y, Momen MA, Ravindernath A and Shibutani S (2001) Identification and quantification of tamoxifen-DNA adducts in the liver of rats and mice. *Chem Res Toxicol* **14**:1006-1013.

Footnotes

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Figure legends

Fig. 1. Formation of TAM-DNA adducts via α -hydroxylation of TAM metabolites.

Fig. 2. Determination of TAM-DNA adduct formed through O-sulfonation or O-acetylation of α -OHTAM.

Calf thymus DNA (25 μ g) was incubated at 37 °C for 1 hr with rat liver cytosol (1 mg protein) in a buffer containing 100 μ M α -OHTAM and with (+) or without (-) 200 μ M PAPS (S1-S3), and in a buffer containing 100 μ M α -OHTAM and with (+) or without (-) 1 mM acetyl-CoA (A1-A3). The DNA (2.5 μ g) recovered from the reaction mixture was digested by enzymes and labeled with 32 P. Half of the 32 P-labeled samples were subjected to PAGE for determination of TAM-DNA adducts, as described in the Materials and Methods. Standards represent as *trans*-forms (fr-1 and fr-2) and *cis*-forms (a mixture of fr-3 and fr-4) of 32 P-labeled dG_{3p}-N²-TAM. A known amount (0.0038 pmol) of dG-N²-TAM-modified oligodeoxynucleotide was mixed with 2.5 μ g of calf thymus DNA (7600 pmol) and served as a standard (5 adduct/10⁷ nucleotides) for determination of the level of TAM-DNA adduct.

Fig. 3. Formation of TAM-DNA adduct in reactions catalyzed by rat and human sulfotransferases or acetyltransferases.

Calf thymus DNA (25 μ g) was incubated at 37 °C for 1 hr with 5 μ g of STa or SULT2A1, in a buffer, pH 6.5 containing 100 μ M α -OHTAM and 200 μ M PAPS or with 5 μ g human NAT I or II in a buffer, pH 7.4, containing 100 μ M α -OHTAM and either with or without 1 mM acetyl-CoA. The DNA (2.5 μ g) recovered was used for 32 P-postlabeling/PAGE analysis and compared their migration with standards of dG_{3p}-N²-

TAM. A known amount of dG- N^2 -TAM-modified oligodeoxynucleotide was mixed with 2.5 μ g of calf thymus DNA (7600 pmol) and served as a standard (5 adduct/ 10^7 nucleotides) for determination of the level of TAM-DNA adduct.

Fig. 4. Inhibitory effect of STa antibody on the formation of TAM-DNA adducts.

(A) Calf thymus DNA (10 μ g) was incubated at 37 °C for 10, 20, 40, or 60 min with rat liver cytosol (1 mg protein) in a buffer, pH 6.5, containing 100 μ M α -OHTAM and 200 μ M PAPS. (B) Anti-STa-serum [0 (-), 10 μ l (+), and 30 μ l (++)] was pre-incubated at 37 °C with rat liver cytosol (1 mg protein) or STa (5 μ g) for 30 min and then incubated at 37 °C for 1 hr in the buffer containing calf thymus DNA (10 μ g), 100 μ M α -OHTAM and 200 μ M PAPS. The DNA (2.5 μ g) recovered was used for 32 P-postlabeling/PAGE analysis and compared their migration with standards of dG $_{3p}$ - N^2 -TAM.

Fig. 5. Formation of TAM-DNA adducts in reactions catalyzed in human liver cytosol.

Calf thymus DNA (25 μ g) was incubated at 37 °C for 1 hr with liver cytosol (1 mg protein) from Caucasian women [H42, 23-71 years-old; H43, 23-71 years-old; H89, 23-71 years-old; PH, a pooled cytosol from 22 humans (9 Caucasian women, 13 Caucasian men and one Hispanic man)] in a buffer, pH 6.5, containing 100 μ M α -OHTAM and either with or without 200 μ M PAPS. The DNA (2.5 μ g) recovered was used for 32 P-postlabeling/PAGE analysis and compared their migration with standards of dG $_{3p}$ - N^2 -TAM. A known amount of dG- N^2 -TAM-modified oligodeoxynucleotide was mixed with 2.5 μ g of calf thymus DNA (7600 pmol) and served as a standard (5 adduct/ 10^7 nucleotides) for determination of the level of TAM-DNA adduct.

Table 1 Formation of TAM-DNA adducts via *O*-sulfonation of α -hydroxylated TAM and its metabolites using rat liver cytosol.

	DNA adducts (adducts/10 ⁷ nucleotides)			Total
	<u>trans-form</u>		<u>cis-form</u>	
	fr-1	fr-2	fr-3&4	
α -OHTAM	11.8 \pm 2.7	85.0 \pm 12.5	6.5 \pm 1.6	103.3 \pm 14.5
α -OH- <i>N</i> -desTAM	6.0 \pm 2.8	42.8 \pm 17.0	3.6 \pm 1.9	52.4 \pm 22.5
α -OHTAM <i>N</i> -oxide	N.D	2.9 \pm 0.4	N.D	2.9 \pm 0.4

Data are expressed as mean values \pm S.D. from three analyses. N.D., not detectable.

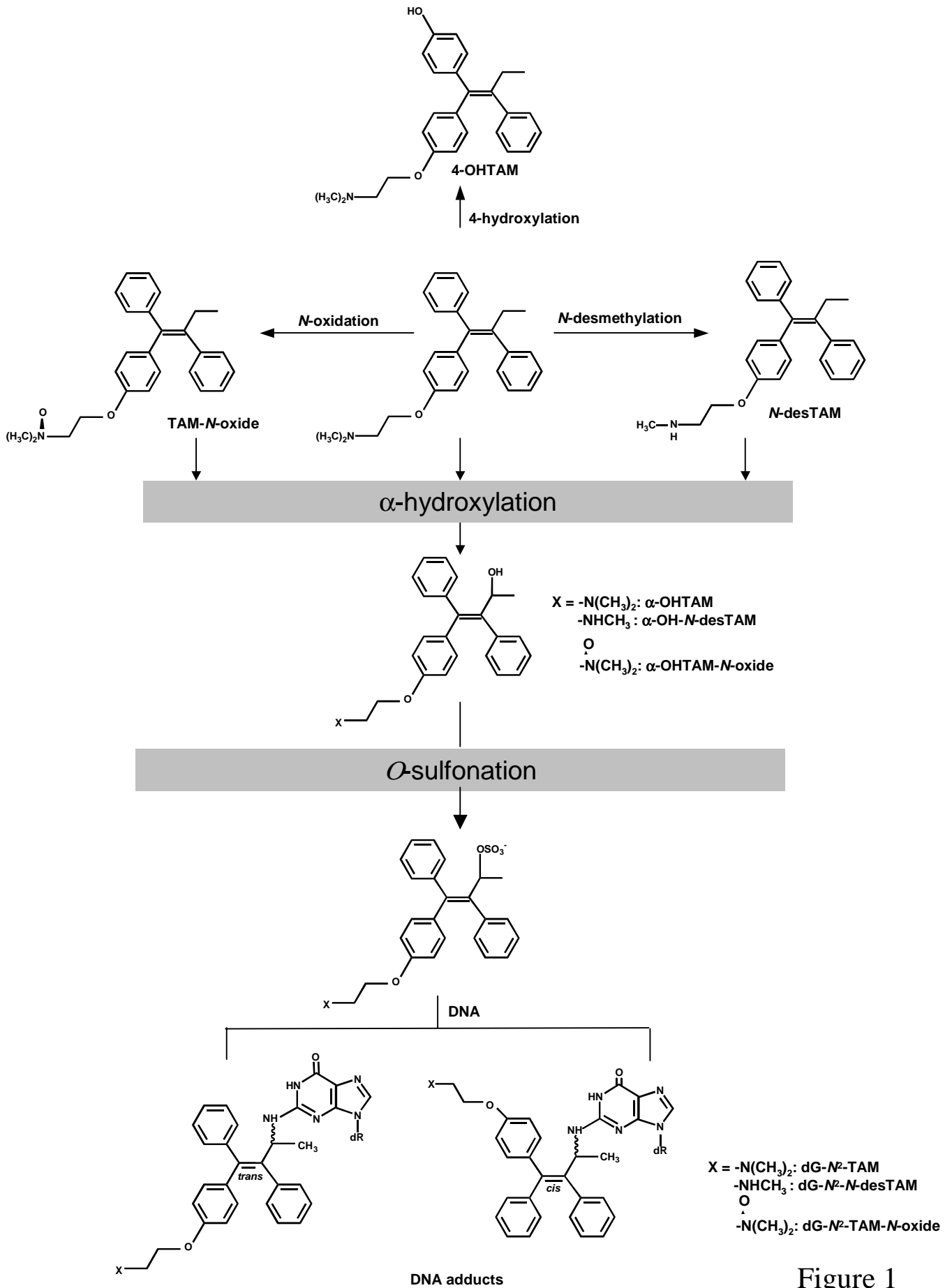


Figure 1

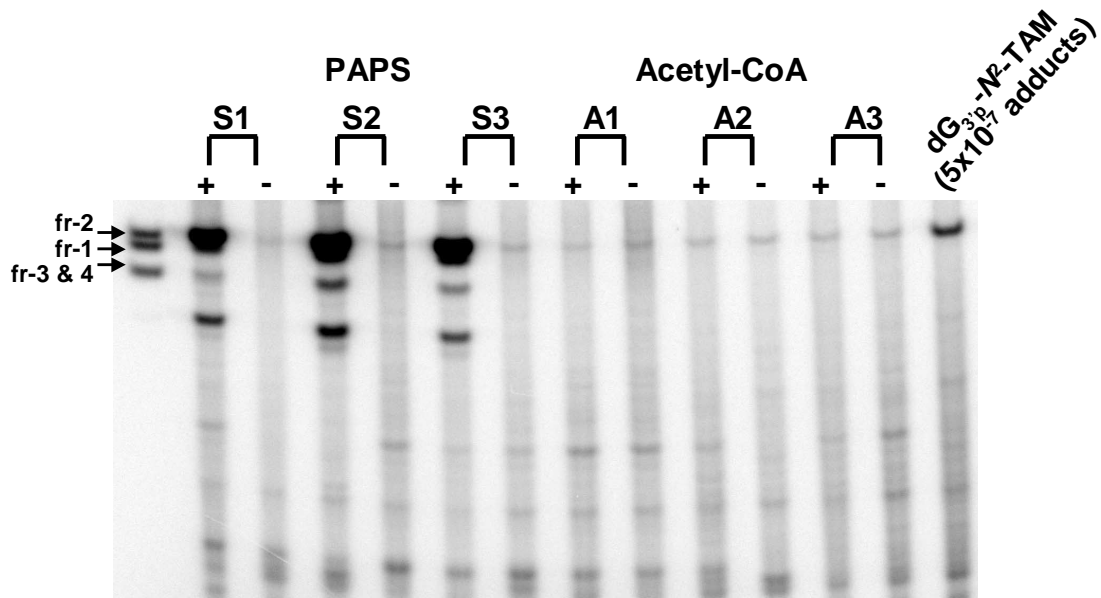


Figure 2

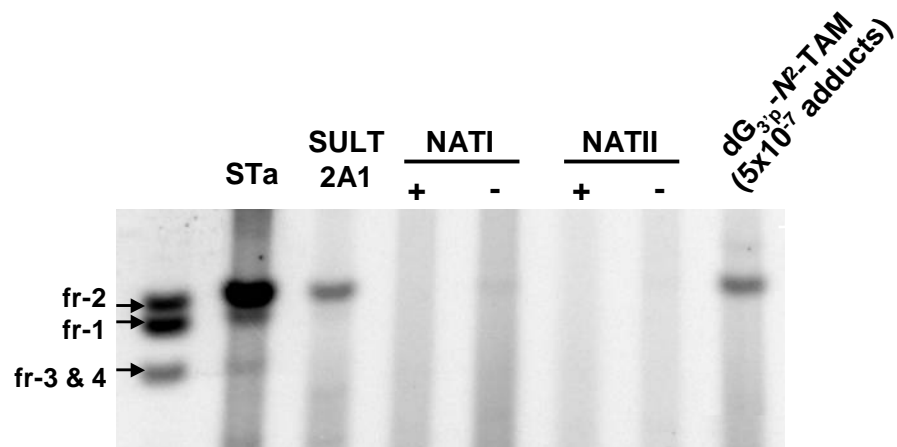


Figure 3

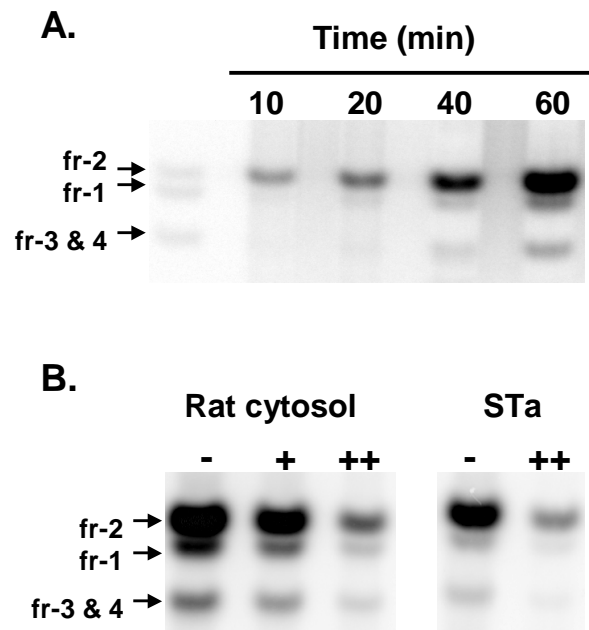


Figure 4

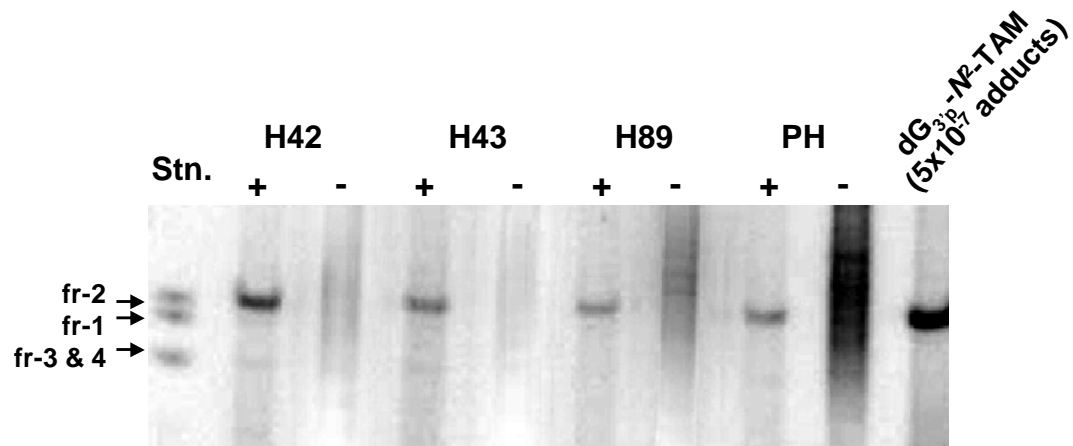


Figure 5